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Modulation of barrier function of bovine aortic and pulmonary artery endothelial cells: dissociation from cytosolic calcium content

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1 Barrier function and cytosolic free calcium content $[Ca^{2+}]_i$ was measured in monolayers of bovine pulmonary artery endothelial cells (BPAEC) and bovine aortic endothelial cells (BAEC).

2 Thrombin (1 u ml^{-1}) increased albumin transfer across monolayers of BPAEC but not BAEC, yet induced biphasic increases in $[Ca^{2+}]_i$ in both endothelial cell types, consisting of a rapid, initial phasic component which decayed to a lower, more sustained plateau phase.

3 4β-Phorbol 12-myristate 13-acetate (PMA; 0.3–3000 nM) increased albumin transfer across monolayers of BPAEC and BAEC, but had no effect on basal levels of $[Ca^{2+}]_i$ in either endothelial cell type.

4 Treatment of BPAEC and BAEC with forskolin (30 μM), an activator of adenylate cyclase, had no effect on resting transfer of albumin, but inhibited that stimulated by PMA (600 nM). It also inhibited the thrombin (1 u ml^{-1})-induced increase in albumin transfer across monolayers of BPAEC, but enhanced the plateau phase of the associated increase in $[Ca^{2+}]_i$.

5 Treatment of BPAEC and BAEC with either atriopeptin II (100 nM), an activator of particulate guanylate cyclase, or 8 bromo cyclic GMP (30 μM) had no effect on resting or PMA (600 nM)-stimulated transfer of albumin. Both agents did, however, inhibit the thrombin (1 u ml^{-1})-induced increase in albumin transfer across monolayers of BPAEC, but had no effect on the associated increase in $[Ca^{2+}]_i$.

6 These data suggest a dissociation between the ability of agents that increase or decrease albumin transfer and their effects on $[Ca^{2+}]_i$. Consequently, activation of protein kinase C may be the major stimulus for trans-endothelial transfer of macromolecular solutes. Endothelial barrier function is enhanced by elevation of either cyclic AMP or cyclic GMP content. Cyclic AMP appears to act by inhibiting the actions of protein kinase C, while cyclic GMP may act to inhibit a key step proximal to activation of this enzyme.

Keywords: Endothelium; vascular permeability, cytosolic calcium; fura-2; cyclic AMP; cyclic GMP; protein kinase C; phorbol esters; thrombin; atrial natriuretic factor

Introduction

The vascular endothelium is the interface between the blood and the interstitium and fulfils the essential function of regulating the exchange of fluid, solutes and cells between these two compartments. This barrier function is subject to dynamic regulation, and is modulated by many factors *in vivo*. For example, increased transfer is stimulated by trans-endothelial endocytosis (Palade, 1960), or, following stimulation by inflammatory mediators such as histamine and bradykinin at post-capillary venules, by endothelial cell contraction and consequent formation of inter-endothelial gaps (Majno & Palade, 1961; Svensjö *et al.*, 1979). Conversely, barrier function can be enhanced by β-adrenoceptor agonists, a property utilised to limit vascular leakage induced by inflammatory mediators (Marciniak *et al.*, 1978; Svensjö *et al.*, 1979).

Important new insights into the mechanisms regulating inflammatory oedema have been gained by the development of endothelial cell culture systems. For example, histamine and thrombin have been shown to increase macromolecular transfer across endothelial monolayers obtained from human umbilical vein (Rotrosen & Gallin, 1986; Killackey *et al.*, 1986) and bovine pulmonary artery (Minnear *et al.*, 1989; Lum *et al.*, 1989).

The precise nature of the effector pathways linking receptor occupation to increases in macromolecular transfer are not, however, fully elucidated. It has been proposed that elevation of cytosolic calcium is the primary trigger on the basis that histamine-induced increases in macromolecular transfer and calcium mobilisation occur over a similar concentration-range (Rotrosen & Gallin, 1986). Furthermore, the calcium ionophore, A23187, induces macromolecular transfer across endothelial monolayers (Shasby *et al.*, 1985; Gudgeon & Martin, 1989) and thrombin-induced transfer is inhibited following inhibition of calcium influx by lanthanum, or buffering of intracellular calcium with quin-2 (Lum *et al.*, 1989). Calcium may not be the only trigger, however, since phorbol esters are known to induce endothelial contraction (Antonov *et al.*, 1986; Grigorian & Ryan, 1987) and macromolecular transfer (Gudgeon & Martin, 1989; Lynch *et al.*, 1990). These actions of phorbol esters are probably mediated by stimulation of protein kinase C since they are mimicked by synthetic diacylglycerols but not by inactive phorbol esters and are blocked by H7, an inhibitor of protein kinase C.

