Stimulation of cyclic GMP production in cultured endothelial cells of the pig by bradykinin, adenosine diphosphate, calcium ionophore A23187 and nitric oxide

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1 The effects of bradykinin, adenosine diphosphate, calcium ionophore A23187 and nitric oxide on the production of adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) were investigated in cultured aortic endothelial cells of the pig.

2 Bradykinin (10^{-7} M), adenosine diphosphate (3×10^{-5} M), nitric oxide (2×10^{-6} M) and A23187 (10^{-6} M) stimulated the production of cyclic GMP. This stimulation reached a maximum within 1 min and declined rapidly with the first three agonists whereas that induced by A23187 was long-lasting.

3 These concentrations of bradykinin, A23187 and nitric oxide had no effect on cyclic AMP production. However, adenosine diphosphate (3×10^{-5} M) slightly but significantly enhanced its production by about 1.7 fold.

4 The basal content of cyclic GMP in endothelial cells was significantly reduced by haemoglobin (10^{-5} M, a scavenger of endothelium-derived relaxing factor(s)) and methylene blue (10^{-5} M, an inhibitor of the activation of soluble guanylate cyclase) and was significantly enhanced by superoxide dismutase (500 u ml^{-1} , a scavenger of superoxide anions). The basal content of cyclic GMP was not affected by N^G -monomethyl-L-arginine (10^{-5} M, a specific inhibitor of the formation of nitric oxide from L-arginine) and was slightly but significantly increased by its D-enantiomer, N^G -monomethyl-D-arginine.

5 The production of cyclic GMP stimulated by bradykinin, adenosine diphosphate, A23187 and nitric oxide was inhibited by haemoglobin (10^{-5} M) and methylene blue (10^{-5} M) but was unaffected by superoxide dismutase (500 u ml^{-1}).

6 The production of cyclic GMP stimulated by bradykinin, adenosine diphosphate or A23187, but not that stimulated by nitric oxide, was significantly reduced by N^G -monomethyl-L-arginine (10^{-5} M). The production of cyclic GMP evoked by nitric oxide, but not that induced by the other three agents, was enhanced significantly by N^G -monomethyl-D-arginine by about 1.5 fold.

7 These data indicate that the endothelium-dependent vasodilators bradykinin, adenosine diphosphate and A23187 activate the production of cyclic GMP in endothelial cells via the synthesis of nitric oxide, which in turn stimulates the soluble guanylate cyclase.

Introduction

Nitric oxide is produced and released from endothelial cells under basal conditions and in response to bradykinin, adenosine triphosphate and the calcium ionophore A23187 (Palmer *et al.*, 1987, 1988a,b; Kelm *et al.*, 1988). Endothelial cells are able to synthesize nitric oxide from the terminal guanidino nitrogen atom(s) of the amino acid L-arginine (Palmer *et al.*, 1988a), suggesting that L-arginine is the physiological precursor for the synthesis of nitric oxide in endothelial cells (Palmer *et al.*, 1988b; Sakuma *et al.*, 1988). Biochemical measurements have shown that endothelium-derived relaxing factor(s), like nitric oxide, enhances directly the activity of soluble guanylate cyclase and hence induces the production of guanosine 3':5'-cyclic monophosphate (cyclic GMP, Förstermann *et al.*, 1986; Ignarro *et al.*, 1986). The production of cyclic GMP mediates, at least partially, the relaxation of vascular tissues, as well as the inhibition of adhesion and aggregation and the disaggregation of platelets which have been observed in response to endothelium-derived relaxing factor(s) (Holzmann, 1982; Rapoport & Murad, 1983; Diamond & Chu, 1983; Murray *et al.*, 1986; Azuma *et al.*, 1986; Radomski *et al.*, 1987; Sneddon & Vane, 1988).

The present experiments were designed to investigate the effects of agents such as bradykinin, adenosine diphosphate and A23187 which enhance the production of endothelium-derived relaxing factor(s) in comparison to exogenous nitric oxide on the production of both cyclic GMP and adenosine 3':5'-cyclic monophosphate (cyclic AMP) in cultured aortic endothelial cells of the pig.

