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Differential control and calcium-dependence of production of endothelium-derived relaxing factor and prostacyclin by pig aortic endothelial cells

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1 Production of endothelium-derived relaxing factor (EDRF) by primary cultures of pig aortic endothelial cells was assessed indirectly by measuring endothelial cyclic GMP content, and prostacyclin production was measured by radioimmunoassay of 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$).

2 The resting level of cyclic GMP fell significantly following removal of extracellular calcium (1 mM EGTA present), but elevations of cyclic GMP content induced by sodium azide (10 μM) or atriopeptin II (10 nM) were similar in the absence and presence of extracellular calcium.

3 Haemoglobin (10 μM) reduced the resting level of cyclic GMP in the presence, but not the absence of extracellular calcium. M&B 22,948 (100 μM), superoxide dismutase (30 u ml^{-1}), bradykinin (0.1 μM), ATP (10 μM) and ionophore A23187 (0.1 μM) each induced an increase in endothelial cyclic GMP content that was reduced in the absence of extracellular calcium.

4 In cascade bioassay experiments using endothelial cells on microcarrier beads and perfused in columns, continuous infusion of bradykinin (0.1 μM) induced release of EDRF, assayed on rabbit aortic rings, that was maximal after 2 min and still detectable up to about 16 min.

5 In the presence of extracellular calcium, the time course of bradykinin (0.1 μM)-stimulated production of EDRF, assessed as endothelial cyclic GMP content was maximal within 1 min, declined thereafter, but was still significant after 30 min. Production of 6-keto $PGF_{1\alpha}$, measured simultaneously, rose rapidly but was complete within 3 min.

6 In the absence of extracellular calcium the resting endothelial content of cyclic GMP fell, but resting production of 6-keto $PGF_{1\alpha}$ was unaffected. The rise in cyclic GMP content stimulated by bradykinin (0.1 μM) was lower than when calcium was present and was complete within 3 min, but the magnitude and time course of bradykinin (0.1 μM)-stimulated production of 6-keto $PGF_{1\alpha}$ was unaffected.

7 In the presence of TMB-8 (100 μM) resting endothelial content of cyclic GMP rose slightly, but production of 6-keto $PGF_{1\alpha}$ fell. The bradykinin (0.1 μM)-stimulated increase in cyclic GMP content was augmented, but the stimulation of 6-keto $PGF_{1\alpha}$ production was blocked. Results from cascade bioassay experiments confirmed that TMB-8 (100 μM) did not inhibit bradykinin-induced production of EDRF.

8 The data suggest that resting production of EDRF but not prostacyclin is dependent upon the presence of extracellular calcium. Bradykinin-stimulated production of EDRF is sustained and requires the presence of extracellular calcium, but stimulated production of prostacyclin is transient and may result from discharge of an intracellular pool of calcium.

9 The vascular endothelial cell appears therefore to control differentially production of EDRF and prostacyclin.

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Introduction

Endothelium-derived relaxing factor (EDRF), recently identified as nitric oxide (Palmer *et al.*, 1987; Ignarro *et al.*, 1987; Furchgott, 1988), and prostacyclin (Moncada *et al.*, 1976), are two powerful vasodilator and anti-aggregatory agents released by the vascular endothelium.

Release of EDRF and prostacyclin occurs spontaneously (Gordon & Martin, 1983; Griffith *et al.*, 1984; Martin *et al.*, 1985), or in response to chemical (Gimbrone & Alexander, 1975; Furchgott & Zawadzki, 1980) or physical stimuli (Grabowski *et al.*, 1985; Holtz *et al.*, 1984). It appears that calcium is involved in the release of both EDRF (Singer & Peach, 1982; Long & Stone, 1985; Griffith *et al.*, 1986) and prostacyclin (Weksler *et al.*, 1978; Seid *et al.*, 1983). Calcium is required for activation of phospholipase A_2 (Hong & Deykin, 1982) which liberates arachidonate from membrane phospholipid, but the calcium-dependent step in EDRF release has not been identified.