The ability of β-adrenoceptor agonists to enhance barrier function *in vivo* has also been demonstrated in endothelial monolayers cultured from human umbilical vein, bovine pulmonary artery and bovine and porcine aorta (Gudgeon & Martin, 1989; Minnear *et al.*, 1989; Martin & Luck, 1991; Langeler & Van Hinsbergh, 1991). Enhancement of barrier function probably results from elevation of endothelial cyclic AMP content since it is mimicked by other stimulants of adenylate cyclase (Stelzner *et al.*, 1989; Yamada *et al.*, 1990; Langeler & Van Hinsbergh, 1991), namely, forskolin, cholera toxin and iloprost, a stable analogue of prostacyclin. It is

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also enhanced by membrane permeant analogues of adenosine 3':5'-cyclic monophosphate (cyclic AMP), and by theophylline and isobutylmethylxanthine, which inhibit phosphodiesterase (Casnocha *et al.*, 1989; Gudgeon & Martin, 1989; Stelzner *et al.*, 1989; Yamada *et al.*, 1990). Enhancement of barrier function has also been reported following treatment with membrane permeant analogues of guanosine 3':5'-cyclic monophosphate (cyclic GMP) or elevation of endothelial cyclic GMP content by atrial natriuretic factors and sodium nitroprusside, which stimulate particulate and soluble guanylate cyclase, respectively (Yamada *et al.*, 1990; Lofton *et al.*, 1991).

The aim of this study was to determine if elevation of cyclic AMP or cyclic GMP content inhibits agonist-stimulated macromolecular transfer across monolayers of endothelial cells cultured from bovine pulmonary artery and aorta by blocking either calcium mobilisation or the stimulation of protein kinase C. A preliminary account of these findings has already been published (Buchan & Martin, 1991a).

Methods

Isolation of bovine aortic and pulmonary artery endothelial cells

Bovine aortic endothelial cells (BAEC) were isolated as described previously (Buchan & Martin, 1991b). Briefly, bovine thoracic aortae were removed shortly after death and flushed with sterile saline containing benzyl penicillin (100 units ml^{-1}) and streptomycin (100 $\mu g\ ml^{-1}$). Following ligation of the proximal end and cannulation of the distal end with an adaptor connected to a syringe, 60 ml of the same saline solution was infused into the lumen, and the aorta was transported back to the laboratory, where all subsequent procedures were carried out in a laminar flow hood. Following ligation of the intercostal arteries, 20 ml of a sterile collagenase solution (0.1%; Type II; Sigma: in Dulbecco's modification of Eagle's Medium (DMEM)) was infused into the lumen and the vessel incubated for 25 min at 37°C in an atmosphere of 5% CO_2 in air. The vessel was then gently massaged and the endothelial cells harvested by centrifugation (200 *g*; 4 min; 10°C) and resuspended in complete culture medium (DMEM containing 10% foetal calf serum, 10% newborn calf serum, 4 mM glutamine, 200 units ml^{-1} benzyl penicillin and 200 $\mu g\ ml^{-1}$ streptomycin). Following a second centrifugation, the cell pellet was resuspended in 50 ml of complete culture medium and seeded into 3 separate 80 cm^2 culture flasks (Gibco). The cells were grown in an atmosphere of 5% CO_2 in air, and typically reached confluence within 4–6 days.

Bovine pulmonary artery endothelial cells (BPAEC) were isolated by a similar method to that outlined above for BAEC. The pulmonary artery was removed, flushed with sterile saline and the proximal end and one of the two distal branches were ligated. The other distal branch was cannulated with an adaptor connected to a syringe and 20 ml of the sterile saline solution was infused into the vessel. At the laboratory, 10 ml of sterile collagenase solution (0.1% in DMEM) was infused into the lumen and the vessel incubated and the cells harvested as indicated above for BAEC. The cells were grown in culture similarly to BAEC except that thymidine (10 μM) was added to enhance growth (Laskey *et al.*, 1990).

Tissue culture materials were obtained from Gibco (Paisley, U.K.) unless otherwise indicated.