Methods

Cell culture

Segments (15 to 20 cm in length) were cut from freshly excised porcine thoracic aortae obtained from a nearby slaughterhouse and placed in ice-cold physiological salt solution (composition in mM: NaCl 118.3, KCl 4.7, CaCl_2 2.5, MgSO_4 1.2, KH_2PO_4 1.2, NaHCO_3 25.0, EDTA 0.026 and glucose 11.1) containing 1% of an antibiotic-antimycotic solution (penicillin $10,000 \text{ u ml}^{-1}$; streptomycin $10,000 \text{ u ml}^{-1}$ and fungizone $25 \mu\text{g ml}^{-1}$). Under aseptic conditions, the aortae were cleaned of connective tissue and washed in 700 ml of Earle's balanced salt solution containing 1% of the antibiotic-antimycotic solution. Each culture was obtained by scraping the intimal surface of four aortae with a sterile scalpel. The cells were collected in 15 ml of Earle's balanced salt solution and then centrifuged at $120g$ for 7 min. The pellet was resuspended in 5 ml of complete tissue culture medium [v/v medium 199 supplemented with 10% Ryan growth supplement, 1% of the antibiotic-antimycotic solution and

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40 $\mu\text{g ml}^{-1}$ heparin] and the collected sheets of endothelial cells were dissociated mechanically, by passing the cell suspension six times through a needle with a diameter of 0.7 mm. The suspension, composed of single and small clumps of cells, was then transferred to ten T-25 flasks. After 24 h of incubation at 37°C in an incubator (5% CO_2 in air, humidified), the culture medium was replaced with fresh medium which was subsequently changed every two to three days. The cells were subcultured without using enzymes. The cultures of endothelial cells were characterized by the presence of Factor VIII-related antigen and by the absorption of a fluorescent acetylated low density lipoprotein (DiI-Ac-LDL; Boulanger *et al.*, 1989).

Measurement of cyclic nucleotides

Endothelial cells (first and second passages) were cultured in 35 mm Petri dishes and were used when they had reached confluence and showed a typical cobblestone pattern. The culture medium was removed by aspiration and replaced twice with 2 ml of warm (37°C) physiological salt solution. After a 30 min equilibration period in the incubator, the incubation medium was replaced with physiological salt solution containing indomethacin (10^{-5} M) and 3-isobutyl-1-methyl xanthine (IBMX, 10^{-4} M) and the cells were then equilibrated for at least 30 min before the addition of a test compound or solvent. The incubation medium was removed by aspiration and the cells were extracted immediately with 0.6 ml of 6% trichloroacetic acid. The cells were then scraped off with a rubber policeman and the suspension collected in an Eppendorf tube. The cells remaining on the Petri dish were collected following a second addition of 0.4 ml of trichloroacetic acid and added to the first extract. The cell suspension was sonicated for 5 s before being centrifuged for 10 min at 2000 *g*. The supernatant was extracted with four volumes of water-saturated ether (anaesthetic grade) and lyophilized. Each sample was resuspended in 0.5 ml of sodium acetate buffer (0.05 M, pH 6.2) and the content of cyclic AMP and of cyclic GMP were determined following their acetylation by a cyclic AMP and a cyclic GMP kit from Biomedical Technologies Inc., (Stoughton, U.S.A.), respectively. The content of cyclic nucleotide was expressed as pmol/ 10^6 cells. The number of cells per dish for each culture was determined by counting with a haemocytometer single cell suspensions obtained after trypsin-EDTA digestion from 6 different dishes.

Preparation of oxyhaemoglobin and nitric oxide

Stock solutions of oxyhaemoglobin and nitric oxide (NO) were prepared as previously reported (Martin *et al.*, 1985; Palmer *et al.*, 1987).

Materials and drugs

Bradykinin, adenosine diphosphate (ADP), A23187, IBMX, methylene blue, superoxide dismutase, haemoglobin and indo-

methacin were obtained from Sigma Chemical Co. (St. Louis, MO). N^G -monomethyl-L-arginine (L-NMMA) and N^G -monomethyl-D-arginine (D-NMMA) were synthesized at the Wellcome Research Laboratories, Langley Court, Beckenham, Kent. Indomethacin was prepared daily in equimolar (10^{-3} M) concentrations of Na_2CO_3 . A23187 (10^{-3} M) and IBMX (10^{-1} M) were dissolved in pure dimethylsulphoxide and further diluted in distilled water. All drug concentrations are expressed in terms of the base. Media 199, Earle's balanced salt solution and the antibiotic-antimycotic solution were obtained from M.A. Bioproducts, Walkersville, MD, U.S.A.; Ryan growth supplement was purchased from Hyclone, Logan, UT, U.S.A.; all plastic flasks and dishes for cell culture were purchased from Falcon Lincoln Park, NJ, U.S.A.

