

Endothelium-derived relaxing factor and atriopeptin II elevate cyclic GMP levels in pig aortic endothelial cells

¹William Martin, David G. White & Andrew H. Henderson

Department of Cardiology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN

1 Two directly-acting stimulants of soluble guanylate cyclase, glyceryl trinitrate (0.1 μM) and sodium azide (10 μM), and a receptor-mediated stimulant of particulate guanylate cyclase, atriopeptin II (10 nM), each elevated the cyclic GMP content of primary cultures of pig aortic endothelial cells without affecting the cyclic AMP content.

2 Two receptor-mediated stimulants of adenylate cyclase, glucagon (1 μM) and isoprenaline (10 μM), had no effect on the cyclic AMP or cyclic GMP content of these cells, but the directly acting stimulant, forskolin (30 μM), induced a small increase in cyclic AMP content.

3 Three agents that release endothelium-derived relaxing factor (EDRF); bradykinin (0.1 μM), ATP (10 μM) and ionophore A23187 (0.1 μM), each markedly elevated the cyclic GMP content of pig aortic endothelial cells, but acetylcholine (1 μM) had no effect. None of these agents had any effect on cyclic AMP content.

4 Two agents that potentiate the actions of EDRF; M & B 22948 (100 μM) and superoxide dismutase (30 units ml^{-1}), each elevated the cyclic GMP content of pig aortic endothelial cells without affecting the cyclic AMP content. Pretreating cells with catalase (100 units ml^{-1}) did not affect the rise in cyclic GMP content induced by superoxide dismutase (30 units ml^{-1}).

5 Pretreatment of pig aortic endothelial cells with haemoglobin (10 μM) reduced the resting content of cyclic GMP and blocked the increase in cyclic GMP content induced by glyceryl trinitrate (0.1 μM), sodium azide (10 μM), bradykinin (0.1 μM), ATP (10 μM), ionophore A23187 (0.1 μM), M & B 22948 (100 μM) and superoxide dismutase (30 units ml^{-1}), but not that induced by atriopeptin II (10 nM).

6 Pretreatment of pig aortic endothelial cells with an inhibitor of soluble guanylate cyclase, methylene blue (20 μM), had no effect on the resting content of cyclic GMP. Methylene blue (20 μM) blocked the increase in cyclic GMP content induced by glyceryl trinitrate (0.1 μM), M & B 22948 (100 μM) and bradykinin (0.1 μM), but not that induced by atriopeptin II (10 nM).

7 The data show that soluble guanylate cyclase, particulate guanylate cyclase and adenylate cyclase are present in pig aortic endothelial cells. They further suggest that EDRF, produced spontaneously or in response to vasoactive agents, elevates endothelial cyclic GMP content by stimulating soluble guanylate cyclase. It is possible that this may serve as a feedback loop by which the endothelial cell modulates EDRF production.

Introduction

The vascular endothelial cell has the ability to regulate vascular smooth muscle reactivity by releasing two powerful vasodilator substances, prostacyclin (Moncada *et al.*, 1977), and endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) now identified as nitric oxide (Furchgott, 1987; Palmer

et al., 1987). Prostacyclin induces vascular relaxation by stimulating adenylate cyclase (Dembinska-Kiec *et al.*, 1979; Miller *et al.*, 1979) whereas EDRF induces relaxation by stimulating a soluble form of the enzyme, guanylate cyclase (Busse *et al.*, 1985; Ignarro *et al.*, 1986). In addition, atrial natriuretic peptides induce vascular relaxation by stimulating a particulate form of guanylate cyclase (Waldman *et al.*, 1984; Mittal, 1985).

¹ Author for correspondence, present address: Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ

Although cyclic GMP and cyclic AMP have well characterized actions in mediating vascular smooth muscle relaxation, it is not yet known if these two cyclic nucleotides have a regulatory function in the vascular endothelial cell. Several vasoactive agents including catecholamines, prostaglandins, acetylcholine, histamine, 5-hydroxytryptamine and angiotensin II have been reported to elevate cyclic AMP or cyclic GMP levels in endothelial cells cultured from a variety of sources including rabbit and bovine aortae and human umbilical vein (Buonassisi & Venter, 1976; Dembinska-Kiec *et al.*, 1979; Makarski, 1981; Brotherton & Hoak, 1982). Recently, atrial natriuretic peptides and nitrovasodilators have been shown to elevate cyclic GMP levels in endothelial cells cultured from human umbilical vein or bovine aorta (Brotherton, 1986; Ganz *et al.*, 1986; Leitman & Murad, 1986). These studies indicate that soluble and particulate forms of guanylate cyclase and adenylate cyclase are present in the vascular endothelial cell.

One possible consequence of the presence of soluble guanylate cyclase in the endothelial cell is that the endothelium may itself be responsive to the EDRF it produces. If so, this might indicate that locally produced EDRF can modulate endothelial cell function through a feedback loop involving soluble guanylate cyclase. Against this possibility are the observations that three EDRF-releasing agents, calcium ionophore A23187, ATP and acetylcholine, have no effect on the cyclic GMP content of bovine or porcine aortic endothelial cells (Peach *et al.*, 1985; Ganz *et al.*, 1986). In contrast, however, Brotherton (1986) has reported that thrombin, bradykinin, histamine and ionophore A23187 each elevate cyclic GMP levels in endothelial cells cultured from human umbilical vein, although the possibility that EDRF was responsible was not considered.