Differences in the mechanisms for production of EDRF and prostacyclin have been identified. For example, endothelium-dependent vasodilatation, resulting from agonist-induced stimulation, is well sustained (Furchgott & Zawadzki, 1980), but agonist-induced production of prostacyclin is transient, being complete within 1–3 min (Gordon & Martin, 1983; Pearson *et al.*, 1983). In addition, basal and stimulated production of EDRF both require the continuous presence of extracellular calcium (Long & Stone, 1985; Griffith *et al.*, 1986), while prostacyclin release can be elicited in its absence (Hallam & Needham, 1987). Studies with TMB-8, an inhibitor of intracellular calcium release suggest that prostacyclin production arises mainly as a consequence of mobilisation of intracellular calcium (Seid *et al.*, 1983).

We now present results of experiments in which the extracellular requirement for calcium and sensitivity to TMB-8 of EDRF release and prostacyclin production are compared.

Methods

Endothelial cell culture

Pig aortic endothelial cells were isolated and grown in culture as previously described (Martin *et al.*, 1988).

For monolayer studies the cells were seeded into Linbro plates each containing 6 wells (9.6 cm^2) and grown to confluence in an incubator at 37°C under an atmosphere of 5% CO_2 in air.

For microcarrier experiments cells were seeded onto 3 ml of Biosilon microcarrier beads (Nunc, 200 μm diameter) in a sterile siliconised Techne microcarrier flask, and grown at 37°C under an atmosphere of 5% CO_2 in air. The beads were stirred at 30 r.p.m. for 2.5 min every 30 min for 3–5 days, during which time the cells grew to confluence, which was confirmed by microscopic examination after staining a sample of the cells with Methyl Violet (0.1%, B.D.H.).

Measurement of cyclic GMP and prostacyclin

Before experimentation the tissue culture medium was removed by aspiration and the cells washed with 2 × 2 ml of Krebs solution containing (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11 and EDTA 0.03, and incubated in 2 ml of Krebs solution at 37°C under an atmosphere of 5% CO_2 in air for at least 90 min. After this time the Krebs solution was removed and replaced either with normal Krebs solution or Krebs in which the calcium had been omitted and to which ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetra acetic acid (EGTA, 1 mM) had been added. Drugs were added to the cells at the concentrations and times indicated in the Results. At the appropriate time the Krebs solution was removed and retained for analysis of prostacyclin, and the cells immediately extracted in 0.5 ml of ice-cold trichloroacetic acid (TCA, 6%), scraped off the multiwell plates and harvested. Cells remaining on the plates were recovered by extracting with a second 0.5 ml of TCA and this was combined with the first. The extracts were spun (13,000 r.p.m., 3 min, room temperature) and the pellet and supernatant separated. The DNA content of the pellet was measured by the fluorescence technique of Kissane & Robins (1958). The supernatant was neutralised and the guanosine 3' : 5'-cyclic monophosphate (cyclic GMP) content measured by radioimmunoassay as previously described (Martin *et al.*, 1985). Cyclic GMP content is expressed as fmol μg^{-1} DNA.

The prostacyclin content of the Krebs solution was determined by radioimmunoassay (measured as 6-keto prostaglandin $F_{1\alpha}$) using an antiserum kindly supplied by Dr A.C. Newby. The cross-reactivity of the antiserum at 50% displacement with prostaglandin E_2 (PGE_2), PGE_1, and $PGF_{2\alpha}$ was 5%, 1.3%, and 1.4%, respectively. Prostacyclin production is expressed as pg 6-keto $PGF_{1\alpha}$ μg^{-1} DNA.

Cascade bioassay

EDRF release from pig aortic endothelial cells grown on microcarrier beads and perfused in

Table 1 Effects of sodium azide and atriopeptin II on the cyclic GMP content of pig aortic endothelial cells in the presence and absence of extracellular calcium

Stimulus	*Ca^{2+} content of Krebs* (mM)	*Cyclic GMP* (fmol μg^{-1} DNA)	n
None (control)	2.5	14.2 ± 4.0	6
None	None	4.8 ± 0.8*	6
Azide	2.5	57.2 ± 13.3*	6
Azide	None	44.0 ± 8.8*	6
None (control)	2.5	15.0 ± 1.6	6
None	None	3.9 ± 0.5***	6
APII	2.5	35.0 ± 3.3***	6
APII	None	34.2 ± 4.3**	6

The cyclic GMP content of pig aortic endothelial cells was determined following exposure to sodium azide (10 μM) for 1.5 min or atriopeptin II (APII, 10 nM) for 3 min in the presence or absence (1 mM EGTA added) of 2.5 mM calcium. Results are expressed as the mean ± s.e.mean. *$P < 0.05$; **$P < 0.005$; ***$P < 0.001$, denotes significant difference from control.

