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Modulation of agonist-induced calcium mobilisation in bovine aortic endothelial cells by phorbol myristate acetate and cyclic AMP but not cyclic GMP

Kevin W. Buchan & [1]William Martin

Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ

1 In bovine aortic endothelial cells (BAEC), thrombin (1 u ml^{-1}), bradykinin (1–10 nM) and adenosine triphosphate (ATP) (0.3 μM–100 μM) each induced a biphasic elevation of cytosolic calcium ($[Ca^{2+}]_i$), consisting of an initial transient followed by a sustained plateau phase.

2 Pretreatment of BAEC with 4β-phorbol 12-myristate 13-acetate (PMA; 100 nM) reduced the magnitude of the initial transient elevation of $[Ca^{2+}]_i$, induced by thrombin (1 u ml^{-1}), low concentrations of bradykinin (1 nM) or ATP (0.3 μM, 3 μM), but not by higher concentrations of the latter two agonists. Addition of PMA (100 nM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), bradykinin (10 nM) or ATP (30 μM) resulted in a fall in $[Ca^{2+}]_i$.

3 The inhibitory effects of PMA (100 nM) were inhibited by staurosporine (100 nM) but not mimicked by the inactive phorbol ester, 4α-phorbol 12,13-didecanoate (4α-PDD; 100 nM). Furthermore, staurosporine (100 nM) increased $[Ca^{2+}]_i$ when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin or bradykinin. In contrast, staurosporine (100 nM) reduced $[Ca^{2+}]_i$when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by ATP (30 μM).

4 Pretreatment with forskolin (10 μM) had no effect on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), bradykinin (1 nM and 10 nM) or ATP (30 μM). In contrast, forskolin (10 μM) and isoprenaline (10 μM) each induced biphasic elevations of $[Ca^{2+}]_i$ when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by the three agonists. Furthermore, in the presence of the inhibitor of calcium influx, nickel chloride (4 mM), these biphasic elevations were reduced to monophasic transient elevations.

5 8 Bromo cyclic GMP (30 μM), a membrane-permeant analogue of guanosine 3′:5′-cyclic monophosphate (cyclic GMP), had no effect on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), bradykinin (10 nM) or ATP (3 μM). Furthermore, 8 bromo cyclic GMP (30 μM) and sodium nitroprusside (1 μM), had no effect when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by the three agonists.

6 N^G-nitro-L-arginine (50 μM), an inhibitor of nitric oxide synthase, had no effect on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), bradykinin (1 nM) or ATP (3 μM), and had no effect on the plateau phase of the increase in $[Ca^{2+}]_i$ induced by these agents.

7 These findings suggest that while activation of protein kinase C inhibits and elevation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) augments calcium mobilisation in bovine aortic endothelial cells, elevation of cyclic GMP appears to have no effect.

Keywords: Endothelial cell; bradykinin; ATP; thrombin; calcium; fura-2; cyclic AMP; phorbol ester; cyclic GMP

Introduction

Endothelium-derived relaxing factor (EDRF) has recently been identified as nitric oxide (Palmer *et al.*, 1987). The primary stimulus for production of EDRF is calcium (Singer & Peach, 1982), reflecting the calcium-dependence of the enzyme nitric oxide synthase (Mayer *et al.*, 1989). Recently, attempts have been made to determine if other intracellular second messenger systems such as protein kinase C and the cyclic nucleotides modulate calcium mobilisation in the vascular endothelium. For example, tumour-promoting phorbol esters, which activate protein kinase C (Castagna *et al.*, 1982) inhibit endothelium-dependent relaxation induced by histamine in guinea-pig pulmonary artery (Weinheimer *et al.*, 1986), acetylcholine and substance P in rabbit aorta (Lewis & Henderson, 1987) and acetylcholine in rabbit pulmonary artery (Cherry & Gillis, 1988). The inability of phorbol esters to inhibit relaxations induced by sodium nitroprusside (Lewis & Henderson, 1987) suggests that these compounds inhibit the production, but not the action, of EDRF. Not all studies on isolated endothelial cells are in agreement, however, since phorbol esters have been reported to inhibit bradykinin-stimulated production of EDRF by bovine (de Nucci *et al.*, 1988) but not by porcine aortic endothelial cells (Smith & Lang, 1990). Nevertheless, the finding that endothelium-dependent relaxation induced by the calcium ionophore, A23187, is unaffected (Weinheimer *et al.*, 1986; Lewis & Henderson, 1987; Cherry & Gillis, 1988) strongly suggests that phorbol esters inhibit EDRF production by blocking receptor-mediated calcium mobilisation, a concept supported by direct measurement of cytosolic calcium using fluorescent probes (Brock & Capasso, 1988; Carter *et al.*, 1989).

Whether the cyclic nucleotides, guanosine 3′:5′-cyclic monophosphate (cyclic GMP) and adenosine 3′:5′-cyclic monophosphate (cyclic AMP), modulate calcium mobilisation in the endothelial cell is less clear. Endothelial cells respond to the EDRF they produce (Martin *et al.*, 1988) as well as to atrial naturiuretic factors (Leitman & Murad, 1986; Martin *et al.*, 1988), and the resulting elevations of cyclic GMP content are thought to inhibit EDRF production (Evans *et al.*, 1988; Hogan *et al.*, 1989). Elevation of cyclic GMP content inhibits thrombin-induced production of inositol 1,4,5-trisphosphate by porcine aortic endothelial cells (Lang & Lewis, 1991), but it is not known if calcium mobilisation is inhibited. Furthermore, elevation of cyclic AMP content has variously been reported in bovine aortic endothelial cells to inhibit

[1] Author for correspondence.

bradykinin-and ATP-stimulated calcium mobilisation (Lückhoff *et al.*, 1990) or to augment ATP-induced mobilisation (Brock *et al.*, 1988), whereas in human umbilical vein endothelial cells it has been reported to have no effect on histamine-stimulated calcium mobilisation (Carson *et al.*, 1989).