We have further investigated the regulatory action of soluble and particulate guanylate cyclase as well as of adenylate cyclase in primary cultures of pig aortic endothelial cells. Furthermore, we have investigated the possibility that these cells are responsive to the EDRF they produce. A preliminary account of these findings has already been published (Martin & White, 1987).

Methods

Endothelial cells culture

Pig aortae were obtained from a local abattoir. Immediately after removal from the pig (within 10 min of death), the aorta was flushed with sterile saline containing benzyl penicillin (100 u ml^{-1}) and streptomycin ($100 \mu\text{g ml}^{-1}$), tied off at one end and cannulated at the other with a 60 ml syringe containing the

same saline solution at ambient temperature. Saline was infused into the lumen before the vessel was transported to the laboratory within 30 min. The endothelial cells were then isolated as previously described (Gordon & Martin, 1983a). Briefly, after ligating the intercostal arteries, collagenase (0.2%, type II, Sigma) was introduced into the aortic lumen and the vessel was incubated at 37°C for 20 min. The endothelial cells from each aorta were then harvested in 60 ml of Medium E199 supplemented with: foetal bovine serum (10%); newborn bovine serum (10%); glutamine (6 mM); benzyl penicillin (100 u ml^{-1}); streptomycin ($100 \mu\text{g ml}^{-1}$) and kanamycin ($100 \mu\text{g ml}^{-1}$), and were seeded into 3 Linbro plates each containing 6 wells (9.6 cm^2). The culture medium was replaced every 2 days and cells were used within 3–7 days. All tissue culture media and supplements were obtained from Flow Laboratories, Irvine, Scotland, except for benzyl penicillin (crystapen; Glaxo) and streptomycin sulphate (Evans Medical Ltd).

The cells were characterized as endothelial cells by several criteria: they grew as a strict monolayer and produced prostacyclin. Furthermore, in randomly selected cultures, no fewer than 98% of the cells were observed to fluoresce following incubation with acetylated low density lipoprotein labelled with 1,1'-dioctadecyl-1-3,3',3'-tetramethyl-indocarbocyanine-perchlorate (CMD UK Ltd; Voyta *et al.*, 1984).

Measurement of cyclic nucleotides

Following removal of the tissue culture medium by aspiration, the endothelial cells were washed with $2 \times 2 \text{ ml}$ 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 disodium ethylenediamine tetraacetic acid 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. Drugs were then added to the cells at the concentrations and times indicated in the Results. At the appropriate time, the Krebs solution was quickly removed and the cells were immediately extracted with 0.5 ml of ice cold 6% trichloroacetic acid (TCA). The cells were then scraped off the multiwell plates and harvested. Cells remaining on the plates were recovered by extracting with a second volume of 0.5 ml TCA and were combined with the first extract. Following centrifugation at 13,000 r.p.m. for 2 min, the pellet and supernatant were separated. The DNA content of the cell pellet was measured by the fluorescence technique of Kissane & Robins (1958). The supernatant was neutralized (to pH 5.5–6) by adding 2 ml of 0.5 M tri-n-octylamine in freon (1,1,2 trichlorotrifluoroethane) and vortex mixing for 90 s. The cyclic GMP and cyclic AMP content of the upper (aqueous) layer was then determined by radioimmunoassay as previously des-

cribed (Martin *et al.*, 1985) using New England Nuclear kits. Cyclic nucleotide content was expressed as $\text{fmol } \mu\text{g}^{-1}$ DNA.

Measurement of prostacyclin

Following removal of the tissue culture medium, the endothelial cells were washed with 2×2 ml of Krebs solution and then incubated in 2 ml of Krebs solution at 37°C under an atmosphere of 5% CO_2 in air. After 60 min the bathing solution was removed and replaced with fresh Krebs solution. After a further 30 min, the Krebs solution was recovered and the cells immediately extracted in 2×0.5 ml volumes of 6% TCA. The prostacyclin content of the Krebs solution was determined by radioimmunoassay (measured as 6-keto-prostaglandin $\text{F}_{1\alpha}$) using an antiserum kindly supplied by Dr A.C. Newby. The DNA content of the cell pellet was determined as described above. Prostacyclin production is expressed as $\text{pg 6-keto-PGF}_{1\alpha} \mu\text{g}^{-1}$ DNA.

Drugs

Acetylcholine chloride, adenosine triphosphate (ATP), ionophore A23187, rat synthetic atriopeptin II, sodium azide, bradykinin triacetate, catalase (bovine liver), *E. coli* endotoxin 055:B5, glucagon, haemoglobin (bovine Type I), isobutylmethylxanthine (IBMX), (\pm)-isoprenaline, methylene blue and superoxide dismutase (bovine erythrocyte) were obtained from Sigma. Forskolin was obtained from Calbiochem and glyceryl trinitrate (10% w/w in lactose) was obtained from Napp Laboratories. Sodium flurbiprofen was a generous gift from Dr R.V. Holland, Boots Pure Drug Co, and M & B 22948 (2-0-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 and forskolin which were dissolved in dimethyl sulphoxide (DMSO); IBMX, which was dissolved in ethanol; and M&B 22948 which was dissolved in 10% triethanolamine.