Measurement of endothelial barrier function

Upon reaching confluence, each flask of BAEC or BPAEC was washed with 2 × 20 ml of sterile saline and incubated with 10 ml of a solution of trypsin (0.05%)/ethylene diamine tetraacetic acid (EDTA; 0.02%) (Flow Laboratories) until the cells had detached, usually 2–4 min. The cell suspension was then added to 2 ml of newborn calf serum to inactivate the trypsin, and twice spun (200 *g*; 4 min; 10°C) followed by resuspension in 5 ml of complete medium for BAEC and complete medium containing thymidine (10 μM) for BPAEC; 100 μl of the cell suspensions was then added to each of 48 Transwell membrane assemblies (Costar; 6.5 mm diameter; 3 μm pore size). These were then placed in 24 well plates, with each well containing 1 ml of complete medium for BAEC or complete medium containing thymidine (10 μM) for BPAEC, and incubated for a further 2–4 days.

For experimentation, membrane assemblies with cells attached were washed twice by immersion in Krebs solution containing (mM): NaCl 118, KCl 4.8, $CaCl_2$ 1.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 2.4, HEPES (N-2-hydroxyethylpiperazine-N'-2 ethanesulphonic acid) 5 at 37°C at pH 7.4, and transferred to 24 well plates. Thereafter, 600 μl of the same Krebs solution was placed in each of the wells which formed the lower chamber and 100 μl of Krebs containing trypan blue-labelled albumin (4%) was placed above the endothelial monolayer. These volumes were chosen so as to avoid creation of a hydrostatic gradient across the monolayer. Drugs were then added to the top and bottom chambers and the plates placed on an orbital shaker and incubated under an atmosphere of air at 37°C. Any monolayers demonstrating visible leakage within the first 5 min were discarded and those remaining (97%) were incubated for 90 min. At the end of this time, a 100 μl aliquot was removed from each of the lower chambers and transfer of trypan blue-labelled albumin across the monolayers quantified by measuring optical density at 590 nm. In the Results, the transfer of albumin is expressed as a percentage of that which would have been achieved at equilibrium.

The trypan blue-labelled albumin complex was prepared by adding trypan blue (180 mg) and bovine serum albumin (4 g; fraction V; Sigma) to 100 ml of Krebs solution. Precipitation with trichloroacetic acid (6%) showed that the trypan blue was >99.8% albumin bound.

Measurement of intracellular free calcium ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured as previously described (Buchan & Martin, 1991b). Briefly, monolayers of first passage BAEC and BPAEC grown on glass coverslips were incubated for 45 min at 37°C with the penta-acetoxymethyl ester form of fura-2 (2 μM) in HEPES (20 mM)-buffered DMEM (Northumbria Biologicals) containing 1% bovine serum albumin (fraction V; Sigma). A coverslip containing fura-2 loaded cells was then transferred to HEPES (10 mM)-buffered Krebs solution, identical to that used for permeability studies except that KH_2PO_4 was omitted, for 20 min at room temperature to maximize conversion to the calcium-sensitive acid form of fura-2. The coverslip was then suspended across the diagonal of a quartz cuvette containing HEPES (10 mM)-buffered Krebs solution in a Perkin Elmer LS3B fluorimeter and maintained at 37°C with continuous stirring. The beam irradiated the cells without passing through the coverslip. The excitation monochromator was computer-driven between 340 and 380 nm every 3.8 s and fluorescence emission was 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 by the equation of Grynkiewicz *et al.* (1985):

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

The maximal (R_{max}) and minimal (R_{min}) fluorescence ratios were determined to be 16.3 and 0.8, respectively. S_f and S_{b2} are the fluorescence values obtained following excitation at 380 nm in the absence of calcium and the presence of saturating levels of calcium, respectively, and the ratio of these two values was calculated to be 7.3. The K_d for the fura-2-calcium complex was assumed to be 225 nM at 37°C.

Drugs

Atriopeptin II, 8 bromo cyclic 3':5' guanosine monophosphate (8 bromo cyclic GMP), 4α-phorbol 12,13-didecanoate (4α-PDD), 4β-phorbol 12-myristate 13-acetate (PMA) and thrombin (bovine) were obtained from Sigma, Poole, Dorset. Forskolin, fura-2 penta-acetoxymethyl ester and ionomycin were obtained from Novabiochem, Cambridge, U.K. Solutions of drugs were made in distilled water except for forskolin, fura-2-acetoxymethyl ester and ionomycin which were dissolved in dimethylsulphoxide 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 the Mann-Whitney test when there was unequal variance in samples. A probability of 0.05 or less was considered significant.