In view of the differing reports on the actions of phorbol esters and cyclic nucleotides we wished to examine more fully the effects of these agents on a single endothelial cell type, namely, bovine aortic endothelial cells (BAEC). Specifically, we wished to examine their effects upon calcium mobilisation induced by a range of agonists over a wide range of concentrations. Furthermore, we wished to examine separately the effects of these regulators on both the initial transient elevation of calcium resulting from intracellular release and the plateau phase resulting from calcium influx.

Methods

Culture of endothelial cells

Bovine aortae were obtained from a local abattoir. Immediately after removal from the animal, the aorta was flushed with sterile saline containing benzyl penicillin (100 u ml^{-1}) and streptomycin (100 $\mu g\,ml^{-1}$). The vessel was ligated at one end and cannulated at the other with a 60 ml syringe containing the same sterile saline. This saline was infused into the lumen and the aorta transported to the laboratory. The endothelial cells were then isolated as described previously (Buchan & Martin, 1991). Briefly, after ligating the intercostal arteries, collagenase (0.1%, type II, Sigma, in Dulbecco's modification of Eagle's medium (DMEM)) was infused into the aortic lumen and the blood vessel incubated for 25 min at 37°C. The endothelial cells from each aorta were then harvested, washed twice and finally resuspended in bicarbonate-buffered DMEM containing foetal bovine serum (10%), newborn bovine serum (10%), glutamine (4 mM), benzyl penicillin (200 u ml^{-1}) and streptomycin (200 $\mu g\,ml^{-1}$) and seeded into 80 cm^2 tissue culture flasks. The culture medium was replaced the day after isolation and every 2 days subsequently. All tissue culture reagents were obtained from Gibco (Paisley, UK). Upon reaching confluence (3–5 days), the cells were detached by exposure to trypsin (0.05%) and EDTA (0.02%) (Flow Laboratories, Irvine, UK), washed twice in DMEM, seeded onto sterile glass coverslips (11 × 42 mm) and grown to confluence, reached after 2–3 days.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured as previously described (Buchan & Martin, 1991). Briefly, confluent bovine aortic endothelial cell monolayers were incubated for 45 min with the penta-acetoxymethyl ester form of fura-2 (2 μM, Novabiochem) at 37°C in HEPES (20 mM)-buffered DMEM (Northumbria Biologicals, Cramlington, UK) containing 1% bovine serum albumin (Fraction V, Sigma). Fura-2 loaded cells were then transferred to HEPES (10 mM)-buffered Krebs solution (pH 7.4) containing (mM): NaCl 118, KCl 4.8, $MgSO_4$ 1, $NaHCO_3$ 2.4, glucose 11, HEPES 10 and $CaCl_2$ 1.8, and left at room temperature for 20 min in order to maximize conversion to the Ca^{2+}-sensitive acid form. For experiments involving nickel, $MgSO_4$ was substituted with $MgCl_2$ (1 mM) and $NaHCO_3$ was omitted. The coverslips were placed in a quartz cuvette containing HEPES (10 mM)-buffered Krebs solution in a Perkin Elmer LS3B fluorimeter, at an angle of 30° to the incident light. The beam irradiated the monolayer without passing through the coverslip. The cuvette was maintained at 37°C and stirred continuously. The excitation monochromator was computer-driven between 340 nm and 380 nm every 3.8 s and fluorescence emission collected at 509 nm. Background auto-fluorescence was determined at the end of each experiment by permeabilizing the cells to divalent cations with ionomycin (1 μM) and adding Mn^{2+} (2 mM) to quench intracellular fura-2 fluorescence. Following subtraction of auto-fluorescence, the corrected fluorescence values obtained following excitation at 340 nm were divided by those obtained at 380 nm, giving a corrected ratio (R). $[Ca^{2+}]_i$ was then calculated by the computer from the equation of Grynkiewicz *et al.* (1985):

$$[Ca^{2+}]_i = K_d \times \frac{(R - R_{min})}{(R_{max} - R)} \cdot \frac{S_{f2}}{S_{b2}}$$

the maximal (R_{max}) and minimal (R_{min}) fluorescence ratios of fura-2 obtained in medium containing saturating concentrations of calcium and in calcium-free medium (with 40 mM EGTA) were determined to be 16.3 and 0.8 respectively. S_{f2} and S_{b2} are the fluorescence values obtained at 380 nm in the absence of calcium and the presence of saturating levels of calcium, respectively, and the S_{f2}/S_{b2} ratio was determined to be 7.3. The K_d for the fura-2-Ca^{2+} complex was assumed to be 225 nM at 37°C.