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

Statistical analysis

Since the resting content of cyclic nucleotides varied substantially from batch to batch, each experiment was performed with its own internal controls. Results are expressed as the mean \pm s.e.mean and comparisons were made by means of Student's *t* test. A probability of 0.05 or less was considered significant. In the results, *n* represents the number of replicate multiwell dishes of cells from a single cell preparation,

and the cyclic GMP and cyclic AMP values shown are from the same preparations of cells.

Results

Haemoglobin and methylene blue

The resting levels of cyclic GMP and cyclic AMP in primary cultures of pig aortic endothelial cells were $11.1 \pm 1.4 \text{ fmol } \mu\text{g}^{-1}$ DNA ($n = 63$) and $738 \pm 97 \text{ fmol } \mu\text{g}^{-1}$ DNA ($n = 63$), respectively.

Treatment of a different batch of pig aortic endothelial cells for 20 min with haemoglobin ($10 \mu\text{M}$), reduced the resting level of cyclic GMP from $6.0 \pm 0.8 \text{ fmol } \mu\text{g}^{-1}$ DNA ($n = 28$) to $2.4 \pm 0.5 \text{ fmol } \mu\text{g}^{-1}$ DNA ($n = 25$; $P < 0.001$). The cyclic AMP content following treatment with haemoglobin ($10 \mu\text{M}$; 20 min) ($562 \pm 75 \text{ fmol } \mu\text{g}^{-1}$ DNA; $n = 29$) did not differ significantly from control values ($423 \pm 39 \text{ fmol } \mu\text{g}^{-1}$ DNA; $n = 28$).

Treatment of pig aortic endothelial cells for 20 min with the selective inhibitor of soluble guanylate cyclase, methylene blue ($20 \mu\text{M}$), had no effect on the resting levels of cyclic GMP or cyclic AMP (Tables 1, 2, 4 and 5).

Stimulants of soluble guanylate cyclase

In two separate experiments glyceryl trinitrate ($0.1 \mu\text{M}$; 3 min) induced 3.3 fold ($n = 6$) and 3.2 fold ($n = 6$) increases in the cyclic GMP content of pig aortic endothelial cells, and sodium azide ($10 \mu\text{M}$; 1.5 min) induced a 4.5 fold ($n = 6$) increase (Table 1). Neither agent had any effect on the cyclic AMP content.

Treatment of endothelial cells for 20 min with haemoglobin ($10 \mu\text{M}$) inhibited the increases in cyclic GMP content induced by glyceryl trinitrate ($0.1 \mu\text{M}$; 3 min) or sodium azide ($10 \mu\text{M}$; 1.5 min; Table 1). Treatment of endothelial cells for 20 min with methylene blue ($20 \mu\text{M}$) inhibited the rise in cyclic GMP content induced by glyceryl trinitrate ($0.1 \mu\text{M}$; 3 min; Table 1).

Receptor-mediated stimulants of particulate guanylate cyclase

In two separate experiments atriopeptin II (10 nM , 3 min) induced 142.6 fold ($n = 6$) and 17.4 fold ($n = 6$) increases in the cyclic GMP content of pig aortic endothelial cells but had no effect on cyclic AMP content (Table 2). Treatment of endothelial cells for 20 min with either haemoglobin ($10 \mu\text{M}$) or methylene blue ($20 \mu\text{M}$) had no effect on the rise in cyclic GMP content induced by atriopeptin II (10 nM ; 3 min; Table 2).

E. coli endotoxin 055:B5 ($2.5 \mu\text{g ml}^{-1}$; 3 min) had no effect on either the cyclic GMP or cyclic AMP content of pig aortic endothelial cells (Table 2).

Table 1 Effects of glyceryl trinitrate and sodium azide on the cyclic nucleotide content of pig aortic endothelial cells

<i>Stimulus</i>	<i>Pretreatment</i>	<i>Cyclic GMP</i> (fmol μg^{-1} DNA)	<i>Cyclic AMP</i> (fmol μg^{-1} DNA)	<i>n</i>
None (Control)	None	2.7 \pm 1.0	299 \pm 29	8
None	Hb, 10 μM	1.9 \pm 0.9	400 \pm 75	4
GTN, 0.1 μM	None	8.8 \pm 1.8*	377 \pm 17	6
GTN, 0.1 μM	Hb, 10 μM	0.9 \pm 0.6	367 \pm 52	4
None (Control)	None	39.2 \pm 8.5	1270 \pm 200	6
None	MB, 20 μM	29.5 \pm 9.9	1430 \pm 88	6
GTN, 0.1 μM	None	125.3 \pm 17.0**	1428 \pm 258	6
GTN, 0.1 μM	MB, 20 μM	21.2 \pm 7.5	1336 \pm 92	6
None (Control)	None	11.4 \pm 1.4	220 \pm 32	6
None	Hb, 10 μM	3.1 \pm 0.8***	231 \pm 35	6
Azide, 10 μM	None	50.9 \pm 3.3***	285 \pm 40	6
Azide, 10 μM	Hb, 10 μM	10.8 \pm 2.4	307 \pm 37	6

Following exposure of pig aortic endothelial cells to glyceryl trinitrate (GTN; 0.1 μM) for 3 min or sodium azide (10 μM) for 1.5 min, the bathing solution was removed and the cyclic GMP and cyclic AMP content of the cells determined. The effects of pretreating cells for 20 min with haemoglobin (Hb; 10 μM) were examined on the resting cyclic nucleotide content and on the content following stimulation with GTN or azide. The effects of pretreating cells for 20 min with methylene blue (MB; 20 μM) were examined on the resting cyclic nucleotide content and on the content following stimulation with GTN. Results are expressed as the mean \pm s.e.mean. * P < 0.05; ** P < 0.005; *** P < 0.001, denotes significant difference from control.