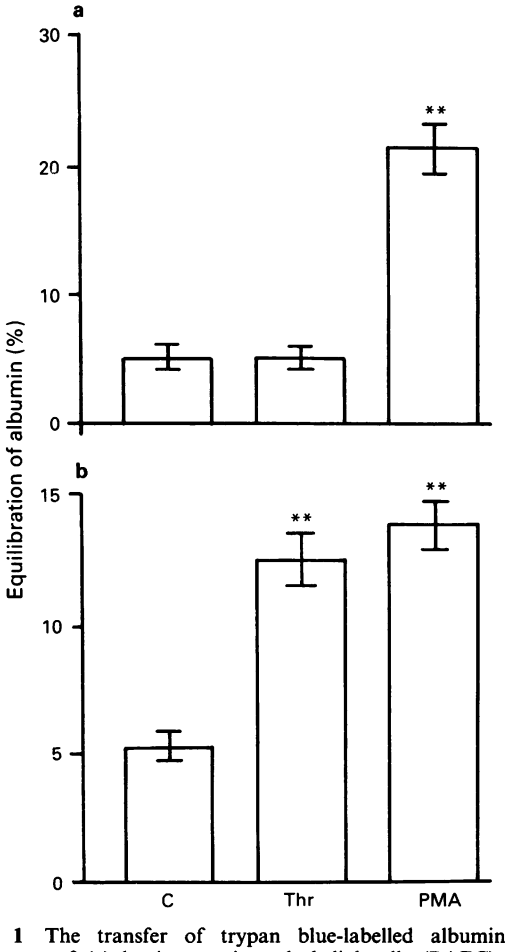

Figure 1 The transfer of trypan blue-labelled albumin across monolayers of (a) bovine aortic endothelial cells (BAEC) and (b) bovine pulmonary artery endothelial cells (BPAEC) during a 90 min incubation period in the absence of drugs (C) and following stimulation with thrombin (Thr; 1 u ml^{-1}) or 4β-phorbol 12-myristate 13-acetate (PMA; 600 nM). Values given are means and vertical bars indicate the s.e.mean of 6 observations. $^{**}P<0.01$ indicates a significant difference from control (C).

Results

Effects of thrombin and a phorbol ester on endothelial barrier function

Resting transfer of trypan blue-labelled albumin across monolayers of BPAEC and BAEC in the 90 min incubation period was typically 2–10% (Figures 1–5). Treatment with thrombin (1 u ml^{-1}) during the 90 min period increased albumin transfer across monolayers of BPAEC, but not BAEC, whereas 4β-phorbol 12-myristate 13-acetate (PMA; 600 nM) increased albumin transfer across monolayers of both endothelial cell types (Figure 1). The ability of PMA to