Statistics

Data are presented as mean $\pm$ s.e.mean of two or three experiments performed on triplicated dishes; *n* represents the number of 35 mm Petri dishes studied. Statistical comparisons were made by Student's paired *t* test. *P* values less than 0.05 were considered to be statistically significant.

Results

Basal production of cyclic GMP

Treatment of endothelial cells for 5 min with haemoglobin (10^{-5} M) reduced significantly, by 27.5%, the content of cyclic GMP in endothelial cells from 0.080 ± 0.003 to 0.058 ± 0.003 pmol/ 10^6 cells (*n* = 5; *P* < 0.005). Incubation of endothelial cells for 30 min with methylene blue (10^{-5} M) also reduced significantly, by 35%, the content of cyclic GMP (from 0.080 ± 0.003 to 0.055 ± 0.002 pmol/ 10^6 cells, *n* = 6, *P* < 0.001). A 5 min incubation period with superoxide dismutase (500 u ml^{-1}) enhanced significantly the content of cyclic GMP by 1.75 fold (from 0.080 ± 0.003 to 0.140 ± 0.011 pmol/ 10^6 cells, *n* = 6, *P* < 0.005). Treatment of endothelial cells with L-NMMA (10^{-5} M, for 30 min) did not affect the basal content of cyclic GMP (0.19 ± 0.03 and 0.15 ± 0.02 pmol/ 10^6 cells, *n* = 9 in the absence or the presence of the compound, respectively) while a 30 min incubation period of the cells with D-NMMA (10^{-5} M) enhanced significantly the production of cyclic GMP by 1.7 fold (from 0.19 ± 0.03 to 0.33 ± 0.04 pmol/ 10^6 cells, *n* = 9, *P* < 0.01).

Stimulated production of cyclic GMP

Kinetic studies Bradykinin (10^{-7} M) stimulated the production of cyclic GMP from 0.26 ± 0.03 to 11.38 ± 1.99 pmol/ 10^6 cells (*n* = 9, *P* < 0.001) within 1 min; after 1 min, the effect of bradykinin on cyclic GMP accumulation declined with time. ADP (3×10^{-5} M), A23187 (10^{-6} M) and NO (2×10^{-6} M) also stimulated the production of cyclic GMP in cultured endothelial cells of the pig (Figure 1); this accumulation of cyclic GMP reached a maximum within less than

Figure 1 Effects of adenosine diphosphate (3×10^{-5} M; a), the calcium ionophore A23187 (10^{-6} M; b) and nitric oxide (2×10^{-6} M; c) on the accumulation of cyclic GMP in cultured porcine aortic endothelial cells as a function of time. The experiments were performed in the presence of indomethacin (10^{-5} M) and isobutyl-1-methylxanthine (10^{-4} M). The results are expressed as the mean of two (c) or three (a,b) different experiments performed on three dishes; s.e.mean shown by vertical bars.

Table 1 Effect of bradykinin, adenosine diphosphate (ADP), the calcium ionophore A23187 and nitric oxide on the accumulation of cyclic AMP in cultured porcine aortic endothelial cells

Time (s)	Cyclic AMP content (pmol/10 ⁶ cells)							
	0	20	30	40	60	120	300	
Control	2.63 ± 0.34	ND	ND	ND	2.79 ± 0.38	ND	2.74 ± 0.26	
Bradykinin (10 ⁻⁷ M)	—	ND	2.87 ± 0.25	ND	2.84 ± 0.33	2.88 ± 0.34	2.64 ± 0.21	
Control	2.63 ± 0.34	ND	ND	2.79 ± 0.38	ND	2.74 ± 0.26		
ADP (3 × 10 ⁻⁵ M)	—	ND	4.28 ± 0.62*	ND	4.51 ± 0.67*	4.84 ± 0.60*	4.51 ± 0.63*	
Control	1.07 ± 0.11	ND	ND	0.95 ± 0.08	ND	0.99 ± 0.10		
A23187 (10 ⁻⁶ M)	—	ND	0.97 ± 0.10	ND	0.97 ± 0.08	0.98 ± 0.07	0.92 ± 0.07	
Control	2.76 ± 0.16	ND	ND	ND	3.17 ± 0.18	ND	3.65 ± 0.28	
Nitric oxide (2 × 10 ⁻⁶ M)	—	3.05 ± 0.16	ND	2.99 ± 0.22	3.16 ± 0.20	2.75 ± 0.51	3.04 ± 0.17	