Receptor-mediated and direct stimulants of adenylate cyclase

Neither glucagon (1 μM , 3 min) nor (\pm)-isoprenaline (10 μM ; 3 min) had any effect on the cyclic GMP or cyclic AMP content of pig aortic endothelial cells (Table 3). Forskolin (30 μM ; 3 min) induced a 4.7 fold

(n = 6) increase in the cyclic GMP content and a 2 fold (n = 6) increase in the cyclic AMP content of pig aortic endothelial cells (Table 3). The rise in cyclic GMP content was a solvent effect since the solvent itself, DMSO (1%; 3 min), induced a 3.2 fold (n = 7) increase in cyclic GMP content. DMSO (1%; 3 min) itself had no effect on the cyclic AMP content (Table 3).

Table 2 Effects of atriopeptin II and *E. coli* endotoxin on the cyclic nucleotide content of pig aortic endothelial cells

<i>Stimulus</i>	<i>Pretreatment</i>	<i>Cyclic GMP</i> (fmol μg^{-1} DNA)	<i>Cyclic AMP</i> (fmol μg^{-1} DNA)	<i>n</i>
None (Control)	None	2.7 \pm 1.0	299 \pm 29	8
None	Hb, 10 μM	1.9 \pm 0.9	279 \pm 54	4
APII, 10 nM	None	385.1 \pm 49.3***	252 \pm 14	6
APII, 10 nM	Hb, 10 μM	314.2 \pm 57.4***	240 \pm 20	6
None (Control)	None	39.2 \pm 8.5	1270 \pm 200	6
None	MB, 20 μM	29.5 \pm 9.9	1430 \pm 88	6
APII, 10 nM	None	683.3 \pm 100.1***	1289 \pm 105	6
APII, 10 nM	MB, 20 μM	744.2 \pm 42.3***	1606 \pm 123	6
None (Control)	None	5.4 \pm 0.7	742 \pm 91	5
Endo, 2.5 $\mu\text{g ml}^{-1}$	None	6.6 \pm 1.7	811 \pm 88	6

Following 3 min exposure of pig aortic endothelial cells to atriopeptin II (APII; 10 nM) or *E. coli* endotoxin (Endo; 2.5 $\mu\text{g ml}^{-1}$), the bathing solution was removed and the cyclic GMP and cyclic AMP content of the cells determined. The effects of pretreating cells for 20 min with haemoglobin (Hb; 10 μM) or methylene blue (MB; 20 μM) was determined on the resting cyclic nucleotide content and the content following stimulation with APII. Results are expressed as the mean \pm s.e.mean. *** P < 0.001, denotes significant difference from control.

Table 3 Effects of glucagon, isoprenaline and forskolin on the cyclic nucleotide content of pig aortic endothelial cells

Stimulus	Pretreatment	Cyclic GMP (fmol μg ⁻¹ DNA)	Cyclic AMP (fmol μg ⁻¹ DNA)	n
None (Control)	None	27.7 ± 6.1	736 ± 63	6
Glucagon, 1 μM	None	27.2 ± 6.6	785 ± 51	6
None (Control)	None	33.1 ± 9.0	1769 ± 198	6
Isoprenaline, 10 μM	None	65.0 ± 8.0	1620 ± 94	6
None (Control)	None	8.4 ± 0.9	273 ± 13	6
None	IBMX, 10 μM	11.4 ± 1.7	326 ± 31	6
Forskolin, 30 μM	None	39.6 ± 5.1***	546 ± 21***	6
Forskolin, 30 μM	IBMX, 10 μM	52.7 ± 7.6***	697 ± 47***	6
None (Control)	None	7.6 ± 1.0	372 ± 51	8
DMSO, 1%	None	24.0 ± 4.5**	433 ± 49	7

Following 3 min exposure of pig aortic endothelial cells to glucagon (1 μM), (±)-isoprenaline (10 μM) or forskolin (30 μM), the bathing solution was removed and the cyclic GMP and cyclic AMP content of the cells determined. The effects of pretreating cells for 20 min with isobutylmethylxanthine (IBMX; 10 μM) were examined on the responses to forskolin. The effects a 3 min exposure to dimethyl sulphoxide (DMSO; 1%), the solvent used to dissolve forskolin, were also determined. Results are expressed as the mean ± s.e.mean. ***P* < 0.005; ****P* < 0.001 denotes significant difference from control.

Pretreatment of pig aortic endothelial cells for 20 min with the phosphodiesterase inhibitor, IBMX (10 μM), had no effect on the resting levels of cyclic GMP or cyclic AMP, but it did potentiate slightly the increases in cyclic GMP and cyclic AMP content induced by forskolin (30 μM; 3 min; Table 3).