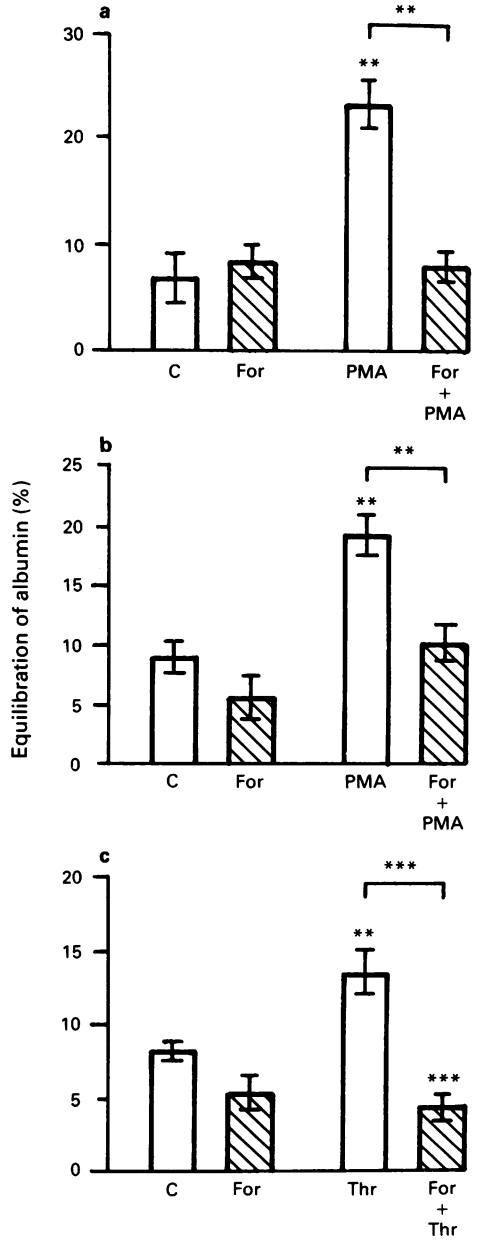

Figure 2 The effects of forskolin (For; 30 μM) on resting transfer of trypan blue-labelled albumin and on transfer stimulated by 4β-phorbol 12-myristate 13-acetate (PMA; 600 nM) across monolayers of (a) bovine aortic endothelial cells (BAEC) and (b and c) bovine pulmonary artery endothelial cells (BPAEC) during a 90 min incubation. The effect of forskolin on albumin transfer stimulated by thrombin (Thr; 1 u ml^{-1}) across monolayers of bovine pulmonary artery endothelial cells is also shown. Values given are means and vertical bars indicate the s.e.mean of 6–12 observations. $^{**}P<0.01$; $^{***}P<0.001$ indicates a significant difference from control (C) or between groups joined by a bracket.

increase albumin transfer was not shared with the inactive phorbol ester, 4α-phorbol 12,13-didecanoate (600 nM; data not shown).

Effects of cyclic nucleotides on endothelial barrier function

Forskolin (30 μM), which activates the catalytic subunit of adenylate cyclase (Seaman & Daly, 1981), had no effect on resting transfer of albumin across monolayers of BPAEC or BAEC, inhibited the increase in albumin transfer stimulated by thrombin (1 u ml^{-1}) and PMA (600 nM) across BPAEC, and inhibited the increase stimulated by PMA (600 nM) across BAEC (Figure 2).

Neither atriopeptin II (100 nM), an activator of particulate guanylate cyclase, nor 8 bromo cyclic GMP (30 μM), a membrane permeant analogue of cyclic GMP, had any effect on resting or PMA (600 nM)-stimulated transfer of albumin across monolayers of BAEC (Figure 3) or BPAEC (Figure 4), but both inhibited that stimulated by thrombin (1 u ml^{-1}) across monolayers of BPAEC (Figures 3 and 4).

Calcium mobilization in endothelial cells

In monolayers of BPAEC and BAEC the basal level of $[Ca^{2+}]_i$ was 106 ± 4 nM ($n = 101$) and 98 ± 4 nM ($n = 127$), respectively. Thrombin (1 u ml^{-1}) induced a biphasic eleva-