Table 2 Effects of haemoglobin, M&B 22,948 and superoxide dismutase on the cyclic GMP content of pig aortic endothelial cells in the presence and absence of extracellular calcium

Stimulus	*Ca^{2+} content of Krebs* (mM)	*Cyclic GMP* (fmol μg^{-1} DNA)	n
None (control)	2.5	17.8 ± 1.6	9
None	None	4.5 ± 0.4***	10
Hb	2.5	3.5 ± 0.5***	6
Hb	None	3.3 ± 0.5***	6
None (control)	2.5	15.4 ± 1.6	6
None	None	3.9 ± 0.5***	6
M&B	2.5	29.2 ± 4.7*	6
M&B	None	5.2 ± 0.08***	6
None (control)	2.5	25.1 ± 4.4	6
None	None	3.7 ± 1.0***	6
SOD	2.5	69.8 ± 14.8*	6
SOD	None	11.0 ± 1.9*	6

The cyclic GMP content of pig aortic endothelial cells was determined following exposure to haemoglobin (Hb, 10 μM) for 20 min, or to M&B 22,948 (M&B, 100 μM) or superoxide dismutase (SOD, 30 u ml^{-1}) for 3 min in the presence or absence (1 mM EGTA added) of 2.5 mM calcium. Results are expressed as the mean ± s.e.mean. *$P < 0.05$; ***$P < 0.001$, denotes significant difference from control.

columns was detected by bioassay using an endothelium-denuded ring of rabbit aorta. The preparation of endothelial cell columns was as previously described (Gordon & Martin, 1983). The perfusate, which contained flurbiprofen (10 μM, to inhibit cyclooxygenase) and superoxide dismutase (30 u ml^{-1}, to potentiate the actions of EDRF), was passed over an endothelium-denuded ring of rabbit aorta that had been suspended under 2 g resting tension and contracted sub-maximally with phenylephrine (0.1–10 μM). Tension was recorded isometrically by Ormed UF1 transducers and displayed on a Lectromed recorder.

Drugs

Adenosine triphosphate (ATP), atriopeptin II (rat synthetic), sodium azide, bradykinin triacetate, haemoglobin (bovine Type 1), ionophore A23187, phenylephrine, superoxide dismutase (bovine erythrocyte) and trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8) were obtained from Sigma. Sodium flurbiprofen was a generous gift from Dr R.V. Holland, Boots Pure Drug Co. and M&B 22,948 (2-O-propoxyphenyl-8-azapurin-6-one) was a generous gift from Dr J.E. Souness, May & Baker Ltd. All drugs were dissolved in twice-distilled water except for A23187 which was dissolved in ethanol, TMB-8 which was dissolved in 1% ethanol and M&B 22,948 which was dissolved in 10% triethanolamine.

Solutions of haemoglobin were reduced to the ferrous form with dithionite before use, as previously described (Martin *et al.*, 1985).

Statistical analysis

The resting content of cyclic GMP and basal production of prostacyclin varied in batches of cells. The validity of statistical comparisons was therefore enhanced by performing each experiment with its own internal controls. In the results, *n* represents the number of replicate dishes of cells used in experiments. Results are expressed as the mean ± s.e.mean and comparisons were made by means of Student's *t* test. A probability of 0.05 or less was considered significant.

Results

Calcium removal and cyclic GMP content

When incubated in normal Krebs solution (calcium 2.5 mM), the resting level of cyclic GMP in primary cultures of pig aortic endothelial cells was 20.9 ± 2.0 fmol μg^{-1} DNA ($n = 59$). Following 20 min incubation in nominally calcium-free Krebs

Table 3 Effects of bradykinin, ATP and ionophore A23187 on the cyclic GMP content of pig aortic endothelial cells in the presence and absence of extracellular calcium