Stimulants of EDRF release

In two separate experiments bradykinin (0.1 μM; 1.5 min) induced 122.8 fold (*n* = 6) and 37.9 fold (*n* = 6) increases in the cyclic GMP content of pig aortic endothelial cells but had no effect on the cyclic AMP content (Table 4). The increase in cyclic GMP content was maximal within 30 s of exposure to bradykinin (0.1 μM) and declined only slightly during 10 min exposure (Figure 1).

ATP (10 μM; 3 min) and calcium ionophore A23187 (0.1 μM; 3 min), induced 19.1 fold (*n* = 6) and 24.2 fold (*n* = 6) increases, respectively, in the cyclic GMP content of pig aortic endothelial cells without affecting the cyclic AMP content (Table 4). In contrast, acetylcholine (1 μM; 1.5 min) had no effect on either the cyclic GMP or cyclic AMP content of pig aortic endothelial cells (Table 4).

Treatment of pig aortic endothelial cells for 20 min with haemoglobin (10 μM) reduced the rises in cyclic GMP content induced by bradykinin (0.1 μM; 1.5 min), ATP (10 μM; 3 min) or A23187 (0.1 μM; 3 min) (Table 4). Furthermore, treatment with methylene blue (20 μM; 20 min) reduced the rise in cyclic

GMP content induced by bradykinin (0.1 μM; 1.5 min; Table 4).

M&B 22948

In two separate experiments the selective cyclic GMP phosphodiesterase inhibitor, M&B 22948 (100 μM; 3 min), induced 4.0 fold (*n* = 6) and 7.0 fold (*n* = 6) increases in the cyclic GMP content of pig aortic endothelial cells but had no effect on the cyclic AMP content (Table 5).

The rise in cyclic GMP content induced by M&B 22948 (100 μM; 3 min) was blocked by pretreatment of pig aortic endothelial cells for 20 min with either haemoglobin (10 μM) or methylene blue (20 μM; Table 5).

Superoxide dismutase and catalase

In two separate experiments, superoxide dismutase (30 units ml⁻¹; 3 min) induced 4.7 fold (*n* = 6) and 2.6 fold (*n* = 6) increases in the cyclic GMP content of pig aortic endothelial cells but had no effect on the cyclic AMP content (Table 5). The rise in cyclic GMP content induced by superoxide dismutase (30 units ml⁻¹; 3 min) was blocked by treating pig aortic endothelial cells for 20 min with haemoglobin (10 μM; Table 5).

When pig aortic endothelial cells were treated for 20 min with catalase (100 units ml⁻¹) no effect on either the cyclic GMP or cyclic AMP content was seen (Table 5). Treatment with catalase (100 units ml⁻¹;

Table 4 Effects of bradykinin, ATP, ionophore A23187 and acetylcholine on the cyclic nucleotide content of pig aortic endothelial cells

<i>Stimulus</i>	<i>Pretreatment</i>	<i>Cyclic GMP</i> (fmol μg^{-1} DNA)	<i>Cyclic AMP</i> (fmol μg^{-1} DNA)	<i>n</i>
None (Control)	None	5.3 \pm 1.0	973 \pm 51	6
None	Hb, 10 μM	5.2 \pm 1.7	616 \pm 49	6
Bradykinin, 0.1 μM	None	650.8 \pm 92.2***	898 \pm 167	6
Bradykinin, 0.1 μM	Hb, 10 μM	158.5 \pm 10.7***	679 \pm 58	6
None (Control)	None	32.3 \pm 6.5	2778 \pm 302	6
None	MB, 20 μM	19.4 \pm 3.1	3706 \pm 414	6
Bradykinin, 0.1 μM	None	1223.4 \pm 520.2*	3370 \pm 248	6
Bradykinin, 0.1 μM	MB, 20 μM	69.8 \pm 12.0*	4065 \pm 547	6
None (Control)	None	9.9 \pm 2.8	947 \pm 149	6
None	Hb, 10 μM	2.3 \pm 0.9*	606 \pm 40	5
ATP, 10 μM	None	188.9 \pm 45.2**	1268 \pm 205	6
ATP, 10 μM	Hb, 10 μM	22.3 \pm 6.5	1540 \pm 336	6
None (Control)	None	5.0 \pm 1.0	308 \pm 131	6
None	Hb, 10 μM	1.9 \pm 0.5*	273 \pm 56	5
A23187, 0.1 μM	None	121.0 \pm 12.1***	362 \pm 48	6
A23187, 0.1 μM	Hb, 10 μM	18.9 \pm 1.4***	287 \pm 37	6
None (Control)	None	28.4 \pm 5.0	486 \pm 35	5
ACh, 1 μM	None	36.4 \pm 1.8	512 \pm 32	7

Following exposure of pig aortic endothelial cells to bradykinin (0.1 μM) for 1.5 min, to ATP (10 μM) or to A23187 (0.1 μM) for 3 min or to acetylcholine (ACh; 1 μM) for 1.5 min, the bathing solution was removed and the cyclic GMP and cyclic AMP content of the cells determined. The effects of pretreating cells for 20 min with haemoglobin (Hb; 10 μM) or methylene blue (MB; 20 μM) are also shown. Results are expressed as the mean \pm s.e.mean. * P < 0.05; ** P < 0.005; *** P < 0.001, denotes significant difference from control.