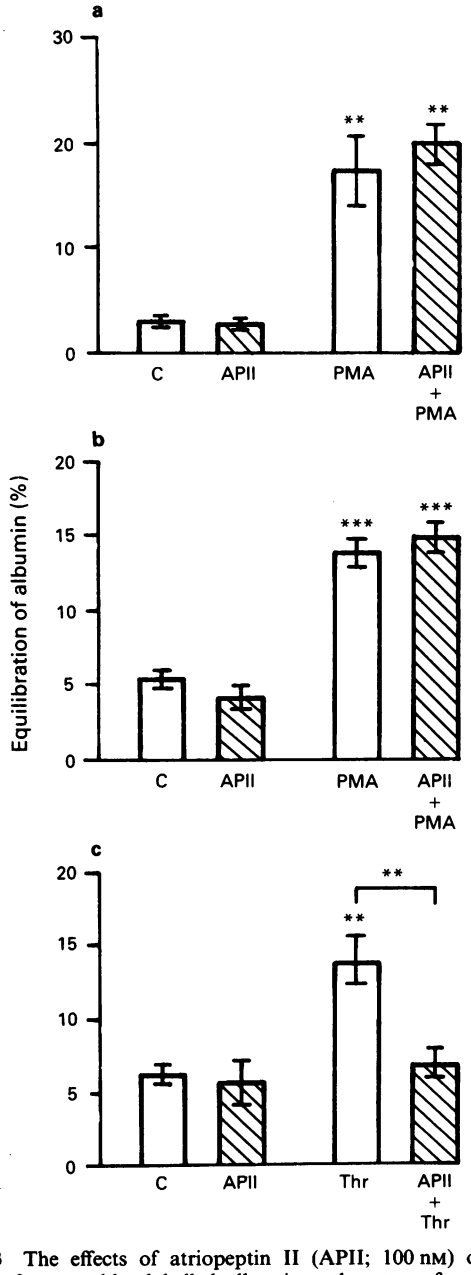

Figure 3 The effects of atriopeptin II (APII; 100 nM) on resting transfer of trypan blue-labelled albumin and on transfer stimulated by 4β-phorbol 12-myristate 13-acetate (PMA; 600 nM) across monolayers of (a) bovine aortic endothelial cells (BAEC) and (b and c) bovine pulmonary artery endothelial cells (BPAEC) during a 90 min incubation. The effect of atriopeptin II on albumin transfer stimulated by thrombin (Thr; 1 u ml^{-1}) across monolayers of bovine pulmonary artery endothelial cells is also shown. Values given are means and vertical bars indicate the s.e.mean of 6 observations. $^{**}P < 0.01$; $^{***}P < 0.001$ indicates a significant difference from control (C) or between groups joined by a bracket.

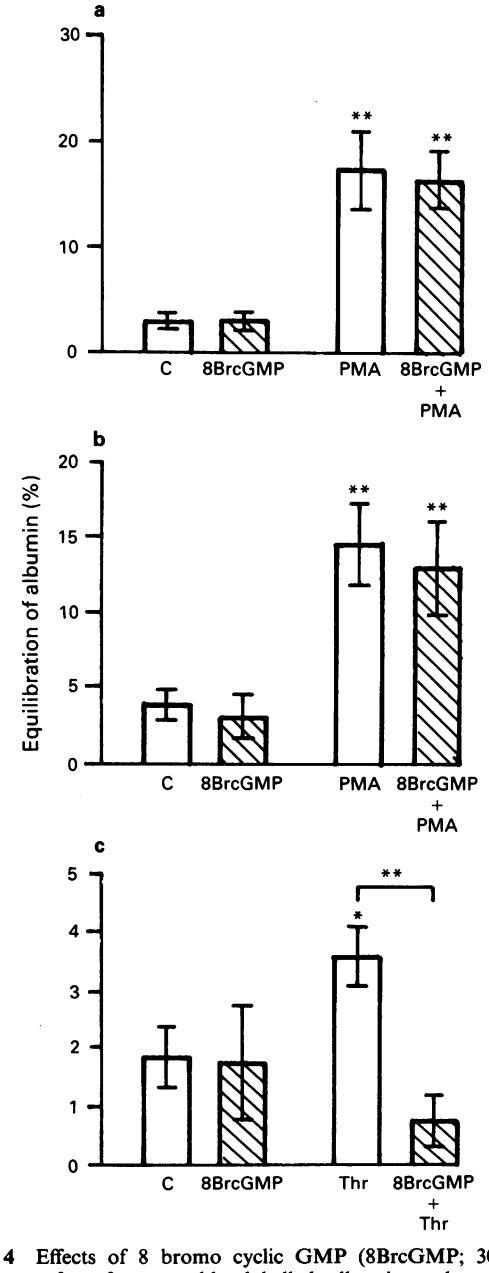

Figure 4 Effects of 8 bromo cyclic GMP (8BrcGMP; 30 μM) on resting transfer of trypan blue-labelled albumin and on transfer stimulated by 4β-phorbol 12-myristate 13-acetate (PMA; 600 nM) across monolayers of (a) bovine aortic endothelial cells (BAEC) and (b and c) bovine pulmonary artery endothelial cells (BPAEC) during a 90 min incubation period. The effect of 8 bromo cyclic GMP on albumin transfer stimulated by thrombin (Thr; 1 u ml^{-1}) across monolayers of bovine pulmonary artery endothelial cells is also shown. Values given are means and vertical bars indicate the s.e.mean of 6 observations. $^{*}P < 0.05$; $^{**}P < 0.01$ indicates a significant difference from control (C) or between groups joined by a bracket.

tion of $[Ca^{2+}]_i$ in both cell types consisting of a large initial peak at around 30 s which then fell to a more sustained plateau within 5 min (Figure 5); the peak and plateau levels were 350 ± 51 nM and 239 ± 21 nM ($n = 9$), respectively, for BPAEC, and 291 ± 30 nM and 180 ± 13 nM ($n = 20$), respectively, for BAEC. In contrast, PMA (1–1000 nM) had no effect on the basal level of $[Ca^{2+}]_i$ in BPAEC or BAEC.