Drugs

Adenosine triphosphate (ATP; sodium salt); bradykinin triacetate, 8 bromo cyclic GMP, ethylene glycol *bis* (β-aminoethyl ethene N,N,N′,N′-tetraacetic acid (EGTA), (±)-isoprenaline hemisulphate, N^G-nitro-L-arginine (L-NNA), 4α-phorbol 12, 13-didecanoate (4α-PDD), 4β-phorbol 12-myristate 13-acetate (PMA), sodium nitroprusside and thrombin (bovine) were obtained from Sigma. Fura-2 penta-acetoxymethyl ester (fura-2/AM), forskolin, ionomycin and staurosporine were obtained from Novabiochem. All drugs were dissolved in distilled water except for fura-2/AM, ionomycin, forskolin and staurosporine which were dissolved in dimethyl sulphoxide (DMSO) and PMA and 4α-PDD which were dissolved in 100% ethanol.

Statistical analysis

Results are expressed as the mean ± s.e.mean and comparisons were made by Student's *t* test or by the Mann-Whitney test when there was unequal variance in samples. A probability of 0.05 or less was considered significant. The term '*n*' refers to the number of experiments conducted, usually on different batches of cells.

Results

Phorbol esters

In the presence of 1.8 mM extracellular calcium, the resting level of $[Ca^{2+}]_i$ in bovine aortic endothelial cells was 88 ± 5 nM ($n = 37$). The addition of thrombin (Figure 1), bradykinin or adenosine triphosphate (ATP) resulted in a biphasic elevation of $[Ca^{2+}]_i$ consisting of a rapid, transient component which peaked within 30 s, followed by a lower sustained component. Pretreatment with 4β-phorbol 12-myristate 13-acetate (PMA; 100 nM, 5 min), an activator of protein kinase C, had no effect on resting $[Ca^{2+}]_i$ (Table 1). It did, however, significantly inhibit the transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), low concentrations of bradykinin (1 nM) or ATP (0.3 μM and 3 μM), but had no effect on that induced by higher concentrations of bradykinin or ATP (Figure 1b, Figure 2, Table 1). 4α-Phorbol 12, 13-didecanoate (4α-PDD; 100 nM, 5 min), which does not activate protein kinase C, had no effect on resting $[Ca^{2+}]_i$ or the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (Figure 1c, Table 1).

Pretreatment with staurosporine (100 nM, 5 min) an inhibitor of protein kinase C, had no effect on resting $[Ca^{2+}]_i$ or the magnitude of the thrombin-induced initial transient elevation of $[Ca^{2+}]_i$ (Table 1), but did inhibit the ability of PMA

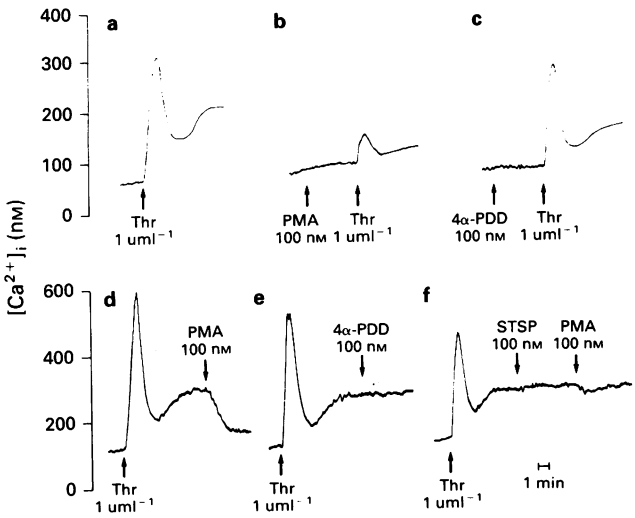

Figure 1 Individual traces showing (a) the biphasic elevation of $[Ca^{2+}]_i$ induced by thrombin (Thr; 1 u ml^{-1}) in bovine aortic endothelial cells. Also shown are the effects of pretreatment with (b) 4β-phorbol 12-myristate 13-acetate (PMA; 100 nM, 5 min) and (c) 4α-phorbol 12, 13 didecanoate (4α-PDD; 100 nM, 5 min) on the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin as well as the effects of (d) PMA (100 nM), (e) 4α-PDD (100 nM) and (f) PMA (100 nM) on the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin in the presence of staurosporine (STSP, 100 nM).

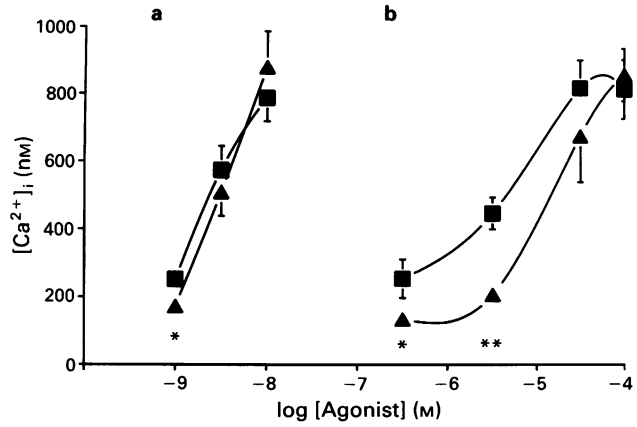

Figure 2 Concentration-effect curves showing the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by (a) bradykinin (BK) and (b) adenosine triphosphate (ATP) in bovine aortic endothelial cells in the absence (squares) and presence (triangles) of 4β-phorbol 12-myristate 13-acetate (PMA; 100 nM, 5 min). Individual points represent the mean of 4–10 observations and vertical bars represent the s.e.mean. Where error bars are not seen, they are encompassed within the symbols. Asterisks indicate a significant difference from the response obtained in the absence of PMA; $*P < 0.05$; $**P < 0.01$.