The experiments were performed in the presence of indomethacin (10⁻⁵ M) and isobutyl-1-methyl xanthine (10⁻⁴ M). The results are expressed as mean ± s.e.mean of two (nitric oxide) or three (ADP, A23187) different experiments performed on three dishes. The asterisks denote a significant difference between the cyclic AMP content of control (0s) and agonist-treated endothelial cells. ND = not determined.

1 min of stimulation and declined rapidly thereafter with ADP and NO (Figure 1a,c). The production of cyclic GMP evoked by A23187 was long-lasting, declining to about 75% of the maximal accumulation of cyclic GMP after 5 min of observation (Figure 1b).

Bradykinin, A23187 or NO did not affect the content of cyclic AMP in endothelial cells (Table 1). ADP (3 × 10⁻⁵ M) significantly increased the content of cyclic AMP to a plateau level of 1.7 fold basal content after 30s of stimulation ($P < 0.001$, Table 1).

Role of endothelium-derived relaxing factor(s) Treatment of endothelial cells with haemoglobin (10⁻⁵ M) for 5 min markedly inhibited the production of cyclic GMP stimulated by bradykinin (10⁻⁷ M, 1 min, $P < 0.001$), by A23187 (10⁻⁶ M, 1 min, $P < 0.005$), by ADP (3 × 10⁻⁵ M, 1 min, $P < 0.005$) and also that measured in response to a 20s incubation period with NO (2 × 10⁻⁶ M, $P < 0.001$) (Figure 2). The production of cyclic GMP in response to an activator of the particulate guanylate cyclase, α -human atrial natriuretic peptide (10⁻⁷ M, 1 min), was not affected by the incubation of endothelial cells with haemoglobin (10⁻⁵ M for 5 min, data not shown).

Figure 2 Effects of modulators of the biological action of endothelium-derived relaxing factor(s) on basal cyclic GMP content (solid columns) and stimulated cyclic GMP production (hatched columns) in the presence of (a) bradykinin (10⁻⁷ M, 1 min); (b) adenosine diphosphate (3 × 10⁻⁵ M, 1 min); (c) A23187 (10⁻⁶ M, 1 min) and (d) nitric oxide (2 × 10⁻⁶ M, 20s). The cells were incubated with haemoglobin (Hb, 10⁻⁵ M) or with superoxide dismutase (SOD, 500 U ml⁻¹) for 5 min or with methylene blue (MB, 10⁻⁵ M) for 30 min before the addition of a test compound or drug-vehicle. All experiments were performed in the presence of indomethacin (10⁻⁵ M) and isobutyl-1-methyl xanthine (10⁻⁴ M). The results are expressed as the mean of two different experiments performed on three dishes; s.e.mean shown by vertical bars.

Incubation of endothelial cells with methylene blue (10⁻⁵ M) reduced markedly the production of cyclic GMP detected in response to a 1 min stimulation period with bradykinin (10⁻⁷ M, $P < 0.001$), ADP (3 × 10⁻⁵ M, $P < 0.01$), A23187 (10⁻⁶ M, $P < 0.01$) and also that evoked by NO (2 × 10⁻⁶ M for 20s, $P < 0.001$) (Figure 2). Methylene blue (10⁻⁵ M) also reduced, but to a lesser extent, the production of cyclic GMP stimulated by α -human atrial natriuretic peptide (10⁻⁷ M for 1 min) from 1.01 ± 0.05 to 0.68 ± 0.02 pmol/10⁶ cells ($n = 6$, $P < 0.001$).

A 5 min incubation period with superoxide dismutase (500 U ml⁻¹) did not affect the production of cyclic GMP in response to bradykinin (10⁻⁷ M, 1 min), ADP (3 × 10⁻⁵ M, 1 min), A23187 (10⁻⁶ M, 1 min) or NO (2 × 10⁻⁶ M, 20s) (Figure 2).