Effects of cyclic nucleotides on thrombin-induced calcium mobilisation in BPAEC

Pretreatment of BPAEC with forskolin (30 μM; 5 min) had no effect on basal levels of $[Ca^{2+}]_i$ or on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}) (data not shown). Addition of forskolin (30 μM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}) did, however, lead to a further rapid increase in $[Ca^{2+}]_i$ of 67 ± 7 nM ($n = 6$), which remained stable for at least 5 min (Figure 5).

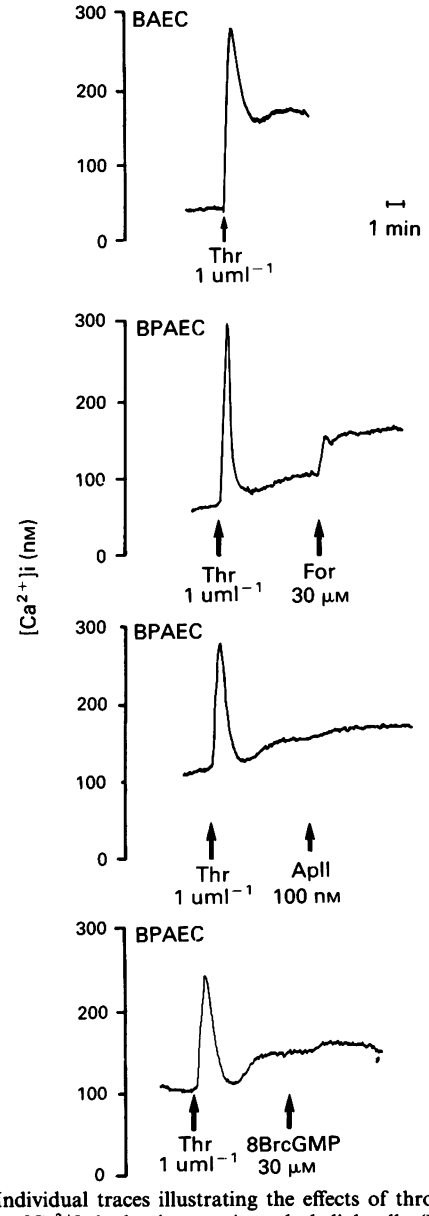

Figure 5 Individual traces illustrating the effects of thrombin (Thr; 1 u ml^{-1}) on $[Ca^{2+}]_i$ in bovine aortic endothelial cells (BAEC) and bovine pulmonary artery endothelial cells (BPAEC). The effects of adding forskolin (For; 30 μM), atriopeptin II (APII; 100 nM) or 8 bromo cyclic GMP (8BrcGMP; 30 μM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (Thr; 1 u ml^{-1}) in bovine pulmonary artery endothelial cells are also shown. Each trace is representative of at least 5 separate observations.

Pretreatment of BPAEC for 5 min with either atriopeptin II (100 nM) or 8 bromo cyclic GMP (30 μM) had no effect on basal levels of $[Ca^{2+}]_i$ or on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}) (data not shown). Atriopeptin II (100 nM) and 8 bromo cyclic GMP (30 μM) were both also without effect when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}) (Figure 5).

Discussion

The major new finding in this study is that changes in endothelial barrier function can be dissociated from changes in levels of cytosolic calcium ($[Ca^{2+}]_i$). Evidence for this comes from the observation that thrombin stimulates calcium mobilization in both BPAEC and BAEC, yet increases albumin transfer across monolayers of only BPAEC. Furthermore, the phorbol ester, PMA, had no effect on basal levels of $[Ca^{2+}]_i$ in BPAEC or BAEC, yet stimulated albumin transfer across monolayers of both endothelial cell types. It is likely that the ability of phorbol esters to stimulate transendothelial transfer of macromolecular solutes results from activation of protein kinase C, since this action is not shared with phorbol esters which do not activate this enzyme, but is mimicked by synthetic diacylglycerols, and blocked by H7 (Gudgeon & Martin, 1989; Lynch *et al.*, 1990). It is possible, therefore, that activation of protein kinase C represents the major pathway by which inflammatory mediators induce plasma leakage.