20 min) had no effect on the rise in cyclic GMP content induced by superoxide dismutase (30 units ml^{-1} ; 3 min; Table 5).

Flurbiprofen

Pretreatment of pig aortic endothelial cells for 60 min with the cyclo-oxygenase inhibitor, flurbiprofen (10 μM), reduced the amount of prostacyclin (measured as 6-keto-prostaglandin $\text{F}_{1\alpha}$) released into the bathing Krebs solution during the subsequent 30 min. (Table 6). Flurbiprofen (10 μM) treatment had no effect on either the cyclic GMP or cyclic AMP content of pig aortic endothelial cells (Table 6).

Discussion

Guanylate cyclase, the enzyme that catalyses the formation of cyclic GMP, exists in both a soluble and a particulate form in a wide range of tissues (Murad *et al.*, 1979). The soluble and presumably cytoplasmic form of the enzyme is activated by EDRF (nitric

oxide) and substances that give rise to nitric oxide, i.e. nitrovasodilators (Katsuki *et al.*, 1977; Craven & De Rubertis, 1978; Busse *et al.*, 1985). The particulate form of guanylate cyclase is activated following receptor activation by atrial natriuretic peptides and by certain bacterial endotoxins (Field *et al.*, 1978; Murad *et al.*, 1979; Waldman *et al.*, 1984).

We found that two stimulants of soluble guanylate cyclase, glyceryl trinitrate and azide, and a receptor-mediated stimulant of particulate guanylate cyclase, atriopeptin II, each elevate cyclic GMP levels in pig aortic endothelial cells. Since less than 2% of the cells in our cultures failed to accumulate low density lipoprotein, contamination with other cell types was minimal. We can be reasonably certain, therefore, that the large increases in cyclic GMP content observed took place in endothelial cells and not in a subpopulation of contaminating cells. Two agents that selectively inhibit the stimulation of soluble but not particulate guanylate cyclase, methylene blue and haemoglobin (Rapoport *et al.*, 1985; Martin *et al.*, 1985; 1986a), each blocked the increases in cyclic GMP content induced by the nitrovasodilators but not by atriopep-

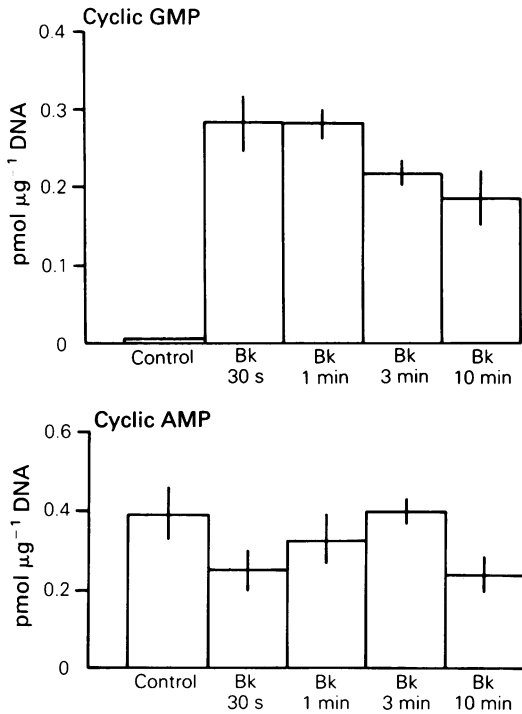


Figure 1 Time course of the effects of bradykinin (Bk, 0.1 μM) on the cyclic GMP and cyclic AMP contents of pig aortic endothelial cells. Results are expressed as the mean of 4–6 observations with s.e.mean shown by vertical lines.

tin II. *E. coli* endotoxin had no effect on the cyclic GMP content of pig aortic endothelial cells, indicating that this cell type, unlike enteric epithelium (Field *et al.*, 1978), may lack receptors for this agent.

It is clear therefore that pig aortic endothelial cells contain both the soluble and particulate form of guanylate cyclase. Vascular endothelial cells from other sources, i.e. human umbilical vein and bovine aorta, also respond with increased levels of cyclic GMP to stimulants of soluble or particulate guanylate cyclase (Brotherton, 1986; Ganz *et al.*, 1986; Leitman & Murad, 1986). It is likely therefore that cyclic GMP acts as an intracellular messenger in endothelial cells but, apart from a possible action in elevating thromboxane A₂ production (Fuller & Worthington, 1984), little is known of the functions of this cyclic nucleotide in the endothelium.

The presence of particulate guanylate cyclase in the vascular endothelium might suggest that this cell type is involved in the physiological actions of atrial natriuretic peptides. Although it is clear that the

vasodilator actions of atrial natriuretic peptides are completely independent of the endothelium (Winqvist *et al.*, 1984), the possibility that the regulation of fluid volume by these peptides (Currie *et al.*, 1983) might involve an action on endothelial permeability warrants investigation.

We found that agents known to induce endothelium-dependent relaxation of piglet aorta and release EDRF from cultured pig aortic endothelial cells, i.e. bradykinin, ATP and ionophore A23187 (Gordon & Martin, 1983a; Peach *et al.*, 1985; Gryglewski *et al.*, 1986b), each induce large elevations in the cyclic GMP content of primary cultures of pig aortic endothelial cells. Analysis of the time course of the bradykinin-induced elevation of cyclic GMP showed that it was maximal within 30 s and began to decline slightly after 3 min but was still substantially above basal levels even at 10 min. Haemoglobin, which blocks the actions of EDRF (Martin *et al.*, 1985), and the inhibitor of soluble guanylate cyclase, methylene blue (Holzmann, 1982), each inhibited these elevations of cyclic GMP content, indicating that they were probably mediated by EDRF.