(100 nM) to attenuate this initial transient elevation of $[Ca^{2+}]_i$ (Table 1).

When PMA (100 nM) was added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}; Figure 1d), bradykinin (10 nM) and ATP (30 μM), falls in $[Ca^{2+}]_i$ of 89 ± 26 nM ($n = 4$), 35 ± 14 nM ($n = 4$) and 161 ± 29 nM ($n = 6$), respectively, were obtained. Addition of 4α-PDD (100 nM) during the thrombin-induced plateau phase of the increase in $[Ca^{2+}]_i$ had no effect on $[Ca^{2+}]_i$ ($n = 4$; Figure 1e).

Staurosporine (100 nM) added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}) and bradykinin (10 nM) resulted in increases in $[Ca^{2+}]_i$ of 10 ± 3 nM ($n = 13$) and 43 ± 12 nM ($n = 4$), respectively. In contrast, staurosporine (100 nM) induced a fall in $[Ca^{2+}]_i$ of 76 ± 14 nM ($n = 4$) when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by ATP (30 μM). Furthermore, staurosporine (100 nM), when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), reduced the PMA (100 nM)-induced fall in $[Ca^{2+}]_i$ to 19 ± 3 nM ($n = 4$; Figure 1f).

Table 1 Effects of phorbol esters and staurosporine on the increase in $[Ca^{2+}]_i$ stimulated by thrombin

Stimulus	*Pretreatment*	$[Ca^{2+}]_i$ (nM)		n
None (control)	None	88 ± 5		37
None	PMA (100 nM)	97 ± 9		10
None	STSP (100 nM)	102 ± 7		13
None	PMA (100 nM) +STSP (100 nM)	122 ± 4*		7
Thr (1 u ml^{-1}) (control)	None	333 ± 29	***	14
Thr (1 u ml^{-1})	PMA (100 nM)	191 ± 20	***	10
Thr (1 u ml^{-1})	STSP (100 nM)	274 ± 34	*	6
Thr (1 u ml^{-1})	PMA (100 nM) +STSP (100 nM)	274 ± 24	*	9
None (control)	None	76 ± 6		12
None	4α-PDD (100 nM)	87 ± 6		6
Thr (1 u ml^{-1})	None	237 ± 18		6
Thr (1 u ml^{-1})	4α-PDD (100 nM)	232 ± 26		6

Bovine aortic endothelial cells were pretreated with either 4β-phorbol 12-myristate 13-acetate (PMA; 100 nM, 5 min), 4α-phorbol 12, 13 didecanoate (4α-PDD; 100 nM, 5 min), staurosporine (STSP; 100 nM, 5 min) or with a combination of STSP (100 nM, 10 min) and PMA (100 nM; 5 min). The effects of these pretreatments on both resting $[Ca^{2+}]_i$ and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (Thr; 1 u ml^{-1}) are shown. Values are the mean ± s.e.mean. Asterisks denote a significant difference from the control or a difference between groups joined by a bracket; ($*P < 0.05$; $***P < 0.001$).

Forskolin and isoprenaline

Pretreatment with forskolin (10 μM, 5 min), an activator of adenylate cyclase, had no effect on resting $[Ca^{2+}]_i$ or on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), bradykinin (1 nM and 10 nM) or ATP (30 μM; Table 2).

Addition of a maximal concentration of forskolin (10 μM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}) resulted in a biphasic elevation of $[Ca^{2+}]_i$ (Figure 3b): the maximum increase was 66 ± 8 nM ($n = 9$) and this decayed to 41 ± 6 nM after 5 min. Forskolin (10 μM) induced similar biphasic elevations of $[Ca^{2+}]_i$ when added during the plateau phases induced by both bradykinin

Table 2 Effects of forskolin on the increases in $[Ca^{2+}]_i$ stimulated by thrombin, bradykinin and ATP

Stimulus	*Pretreatment*	$[Ca^{2+}]_i$ (nM)	n
None (control)	None	97 ± 5	51
None	For (10 μM)	110 ± 8	22
Thr (1 u ml^{-1})	None	347 ± 34*	11
Thr (1 u ml^{-1})	For (10 μM)	362 ± 52*	9
BK (1 nM)	None	298 ± 43*	6
BK (1 nM)	For (10 μM)	264 ± 26*	5
BK (10 nM)	None	809 ± 104*	8
BK (10 nM)	For (10 μM)	708 ± 122*	4
ATP (30 μM)	None	1038 ± 77*	4
ATP (30 μM)	For (10 μM)	1153 ± 112*	4

The effects of pretreatment of bovine aortic endothelial cells with forskolin (For; 10 μM, 5 min) on resting $[Ca^{2+}]_i$ and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (Thr; 1 u ml^{-1}), bradykinin (BK; 1 nM and 10 nM) and adenosine triphosphate (ATP; 30 μM) are shown. Values are the mean ± s.e.mean. Asterisks denote a significant difference from control; $*P < 0.001$.