At present, it is not clear how activation of protein kinase C inhibits endothelial barrier function, but it is likely to result from endothelial contraction (Antonov *et al.*, 1986; Grigorian & Ryan, 1987) and formation of inter-endothelial gaps. In vascular smooth muscle, activation of protein kinase C induces contraction by increasing the sensitivity of the contractile proteins to calcium (Itoh *et al.*, 1988), and it is possible that a similar mechanism operates in the endothelium. Alternatively, activation of protein kinase C may be responsible for the loss of peripheral bands of F-actin and the resultant disruption of cell-cell contacts (Garcia *et al.*, 1986; Minnear *et al.*, 1989) in a manner similar to that described for a kidney epithelial cell line (Schliva *et al.*, 1984).

Our finding that forskolin, which directly activates the catalytic subunit of adenylate cyclase (Seaman & Daly, 1981), inhibits increases in albumin transfer stimulated by PMA across monolayers of BPAEC and BAEC as well as that stimulated by thrombin across BPAEC is consistent with previous reports of elevated levels of cyclic AMP enhancing endothelial barrier function *in vivo* and *in vitro* (Marciniak *et al.*, 1978; Svensjö *et al.*, 1979; Killackey *et al.*, 1986; Minnear *et al.*, 1989; Gudgeon & Martin, 1989; Carson *et al.*, 1989; Langeler & Van Hinsbergh, 1991). Furthermore, our observation that atriopeptin II, a stimulant of endothelial particulate guanylate cyclase (Schini *et al.*, 1988; Martin *et al.*, 1988), and 8 bromo cyclic GMP inhibit thrombin-stimulated transfer of albumin across monolayers of BPAEC is also consistent with the ability of cyclic GMP to enhance barrier function (Yamada *et al.*, 1990; Lofton *et al.*, 1991).

The mechanisms by which elevations of cyclic AMP or cyclic GMP enhance endothelial barrier function are not clear, but are unlikely to result from inhibition of calcium mobilization, since atriopeptin II and 8 bromo cyclic GMP had no effect on thrombin-induced calcium mobilization and forskolin actually augmented this. This proposal is supported by the observation that elevation of cyclic AMP content inhibits histamine-induced transfer of albumin across monolayers of human umbilical vein endothelial cells but does not block the associated increase in $[Ca^{2+}]_i$ (Carson *et al.*, 1989). It is possible, however, that inhibition of barrier function is exerted through blockade of protein kinase C, since elevation of cyclic AMP content inhibits albumin transfer stimulated

by both thrombin and PMA. In contrast, elevation of cyclic GMP content inhibits albumin transfer stimulated by thrombin, but not PMA, suggesting a different mechanism of action from cyclic AMP. One possible explanation for this is that cyclic GMP may block the ability of thrombin to stimulate protein kinase C, and consistent with this is the ability of cyclic GMP to inhibit production of inositol (1,4,5) trisphosphate in porcine aortic endothelium (Lang & Lewis, 1991). We do not favour this explanation, however, since 8 bromo cyclic GMP and atriopeptin II had no effect on thrombin-induced mobilization of calcium in BPAEC, which presumably involves hydrolysis of phosphatidylinositol-4,5-bisphosphate (Jaffe *et al.*, 1987). It is possible, however, that albumin transfer is stimulated by activation of protein kinase C resulting from hydrolysis of phosphatidylcholine and not phosphatidylinositol-4,5-bisphosphate. This is suggested since diacylglycerol production from the former source is better sustained (Billah & Anthes, 1990) and would be more consistent with the relatively long time course (90 min) required to observe albumin transfer. If elevations of $[Ca^{2+}]_i$ and sustained production of diacylglycerol are subject to differential regulation in the endothelial cell, as in the neutrophil (Cronstein *et al.*, 1988; Cronstein & Haines, 1992), then it might be possible to explain our ability to block the increase in albumin transfer but not $[Ca^{2+}]_i$ stimulated by thrombin in BPAEC. Direct assessment of the differential effects of elevated levels of cyclic GMP on hydrolysis of phosphatidylinositol-4,5-bisphosphate and phosphatidylcholine will be required to test this hypothesis. A related problem that is also difficult to explain at present is the ability of thrombin to stimulate albumin transfer across monolayers of BPAEC but not BAEC. On the basis of the above scheme, it is possible that following stimulation with thrombin, only BPAEC generates diacylglycerol from phosphatidylcholine in the sustained manner necessary to stimulate albumin transfer. Alternatively, the two cell types could generate diacylglycerols, or contain different forms of protein kinase C (Thompson *et al.*, 1991).

In conclusion, the results of this study show that changes in endothelial barrier function can be dissociated from alterations in cytosolic calcium content and suggest protein kinase C as the primary regulator.

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