Our work shows that stimulation of EDRF production increases the cyclic GMP content of pig aortic endothelial cells. The work of Brotherton (1986) indicates that this is probably also the case for human umbilical vein endothelial cells. Why previous reports (Peach *et al.*, 1985; Ganz *et al.*, 1986) failed to show such increases in cyclic GMP content in response to EDRF releasing agents is not clear. It may be related to the time the endothelial cells spent in culture. We and Brotherton (1986) used only primary cultures but Ganz *et al.* (1986) used subcultured cells. It has been shown that endothelial cell responsiveness declines with time in culture (Pearson *et al.*, 1983; Ager & Martin, 1984; Needham *et al.*, 1987).

This loss of endothelial responsiveness with time in culture may explain our results with acetylcholine. Despite being a powerful endothelium-dependent relaxant of piglet aorta (Gordon & Martin, 1983a), acetylcholine had no effect on the cyclic GMP content of primary cultures of pig aortic endothelial cells. This observation is consistent with our previous finding that acetylcholine and the cholinesterase-resistant agonist carbachol, in contrast to other EDRF releasing agents, do not evoke calcium-activated potassium efflux or prostacyclin production by these cells (Gordon & Martin, 1983a, b). Cocks *et al.* (1985) have also found muscarinic responses to be lacking in endothelial cells cultured from bovine aorta. It is possible therefore that the muscarinic receptor or its coupling to subsequent biochemical events is lost in culture. An alternative explanation proposed by Stephenson & Summers (1987) is that as muscarinic receptors cannot be found on the endothelium of a wide range of blood vessels by autoradiographic techniques, endothelium-

Table 5 Effects of M&B 22948 and superoxide dismutase on the cyclic nucleotide content of pig aortic endothelial cells

<i>Stimulus</i>	<i>Pretreatment</i>	<i>Cyclic GMP</i> (fmol μg^{-1} DNA)	<i>Cyclic AMP</i> (fmol μg^{-1} DNA)	<i>n</i>
None (Control)	None	2.1 \pm 0.6	334 \pm 23	5
None	Hb, 10 μM	1.9 \pm 0.9	348 \pm 73	4
M&B, 100 μM	None	8.4 \pm 2.4*	337 \pm 38	6
M&B, 100 μM	Hb, 10 μM	2.1 \pm 1.6	262 \pm 23	5
None (Control)	None	32.3 \pm 6.5	2778 \pm 302	6
None	MB, 20 μM	19.4 \pm 3.1	3706 \pm 414	6
M&B, 100 μM	None	225.0 \pm 64*	2797 \pm 321	6
M&B, 100 μM	MB, 20 μM	31.3 \pm 0.1	3022 \pm 251	6
None (Control)	None	5.8 \pm 2.0	212 \pm 30	5
None	Hb, 10 μM	0.8 \pm 0.3*	213 \pm 10	5
SOD, 30 u ml ⁻¹	None	27.2 \pm 8.3*	216 \pm 23	6
SOD, 30 u ml ⁻¹	Hb, 10 μM	2.1 \pm 0.9	157 \pm 26	6
None (Control)	None	8.3 \pm 0.8	255 \pm 66	6
None	CAT, 100 u ml ⁻¹	9.8 \pm 0.8	268 \pm 38	5
SOD, 30 u ml ⁻¹	None	21.6 \pm 2.4***	300 \pm 66	6
SOD, 30 u ml ⁻¹	CAT, 100 u ml ⁻¹	22.7 \pm 0.9***	320 \pm 78	5

Following 3 min exposure of pig aortic endothelial cells to M&B 22948 (100 μM) or to superoxide dismutase (SOD; 30 units ml⁻¹), the bathing solution was removed and the cyclic GMP and cyclic AMP content determined. The effects of pretreating cells for 20 min with haemoglobin (Hb; 10 μM), methylene blue (MB; 20 μM), or catalase (CAT; 100 units ml⁻¹) are also shown. Results are expressed as the mean \pm s.e.mean. * P < 0.05; *** P < 0.001, denotes significant difference from control.

dependent relaxation might result from an indirect mechanism through receptors located on the smooth muscle.

We found that haemoglobin reduced the resting level of cyclic GMP in pig aortic endothelial cells. The effect of methylene blue was not statistically significant. These observations suggest that the resting level of cyclic GMP in pig aortic endothelial cells may be influenced by spontaneously released EDRF. This would be consistent with previous observations in the

isolated aorta of the rat and rabbit where the resting cyclic GMP content of the vascular wall is related to spontaneously released EDRF (Rapoport & Murad, 1983; Martin *et al.*, 1986b, c).