Stimulus	*Ca^{2+} content of Krebs* (mM)	*Cyclic GMP* (fmol μg^{-1} DNA)	n
None (control)	2.5	9.1 ± 1.4	7
None	None	6.9 ± 2.3	5
Bk	2.5	86.4 ± 13.2***	8
Bk	None	24.2 ± 5.7*	6
None (control)	2.5	12.3 ± 1.9	6
None	None	9.8 ± 2.6	6
ATP	2.5	41.0 ± 6.5**	6
ATP	None	19.8 ± 2.5*	6
None (control)	2.5	31.6 ± 8.3	6
None	None	4.2 ± 0.9*	6
A23187	2.5	288.2 ± 23.2***	5
A23187	None	106.4 ± 17.8*	5

The cyclic GMP content of pig aortic endothelial cells was determined following exposure to bradykinin (Bk, 0.1 μM) for 1.5 min or to ATP (10 μM) or ionophore A23187 (0.1 μM) for 3 min in the presence or absence (1 mM EGTA added) of 2.5 mM calcium. Results are expressed as the mean ± s.e. mean. $*P < 0.05$; $**P < 0.005$; $***P < 0.001$, denotes significant difference from control.

(EGTA 1 mM present) the resting level of cyclic GMP was significantly lower ($P < 0.001$) at 7.1 ± 1.0 fmol μg^{-1} DNA ($n = 46$).

Sodium azide (10 μM, 1.5 min) and atriopeptin II (10 nM, 3 min) each elevated the cyclic GMP content to levels that were similar in the presence and absence of extracellular calcium (Table 1).

Haemoglobin, M&B 22,948 and superoxide dismutase

Treating endothelial cells for 20 min with the EDRF blocking agent, haemoglobin (10 μM), in the presence but not the absence of extracellular calcium, led to a reduction in cyclic GMP content (Table 2). The cyclic GMP content of endothelial cells obtained in the absence of calcium was not significantly different from that obtained following haemoglobin (10 μM) treatment in the presence of calcium (Table 2).

Treating endothelial cells for 3 min with either M&B 22,948 (100 μM) or superoxide dismutase (30 u ml^{-1}), two agents known to potentiate the actions of EDRF, induced rises in cyclic GMP content that were inhibited in the absence of extracellular calcium (Table 2).

Bradykinin, ATP and ionophore A23187

Three agents known to elicit EDRF production, bradykinin (0.1 μM, 1.5 min), ATP (10 μM, 3 min) and ionophore A23187 (0.1 μM, 3 min), induced rises in endothelial cyclic GMP content that were inhibited in the absence of extracellular calcium (Table 3).

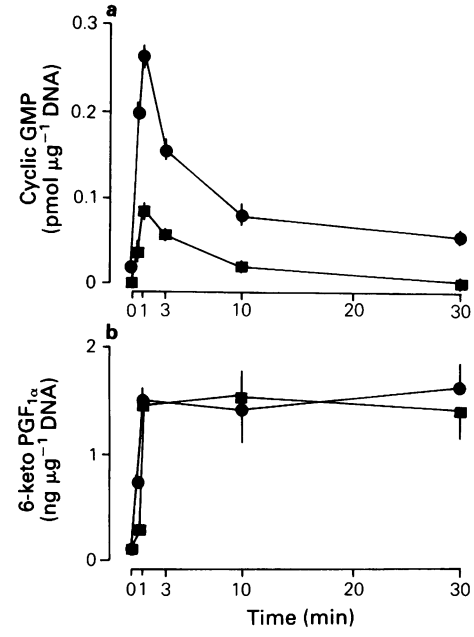

Figure 1 Time courses showing the simultaneous effects of bradykinin (0.1 μM) on endothelial cyclic GMP content (a) and 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) production (b) measured in the absence (●) and presence (■) of haemoglobin (10 μM). Results are presented as the mean of 6 observations with s.e.mean shown by vertical lines.

Effects of haemoglobin on cyclic GMP accumulation and prostacyclin production

In the presence of calcium (2.5 mM), the resting content of cyclic GMP in endothelial cells was 20·7 ± 3.8 fmol μg^{-1} DNA ($n = 6$) and the resting production of 6-keto $PGF_{1\alpha}$, measured over 40 min, was 126 ± 21 pg μg^{-1} DNA ($n = 5$). Bradykinin (0.1 μM) induced a maximum increase in cyclic GMP within 1 min, which declined thereafter but even at 10 min and 30 min was still significant (Figure 1). Production of 6-keto $PGF_{1\alpha}$, measured simultaneously, also rose rapidly but this was complete within 1 min (Figure 1).