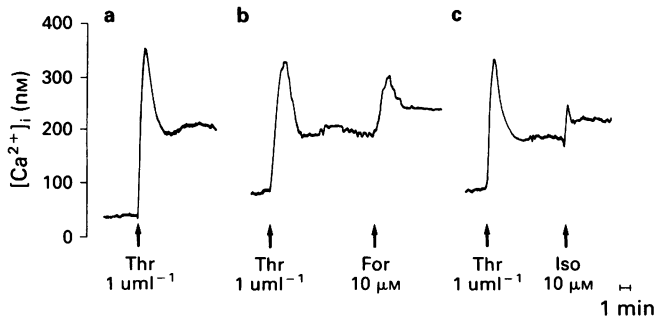

Figure 3 Individual traces showing (a) the biphasic elevation of $[Ca^{2+}]_i$ induced by thrombin (Thr; 1 u ml^{-1}) in bovine aortic endothelial cells and the effects of (b) forskolin (For; 10 μM) and (c) isoprenaline (Iso; 10 μM) when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin.

(10 nM) and ATP (3 μM): with bradykinin (10 nM) the maximum increase was 101 ± 8 nM ($n = 4$) which decayed to 35 ± 8 nM after 5 min, while with ATP (3 μM) the maximum increase was 74 ± 13 nM ($n = 7$) and this decayed to 26 ± 7 nM within 5 min. A maximal concentration of isoprenaline (10 μM) induced a biphasic elevation of $[Ca^{2+}]_i$ when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}; Figure 3c): the maximum increase was 61 ± 6 nM ($n = 6$) and this decayed to 25 ± 6 nM after 5 min. In the presence of nickel (4 mM), which blocks agonist-induced influx of calcium (Hallam *et al.*, 1988), the first component of the biphasic response induced by forskolin or isoprenaline was unaffected, but the second prolonged phase was abolished (data not shown).

In the presence of staurosporine (100 nM), the ability of forskolin (10 μM) to enhance the thrombin (1 u ml^{-1})-induced plateau phase of the increase in $[Ca^{2+}]_i$ was not significantly attenuated: the maximum increase was 53 ± 7 nM ($n = 8$) and this decayed to 33 ± 3 nM after 5 min.

Pretreatment with forskolin (10 μM, 5 min) did not affect the ability of PMA (100 nM) to induce a fall in the plateau phase of the thrombin (1 u ml^{-1})-induced increase in $[Ca^{2+}]_i$: the fall induced by PMA in untreated and forskolin-treated cells was 88 ± 8 nM ($n = 5$) and 114 ± 18 nM ($n = 5$), respectively.

8 Bromo cyclic GMP, sodium nitroprusside and N^G-nitro L-arginine

Pretreatment with 8 bromo cyclic GMP (30 μM, 5 min) had no effect on resting $[Ca^{2+}]_i$ or the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), bradykinin (10 nM) or ATP (3 μM) (Table 3). Furthermore, neither 8 bromo cyclic GMP (30 μM) nor sodium nitroprusside (1 μM) had any effect when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), bradykinin (10 nM) or ATP (30 μM) (data not shown, $n > 4$ for each).

Table 3 Effects of 8 bromo cyclic GMP on the increases in $[Ca^{2+}]_i$ stimulated by thrombin, bradykinin and ATP

Stimulus	*Pretreatment*	$[Ca^{2+}]_i$ (nM)	n
None (control)	None	99 ± 6	36
None	8BrcGMP (30 μM)	114 ± 8	15
Thr (1 u ml^{-1})	None	228 ± 16*	9
Thr (1 u ml^{-1})	8 BrcGMP (30 μM)	238 ± 28*	6
BK (10 nM)	None	909 ± 104*	8
BK (10 nM)	8 BrcGMP (30 μM)	952 ± 245*	4
ATP (3 μM)	None	745 ± 101*	4
ATP (3 μM)	8 BrcGMP (30 μM)	892 ± 99*	5

The effects of pretreatment of bovine aortic endothelial cells with 8 bromo cyclic GMP (8 BrcGMP; 30 μM, 5 min) on resting $[Ca^{2+}]_i$ and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (Thr; 1 u ml^{-1}), bradykinin (BK; 10 nM) and adenosine triphosphate (ATP; 3 μM) are shown. Values are mean ± s.e.mean. Asterisks denote a significant difference from control; *$P < 0.001$.

Table 4 Effects of N^G-nitro-L-arginine on the increases in $[Ca^{2+}]_i$ stimulated by thrombin, bradykinin and ATP

Stimulus	*Pretreatment*	$[Ca^{2+}]_i$ (nM)	n
None (control)	None	118 ± 5	36
None	L-NNA (50 μM)	124 ± 5	18
Thr (1 u ml^{-1})	None	299 ± 35*	6
Thr (1 u ml^{-1})	L-NNA (50 μM)	285 ± 39*	6
BK (1 nM)	None	515 ± 78*	6
BK (1 nM)	L-NNA (50 μM)	395 ± 16*	6
ATP (3 μM)	None	528 ± 61*	6
ATP (3 μM)	L-NNA (50 μM)	549 ± 113*	6

The effects of pretreatment of bovine aortic endothelial cells with N^G-nitro-L-arginine (L-NNA; 50 μM, 5 min) on resting $[Ca^{2+}]_i$ and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (Thr; 1 u ml^{-1}), bradykinin (BK; 1 nM) and adenosine triphosphate (ATP; 3 μM) are shown. Values are the mean ± s.e.mean. Asterisks denote a significant difference from control; *$P < 0.001$.