The possibility that spontaneously released EDRF exerts a tonic influence on the resting level of cyclic GMP in pig aortic endothelial cells is further supported by the experiments with M&B 22948. This selective cyclic GMP phosphodiesterase inhibitor, which is known to enhance the actions of EDRF by blocking

Table 6 Effects of flurbiprofen on the production of prostacyclin and the cyclic nucleotide content of pig aortic endothelial cells

<i>Stimulus</i>	<i>Pretreatment</i>	<i>Cyclic GMP</i> (fmol μg^{-1} DNA)	<i>Cyclic AMP</i> (fmol μg^{-1} DNA)	<i>6-keto-PGF_{1α}</i> (pg μg^{-1} DNA)	<i>n</i>
None (Control)	None	11.1 \pm 1.8	361 \pm 91	310 \pm 36	6
None	FBP, 10 μM	12.1 \pm 3.9	408 \pm 139	35 \pm 4***	6

Following 60 min exposure of pig aortic endothelial cells to flurbiprofen (FBP, 10 μM), the bathing solution was removed and fresh solution containing FBP (10 μM) was added. Following a further 30 min exposure, the bathing medium was removed. The 6-keto-PGF_{1 α} content of the bathing medium, and the cyclic GMP and cyclic AMP content of the cells were then determined. Results are expressed as the mean \pm s.e.mean. *** P < 0.001, denotes significant difference from control.

the hydrolysis of cyclic GMP (Griffith *et al.*, 1985; Marin *et al.*, 1986b), increased the cyclic GMP content of pig aortic endothelial cells. Furthermore, this elevation of cyclic GMP content was blocked by haemoglobin or methylene blue, indicating that it was mediated by EDRF. In addition, superoxide dismutase, which prolongs the half-life and biological actions of EDRF by removal of destructive oxygen-derived free radicals (Gryglewski *et al.*, 1986a; Rubanyi & Vanhoutte, 1986a, b), also elevated the cyclic GMP content of pig aortic endothelial cells. The increase in cyclic GMP content induced by superoxide dismutase was inhibited by haemoglobin but not by catalase, confirming that it arose from a potentiation of the action of spontaneously released EDRF rather than from an increase in EDRF production stimulated by hydrogen peroxide formed by dismutation of superoxide radicals (Rubanyi & Vanhoutte, 1986a).

Two receptor-mediated stimulants of adenylate cyclase, glucagon and isoprenaline, had no effect on the cyclic AMP content of pig aortic endothelial cells. In contrast, β -adrenoceptor stimulants elevate cyclic AMP levels in endothelial cells cultured from rabbit or bovine aorta (Buonassisi & Venter, 1976; Makarski, 1981). Two explanations could account for our inability to increase cyclic GMP levels with glucagon or isoprenaline: firstly, receptors for these agents may be absent. Secondly, the cyclic AMP content of these cells was about 70 times higher than the cyclic GMP content, so it could be argued that small changes might be obscured by the large background level. However, we did increase the cyclic AMP content of pig aortic endothelial cells slightly (2 fold) in response to high concentrations of forskolin, which directly activates the catalytic subunit of adenylate cyclase (Seaman & Daly, 1981). The phosphodiesterase inhibitor, isobutylmethylxanthine, had no effect on the resting level of cyclic AMP in pig aortic endothelial cells and it augmented only slightly the elevation of cyclic AMP content induced by forskolin. It is unlikely therefore that high phosphodiesterase activity can account for our ability to induce only modest increases in cyclic AMP content with powerful stimuli such as forskolin.

As with cyclic GMP, little is known of the

physiological functions of cyclic AMP in the vascular endothelial cell. Early speculation that an increase in cyclic AMP concentrations inhibits the production of prostacyclin by human umbilical vein endothelium (Hopkins & Gorman, 1981; Brotherton & Hoak, 1982) has now been shown to be incorrect (Brotherton *et al.*, 1982). More recently, however, Krulewicz & Fanburg (1986) have suggested that cyclic AMP may regulate angiotensin converting enzyme activity in bovine aortic endothelial cells.

Prostacyclin exerts its vasodilator action by activating receptors linked to adenylate cyclase (Dembinska-Kiec *et al.*, 1979; Miller *et al.*, 1979). In contrast to the stimulatory effects of EDRF on cyclic GMP levels, we found no evidence that the cyclic AMP content of pig aortic endothelial cells was modulated by the prostacyclin they produce. Stimulants of prostacyclin production, i.e. bradykinin, ATP or ionophore A23187 (Gordon & Martin, 1983b; Gryglewski *et al.*, 1986b), had no effect on the cyclic AMP content of pig aortic endothelial cells. Furthermore, the possibility that the higher content of cyclic AMP than cyclic GMP might reflect the action of spontaneously released prostacyclin seems unlikely since the cyclooxygenase inhibitor, flurbiprofen, inhibited prostacyclin production but had no effect on the levels of cyclic AMP. We cannot, however, discount the possibility that small changes in cyclic AMP content occurred but were obscured by the high basal level.

In conclusion, in endothelial cells cultured from pig aorta, large increases in cyclic GMP content were induced by stimulants of soluble or particulate guanylate cyclase, but stimulants of adenylate cyclase induced only modest elevations of cyclic AMP content. Furthermore, our data suggest that EDRF, produced either spontaneously or in response to vasoactive agents, increased the endothelial content of cyclic GMP by activating soluble guanylate cyclase. The possibility that this constitutes a feedback loop by which the endothelial cell modulates EDRF production and/or other endothelial functions is now under investigation.

This work was supported by the British Heart Foundation.

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(Received August 7, 1987.

Accepted September 14, 1987.)