When the EDRF-blocking agent, haemoglobin (10 μM), was present during the 40 min incubation period, the resting cyclic GMP content of the endothelial cells fell significantly, but resting 6-keto $PGF_{1\alpha}$ production was unaffected. Bradykinin (0.1 μM) induced a significantly smaller rise in cyclic GMP content in the presence of haemoglobin

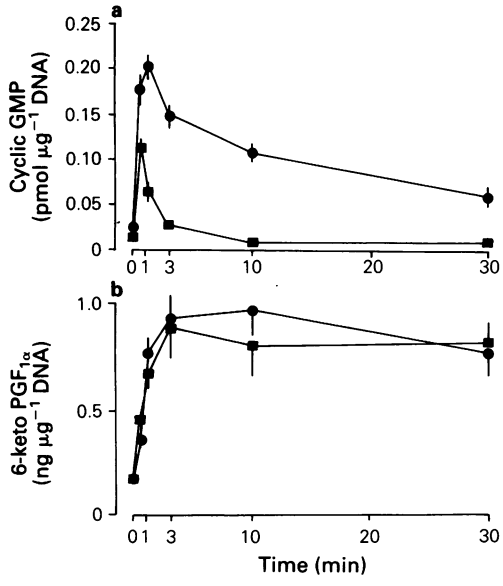

Figure 2 Time courses showing the simultaneous effects of bradykinin (0.1 μM) on endothelial cyclic GMP content (a) and 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) production (b) measured in the presence (●) and absence (1 mM EGTA added, ■) of 2.5 mM extracellular calcium. Results are presented as the mean of 12–31 observations with s.e.mean shown by vertical lines.

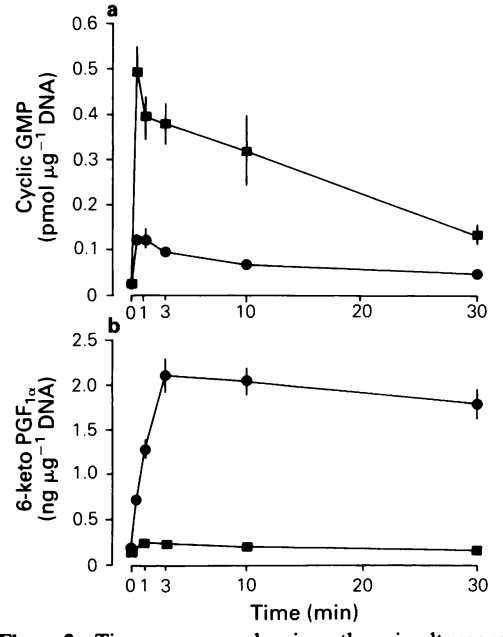

Figure 3 Time courses showing the simultaneous effects of bradykinin (0.1 μM) on endothelial cyclic GMP content (a) and 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) production (b) measured in the absence (●) and presence (■) of trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8, 100 μM). Results are presented as the mean of 17–24 observations with s.e.mean shown by vertical lines.

(10 μM), but the stimulation of 6-keto $PGF_{1\alpha}$ production was unaffected (Figure 1).

Calcium-dependence of cyclic GMP accumulation and prostacyclin production

In the absence of calcium the resting content of cyclic GMP fell significantly, but resting 6-keto $PGF_{1\alpha}$ production was unaffected (Figure 2). Bradykinin (0.1 μM) induced a rise in cyclic GMP content that was significantly smaller in magnitude than that obtained in the presence of calcium (2.5 mM) and was complete within 3 min, but stimulated production of 6-keto $PGF_{1\alpha}$ was unaffected (Figure 2).

Effects of TMB-8 on cyclic GMP accumulation and prostacyclin production

When the inhibitor of intracellular calcium release, TMB-8 (100 μM), was present during the 40 min incubation period the resting cyclic GMP content rose slightly and production of 6-keto $PGF_{1\alpha}$ fell (Figure 3). Bradykinin (0.1 μM) induced a significantly greater rise in cyclic GMP content in the presence of TMB-8 (100 μM), but the stimulation of 6-keto $PGF_{1\alpha}$ production was significantly smaller (Figure 3).