Pretreatment with the inhibitor of nitric oxide synthase, N^G-nitro L-arginine (L-NNA; 50 μM, 5 min) had no effect on resting $[Ca^{2+}]_i$ or the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}), bradykinin (1 nM) or ATP (3 μM) (Table 4). Furthermore, L-NNA (50 μM) had no effect when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}; $n = 3$), bradykinin (10 nM; $n = 4$) or ATP (30 μM; $n = 1$) (data not shown).

Discussion

Agonist-induced elevations of intracellular calcium ($[Ca^{2+}]_i$) in the vascular endothelium are biphasic, consisting of a large transient followed by a lower, more sustained, plateau phase (Hallam & Pearson, 1986; Colden-Stanfield *et al.*, 1987; Brock & Capasso, 1988; Buchan & Martin, 1991). The initial transient results from the release of calcium from intracellular stores and our novel observations show that the ability of the phorbol ester, PMA, to inhibit this component in bovine aortic endothelial cells depends upon the agonist studied. Specifically, PMA inhibited the ability of a maximally active concentration of thrombin or low concentrations of bradykinin and ATP to induce release of calcium from intracellular stores but, in contrast, had not effect on release induced by high concentrations of bradykinin or ATP. Therefore, in bovine aortic endothelial cells, the ability of PMA to inhibit agonist-induced release of calcium from intracellular stores depends not only on the agonist studied, but on the agonist concentration. Our findings in bovine aortic endothelial cells contrast with the ability of phorbol esters to inhibit intracellular release of calcium induced by maximal concentrations of thrombin, bradykinin and ATP in human umbilical vein endothelial cells (Brock & Capasso, 1988, Carter *et al.*, 1989) and highlights a species or site difference.

The actions of PMA probably result from activation of protein kinase C as they were attenuated by staurosporine, an inhibitor of this enzyme, and were not mimicked by the inactive phorbol ester, 4α-phorbol 12, 13-didecanoate. The transient elevation of $[Ca^{2+}]_i$ is likely to result from inositol 1,4,5-trisphosphate (IP_3)-induced release of calcium from intracellular stores (Berridge, 1984). The ability of phorbol esters to inhibit thrombin- and histamine-induced IP_3 production in

human umbilical vein endothelial cells (Brock & Capasso, 1988) is therefore likely to account for their ability to inhibit mobilisation of intracellular calcium. Our finding that PMA inhibits differentially release of intracellular calcium stimulated by a range of agonists may reflect variations in the extent to which receptor-mediated IP_3 production can be inhibited by protein kinase C. Alternatively, at low agonist concentrations, where IP_3 generation may be limited, other mechanisms may contribute to the inhibitory actions of PMA: for example, stimulation of protein kinase C may promote IP_3 breakdown (Connolly *et al.*, 1986) or raise the threshold for activation of the IP_3 receptor (Willems *et al.*, 1989).

The sustained plateau phase of the increase in $[Ca^{2+}]_i$ induced by agonists results from calcium influx (Hallam *et al.*, 1988) through receptor- but not voltage-operated channels (Hallam & Pearson, 1986; Colden-Stanfield *et al.*, 1987; Johns *et al.*, 1987). Our study shows that addition of PMA during the sustained plateau phase of the increase in $[Ca^{2+}]_i$ induced by maximal concentrations of thrombin, bradykinin and ATP in bovine aortic endothelial cells results in a fall in $[Ca^{2+}]_i$ with each of the agonists examined. Therefore, this component of the biphasic elevation of $[Ca^{2+}]_i$ is much more sensitive to the inhibitory actions of PMA than is the intracellular release component. As observed with the intracellular release of calcium, the sustained plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin was less sensitive to the inhibitory actions of PMA than those induced by thrombin or ATP. As EDRF production is calcium-dependent (Singer & Peach, 1982), the ability of phorbol esters to inhibit EDRF release (Weinheimer *et al.*, 1986; Lewis & Henderson, 1987; Cherry & Gillis, 1988; de Nucci *et al.*, 1988; Smith & Lang, 1990) is likely to result from their ability to inhibit endothelial calcium mobilisation. However, differential agonist sensitivity to the inhibitory actions of the phorbol esters on EDRF release appears to occur: for example, Smith & Lang (1990) have shown that bradykinin-induced production of EDRF by pig aortic endothelial cells is unaffected, whereas that induced by substance P or ATP is inhibited. This differential sensitivity may be explained by our finding that bradykinin-induced calcium mobilisation in bovine aortic endothelial cells is less sensitive to the inhibitory actions of phorbol esters than that induced by other agonists.

Our finding that staurosporine increases the plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin and thrombin may reflect the ability of these agonists to activate protein kinase C themselves and so inhibit their own actions by a negative feedback loop. In contrast, staurosporine induced a fall in the plateau phase of the increase in $[Ca^{2+}]_i$ induced by ATP. This may result from the ability of staurosporine to interact with the ATP binding sites of other proteins (Davis *et al.*, 1989).