Cascade bioassay

In cascade bioassay experiments infusion of bradykinin (0.1 nM–0.1 μM) into columns of endothelial cells for 3 min periods induced concentration-dependent relaxations of rabbit aortic rings (Figure 4). In the presence of haemoglobin (10 μM) or methylene blue (20 μM) dilator responses were completely abolished (data not shown).

Continuous infusion of bradykinin (0.1 μM) induced a $71 \pm 8\%$ ($n = 5$) relaxation that was maximal after 2.0 ± 0.2 min and complete after 16.0 ± 0.3 min ($n = 5$) (Figure 5).

When TMB-8 (100 μM) was infused continuously into endothelial cell columns a $73 \pm 3\%$ ($n = 8$) relaxation of phenylephrine-induced tone was observed in rabbit aortic rings. When tone was raised to its original level by increasing the concentration of phenylephrine, infusion of bradykinin (0.1 nM–0.1 μM) induced concentration-dependent relaxations that were similar to those obtained in the absence of TMB-8 (Figure 4).

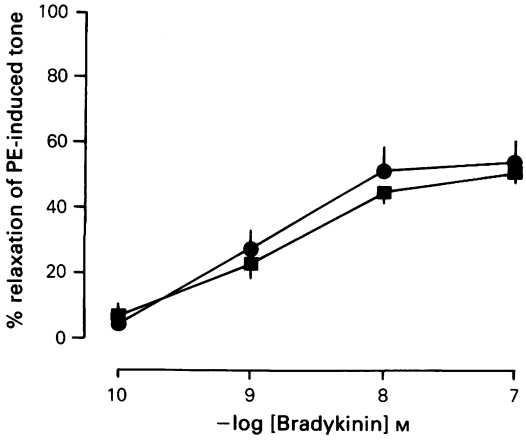

Figure 4 Log concentration-response curves obtained in cascade bioassay experiments showing the relaxation of phenylephrine (PE)-contracted, endothelium-denuded rings of rabbit aorta obtained when bradykinin (0.1 nM–0.1 μM) was infused for 3 min periods into columns of pig aortic endothelial cells on microcarrier beads in the absence (●) and presence (■) of trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8, 100 μM). Results are presented as the mean of 8 observations with s.e.mean shown by vertical lines.

Discussion

EDRF induces vasodilatation by elevating smooth muscle cyclic GMP content (Rapoport & Murad, 1983). Moreover, agents which evoke EDRF release elevate endothelial cyclic GMP content (Adams Brotherton, 1986; Martin *et al.*, 1988). These elevations are inhibited by haemoglobin or methylene blue and potentiated by M&B 22,948 or superoxide dismutase (Martin *et al.*, 1988), which indicates that they are produced by the action of EDRF on endothelial soluble guanylate cyclase. The ability of haemoglobin to lower the resting level of cyclic GMP in endothelial cells suggests that, even in the resting state basal production of EDRF is sufficient to activate endothelial soluble guanylate cyclase. Thus, the measurement of endothelial cyclic GMP content provides an indirect, but sensitive means of assessing EDRF production.

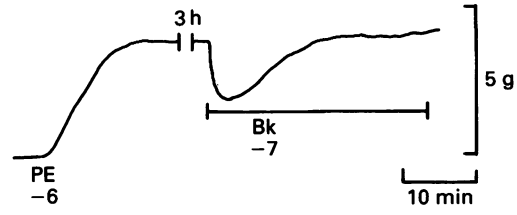

Figure 5 A typical trace obtained in a cascade bioassay experiment showing relaxation of a phenylephrine (PE)-contracted, endothelium-denuded ring of rabbit aorta obtained when bradykinin (Bk) was infused constantly for 30 min into a column of pig aortic endothelial cells on microcarrier beads. Molar concentrations are expressed in log units.

Many studies have shown that basal and agonist-stimulated production of EDRF are dependent on the presence of extracellular calcium (Singer & Peach, 1982; Long & Stone, 1985; Miller *et al.*, 1985; Griffith *et al.*, 1986; Luckhoff *et al.*, 1988). We found that calcium removal lowers resting levels of cyclic GMP in pig aortic endothelial cells to the same extent as the EDRF blocking agent, haemoglobin (Martin *et al.*, 1985). Furthermore, calcium removal prevents the rises in cyclic GMP induced by the EDRF potentiating agents, M&B 22,948 and superoxide dismutase (Martin *et al.*, 1986; Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986), as well as those induced by the EDRF releasing agents, bradykinin, ATP, and ionophore A23187. Thus, measuring endothelial cyclic GMP content has provided additional evidence to support the conclusion that basal and stimulated release of EDRF requires extracellular calcium.