Our study also provides novel evidence that cyclic AMP enhances agonist-induced mobilisation of calcium in bovine aortic endothelial cells. Forskolin, a direct activator of adenylate cyclase, and isoprenaline, a β-adrenoceptor agonist, each induced biphasic elevations of $[Ca^{2+}]_i$ when added during the plateau phase of agonist-induced increases in $[Ca^{2+}]_i$; this consisted of a large transient elevation followed by a lower more sustained plateau phase. Neither forskolin nor isoprenaline had any effect on basal $[Ca^{2+}]_i$. The initial transient and sustained phase of the increases in $[Ca^{2+}]_i$ are likely to result from intracellular calcium release and calcium influx, respectively, since only the latter was abolished in the presence of nickel, a calcium entry blocker (Hallam *et al.*, 1988). Therefore, in bovine aortic endothelial cells, cyclic AMP appears to enhance agonist-induced mobilisation of calcium from both intracellular and extracellular stores. The mechanisms underlying this augmentation are unclear but from our finding that pretreatment with forskolin did not attenuate the ability of PMA to inhibit the plateau phase of agonist-induced increases in $[Ca^{2+}]_i$, it is unlikely that cyclic AMP mediates its actions via inhibition of protein kinase C activity.

Although elevating intracellular cyclic AMP during the plateau phase of the agonist-induced increase in $[Ca^{2+}]_i$ clearly augments intracellular release of calcium, our finding in bovine aortic endothelial cells and those of Carson *et al.*, (1989) in human umbilical vein endothelial cells show that agents which elevate endothelial cyclic AMP content have no effect on the magnitude of the initial agonist-induced transient elevation of $[Ca^{2+}]_i$. This inability to observe an augmentation of the initial transient elevation of calcium is likely to result from the small magnitude of this augmentation relative to the magnitude of the discharge that normally occurs. In one study, elevating cyclic AMP content has been reported to augment slightly the ATP-induced initial transient elevation of $[Ca^{2+}]_i$ in bovine aortic endothelial cells (Brock *et al.*, 1988). All of these findings clearly conflict with those of Lückhoff *et al.* (1990) who reported that elevating cyclic AMP content inhibits endothelial calcium mobilisation. We have no explanation for this anomaly.

Elevation of endothelial cyclic GMP, by 8 bromo cyclic GMP (Evans *et al.*, 1988) or atrial natriuretic factor (Hogan *et al.*, 1989) is reported to inhibit release of EDRF. Agonists which induce EDRF release also elevate endothelial cyclic GMP content (Martin *et al.*, 1988) and, as a consequence, EDRF release may be regulated via a negative feedback mechanism involving cyclic GMP. Cyclic GMP has been demonstrated to inhibit IP_3 production in pig aortic endothelial cells (Lang & Lewis, 1991), but we found in bovine aortic endothelial cells that elevation of cyclic GMP content does not modulate either basal $[Ca^{2+}]_i$ or elevations of $[Ca^{2+}]_i$ induced by thrombin, bradykinin or ATP. As these agonists are known to increase endothelial cyclic GMP content via the generation of EDRF, we considered whether endogenously generated cyclic GMP was capable of inhibiting endothelial calcium mobilisation. N^G-nitro-L-arginine, an inhibitor of EDRF production (Moore *et al.*, 1990) did not, however, modulate either the magnitude of the agonist-induced initial transient elevation of $[Ca^{2+}]_i$ or the plateau phase of the increase in $[Ca^{2+}]_i$. These findings suggest cyclic GMP does not modulate calcium mobilisation, at least in bovine aortic endothelial cells.

In conclusion, our study suggests that stimulation of protein kinase C inhibits, elevation of cyclic AMP enhances and elevation of cyclic GMP has no effect on calcium mobilisation in bovine aortic endothelial cells.

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References

BERRIDGE, M.J. (1984). Inositol trisphosphate and diacylglycerol as second messengers. *Biochem. J.*, **220**, 345–360.

BROCK, T.A. & CAPASSO, E.A. (1988). Thrombin and histamine activate phospholipase C in human endothelial cells via a phorbol ester-sensitive pathway. *J. Cell. Physiol.*, **136**, 54–62.

BROCK, T.A., DENNIS, P.A., GRIENDLING, K.K., DIEHL, T.S. & DAVIS, P.F. (1988). GTPγS loading of endothelial cells stimulates phospholipase C and uncouples ATP receptors. *Am. J. Physiol.*, **255**, C667–C673.

BUCHAN, K.W. & MARTIN, W. (1991). Bradykinin induces elevation of cytosolic calcium through mobilisation of intracellular and extracellular pools in bovine aortic endothelial cells. *Br. J. Pharmacol.*, **102**, 35–40.

CARSON, M.R., SHASBY, S.S. & SHASBY, D.M. (1989). Histamine and inositol phosphate accumulation in endothelium: cAMP and a G-protein. *Am. J. Physiol.*, **257**, L259–L264.

CARTER, T.D., HALLAM, T.J. & PEARSON, J.D. (1989). Protein kinase C activation alters the sensitivity of agonist-stimulated endothelial

cell prostacyclin production to intracellular Ca^{2+}. *Biochem. J.*, **262,** 431–437.

CASTAGNA, M., TAKAI, Y., KAIBUCHI, K., SANO, X., KIKKAWA, U. & NISHIZUKA, Y. (1982). Direct activation of calcium-activated phospholipid-dependent protein kinase by tumour-promoting phorbol esters. *J. Biol. Chem.*, **257,** 7847-7851.