It could be argued that removal of calcium did not prevent release of EDRF, but inhibited the subsequent stimulation of soluble guanylate cyclase. This is unlikely since sodium azide, a stimulant of soluble guanylate cyclase, and atriopeptin II, a receptor-mediated stimulant of particulate guanylate cyclase, induced rises in endothelial cyclic GMP content that were similar in the absence and presence of extracellular calcium.

In cascade bioassay experiments with endothelial cells on microcarrier beads, continuous infusion of bradykinin induced release of EDRF that was maximal at about 2 min and detectable for up to 16 min. EDRF production, assessed by measuring endothelial cyclic GMP content followed a similar time course: stimulation of production was maximal at 1 min and was still detectable after 30 min. Bradykinin-stimulated production of prostacyclin, measured simultaneously, also rose rapidly but, in contrast to the prolonged production of EDRF, was complete within 1–3 min, as demonstrated previously (Gordon & Martin, 1983; Pearson *et al.*, 1983). Removal of extracellular calcium differentially affected production of EDRF and prostacyclin: resting levels of cyclic GMP fell consistent with inhibition of basal EDRF production, but resting production of prostacyclin was unaffected. Furthermore, following treatment with bradykinin, stimulation of EDRF production, assessed as endothelial cyclic GMP content, still increased rapidly but reached a lower maximum and was less well sustained than that obtained in the presence of calcium, being complete within 3 min. The lower sensitivity of cascade

bioassay techniques probably explains why this transient production of EDRF in the absence of extracellular calcium has not been previously observed (Long & Stone, 1985; Griffith *et al.*, 1986; Luckhoff *et al.*, 1988). The magnitude and time course of bradykinin-stimulated production of prostacyclin was, however, similar to that obtained in the presence of calcium (2.5 mM). Thus, basal production of EDRF but not prostacyclin requires the presence of extracellular calcium. Also, bradykinin-stimulated production of EDRF is well sustained and requires the presence of extracellular calcium, but stimulated production of prostacyclin is transient and independent of the presence of extracellular calcium.

The inability of extracellular calcium removal to inhibit bradykinin-stimulated production of prostacyclin is consistent with the proposal that production is dependent mainly on release of an intracellular calcium pool (Seid *et al.*, 1983; Hallam & Needham, 1987). Direct measurement of intracellular calcium levels in endothelial cells using fura 2 or quin 2 supports this proposal (Hallam & Needham, 1987; Colden-Stanfield *et al.*, 1987). The transient rise in endothelial cyclic GMP levels induced by bradykinin in the absence of extracellular calcium may, likewise, reflect EDRF production triggered after the discharge of the intracellular pool of calcium.

If agonist-induced production of prostacyclin is controlled mainly by intracellular calcium release and production of EDRF is controlled by calcium influx, then prostacyclin production but not sustained EDRF production should be blocked by inhibitors of intracellular calcium release. Using TMB-8, we found that both basal and bradykinin-stimulated production of prostacyclin was inhibited, thus confirming previous data (Seid *et al.*, 1983). Basal and bradykinin-induced EDRF production, assessed as endothelial cyclic GMP content, were not inhibited, in fact the effect of bradykinin appeared to be potentiated. Independent confirmation that TMB-8 did not inhibit EDRF production was obtained in cascade bioassay experiments with endothelial cells on microcarrier beads. Whether TMB-8 actually increases agonist-induced production of EDRF, as suggested by experiments in which endothelial cyclic GMP was measured, warrants further investigation.

In conclusion, our data show that agonist-induced production of prostacyclin is transient but production of EDRF is more sustained. They further suggest that prostacyclin production is triggered by discharge of an intracellular store of calcium and EDRF production maintained by influx of extracellular calcium. Thus the vascular endothelial cell appears to exert differential control of production of these two powerful vasoactive agents by utilizing distinct calcium pools.

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