CHERRY, P.D. & GILLIS, N. (1988). Antagonism of acetylcholine-mediated relaxation of rabbit pulmonary arteries by phorbol myristate acetate. *J. Pharmacol. Exp. Ther.*, **247,** 542–546.

COLDEN-STANFIELD, M., SCHILLING, W.P., RITCHIE, A.K., ESKIN, S.G., NAVARRO, L.T. & KUNZE, D.L. (1987). Bradykinin-induced increases in cytosolic calcium and ionic currents in cultured bovine aortic endothelial cells. *Circ. Res.*, **61,** 632–640.

CONNOLLY, T.M., LAWING, W.J. & MAJERUS, P.W. (1986). Protein kinase C phosphorylates human platelet inositol trisphosphate 5'-phosphomonesterase, increasing the phosphatase activity. *Cell*, **46,** 951–958.

DAVIS, P.D., HALL, C.H., KEECH, E., LAWTON, G., NIXON, J.S., SEDGWICK, A.D., WADSWORTH, J., WESTMACOTT, D. & WILKINSON, S.E. (1989). Potent selective inhibitors of protein kinase C. *FEBS. Lett.*, **259,** 61–63.

DE NUCCI, G., GRYGLEWSKI, R.J., WARNER, T.D. & VANE, J.R. (1988). Receptor-mediated release of endothelium-derived relaxing factor and prostacylin from bovine aortic endothelial cells is coupled. *Proc. Natl. Acad. Sci. U.S.A.*, **85,** 2334–2338.

EVANS, H.G., SMITH, J.A. & LEWIS, M.J. (1988). Release of endothelium-derived relaxing factor is inhibited by 8-bromo-cyclic guanosine monophosphate. *J. Cardiovasc. Pharmacol.*, **12,** 672–677.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260,** 3440–3450.

HALLAM, T.J. & PEARSON, J.D. (1986). Exogenous ATP raises cytoplasmic free calcium in fura-2 loaded piglet aortic endothelial cells. *FEBS. Lett.*, **207,** 95–99.

HALLAM, T.J., JACOB, R. & MERRITT, J.E. (1988). Evidence that agonists stimulate bivalent cation influx into human endothelial cells. *Biochem. J.*, **255,** 179–184.

HOGAN, J.C., SMITH, J.A., RICHARDS, A.C. & LEWIS, M.J. (1989). Atrial natriuretic peptide inhibits the release of endothelium-derived relaxing factor from blood vessels of the rabbit. *Eur. J. Pharmacol.*, **165,** 129–134.

JOHNS, A., LATEGAN, T.W., LODGE, N.J., RYAN, U.S., VAN BREEMEN, C. & ADAMS, D.J. (1987). Calcium entry through receptor-operated channels in bovine pulmonary artery endothelial cells. *Tissue Cell*, **19,** 733–745.

LANG, D. & LEWIS, M.J. (1991). Inhibition of inositol 1,4,5-trisphosphate formation by cyclic GMP in cultured aortic endothelial cells of the pig. *Br. J. Pharmacol.*, **102,** 277–281.

LEITMAN, D.C. & MURAD, F. (1986). Comparison of binding and cyclic GMP accumulation by atrial natriuretic peptides in endothelial cells. *Biochim. Biophys. Acta*, **885,** 74–79.

LEWIS, M.J. & HENDERSON, A.H. (1987). A phorbol ester inhibits the release of endothelium-derived relaxing factor. *Eur. J. Pharmacol.*, **137,** 167–171.

LÜCKHOFF, A., MÜLSCH, A. & BUSSE, R. (1990). cAMP attenuates autacoid release from endothelial cells: relation to internal calcium. *Am. J. Physiol.*, **258,** H960-H966.

MARTIN, W., WHITE, D.G. & HENDERSON, A.H. (1988). Endothelium-derived relaxing factor and atriopeptin II elevate cyclic GMP levels in pig aortic endothelial cells. *Br. J. Pharmacol.*, **93,** 229–239.

MAYER, B., SCHMIDT, K., HUMBERT, P. & BÖHME, E. (1989). Biosynthesis of endothelium-derived relaxing factor: a cytosolic enzyme in porcine aortic endothelial cells converts L-arginine into an activator of soluble guanylate cyclase. *Biochem. Biophys. Res. Commun.*, **164,** 678–685.

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-N^G-nitro arginine, a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilation in vitro. *Br. J. Pharmacol.*, **99,** 408–412.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327,** 524–526.

SINGER, H.A. & PEACH, M.J. (1982). Calcium- and endothelial-mediated vascular smooth muscle relaxation in rabbit aorta. *Hypertension*, **4** (suppl II), 19–25.

SMITH, J.A. & LANG, D. (1990). 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. *Br. J. Pharmacol.*, **99,** 565–571.

WEINHEIMER, G., WAGNER, B. & OSWALD, H. (1986). Interference of phorbol esters with endothelium-dependent vascular smooth muscle relaxation. *Eur. J. Pharmacol.*, **130,** 319–322.

WILLEMS, P.H.G.M., VAN DEN BROEK, B.A.M., VAN OS, C.H. & DE PONT, J.J.H.H.M. (1989). Inhibition of inositol 1,4,5-trisphosphate-induced Ca^{2+} release in permeabilized pancreatic acinar cells by hormonal and phorbol ester pre-treatment. *J. Biol. Chem.*, **264,** 9762-9767.

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