Treatment of endothelial cells with L-NMMA (10⁻⁵ M, 30 min) significantly reduced the production of cyclic GMP stimulated by 1 min of exposure to bradykinin (10⁻⁷ M, $P < 0.001$), ADP (3 × 10⁻⁵ M, $P < 0.001$) and also to A23187 (10⁻⁶ M, $P < 0.01$) (Figure 3). The production of cyclic GMP stimulated by NO (2 × 10⁻⁶ M, 20s) was not affected by the treatment of endothelial cells with L-NMMA (10⁻⁵ M) (Figure 3). Incubation of endothelial cells with D-NMMA (10⁻⁵ M, 30 min) did not affect the production of cyclic GMP stimulated by a 1 min incubation of endothelial cells with bradykinin (10⁻⁷ M), ADP (3 × 10⁻⁵ M) or with A23187 (10⁻⁶ M)

Figure 3 Effect of N^G-monomethyl-L-arginine (L-NMMA, 10⁻⁵ M) and N^G-monomethyl-D-arginine (D-NMMA, 10⁻⁵ M) on basal and on bradykinin (BK, 10⁻⁷ M, 1 min), adenosine diphosphate (ADP, 3 × 10⁻⁵ M, 1 min), nitric oxide (NO, 2 × 10⁻⁶ M, 20s), A23187 (10⁻⁶ M, 1 min) or dimethylsulphoxide (DMSO, 0.1%, the solvent of the calcium ionophore, 1 min)-stimulated production of cyclic GMP (solid columns). The cells were incubated with L-NMMA (open columns) or D-NMMA (hatched columns) for 30 min before the addition of a test compound or solvent. All experiments were performed in the presence of indomethacin (10⁻⁵ M) and isobutyl-1-methyl xanthine (10⁻⁴ M). The results are expressed as the mean of three different experiments performed on three dishes; s.e.mean shown by vertical bars.

while that stimulated by NO (2×10^{-6} M, 20 s) was enhanced significantly by 1.5 fold (from 2.97 ± 0.24 to 4.35 ± 0.26 pmol/ 10^6 cells, $n = 9$, $P < 0.01$) (Figure 3).

Discussion

The present study indicates that bradykinin, ADP and A23187 markedly enhanced the production of cyclic GMP in cultured aortic endothelial cells of the pig. The enhanced production of cyclic GMP was almost abolished by treatment of endothelial cells with haemoglobin, a compound which binds endothelium-derived relaxing factor(s) (Martin *et al.*, 1985; Ignarro *et al.*, 1987); it was inhibited following the treatment of the cells with methylene blue, an inhibitor of the activation of soluble guanylate cyclase (Arnold *et al.*, 1977; Ignarro *et al.*, 1986). Treatment of endothelial cells with superoxide dismutase, a compound which protects endothelium-derived relaxing factor(s) from degradation probably by scavenging superoxide anions (Rubanyi & Vanhoutte, 1986; Gryglewski *et al.*, 1986), did not affect the production of cyclic GMP stimulated by bradykinin, A23187 and ADP. These observations indicate that under our experimental conditions, the agonist-stimulated production of endothelium-derived relaxing factor is not affected by superoxide anions. Nevertheless, taken together the data suggest that the enhanced production of cyclic GMP detected in response to bradykinin, ADP and to A23187 is mediated by an increased production of endothelium-derived relaxing factor(s) which in turn enhances the activity of endothelial soluble guanylate cyclase. The production of cyclic GMP in response to agents which stimulate the release of endothelium-derived relaxing factor(s) has also been observed in cultured human umbilical vein and porcine aortic endothelial cells (Brotherton, 1986; Martin *et al.*, 1988) although not in bovine aortic endothelial cells (Ganz *et al.*, 1986; Loeb *et al.*, 1987). The lack of agonist-stimulated accumulation of cyclic GMP may be related to the species, to the culture conditions (e.g., high versus low passages) and/or to the experimental conditions (e.g., presence or absence of a phosphodiesterase inhibitor). It is noteworthy that with increasing times in culture, vascular cells lose the ability to induce biological responses (Goldsmith *et al.*, 1984; Loeb *et al.*, 1987). In the case of cultured vascular smooth muscle cells, the production of cyclic GMP in response to sodium nitroprusside was completely lost after five passages indicating that the activity of soluble guanylate cyclase is labile during long-term culture (Loeb *et al.*, 1987). Furthermore, cultured endothelial cells possess phosphodiesterase activities that are likely to participate in the regulation of their cyclic nucleotide content (Lugnier & Schini, 1988) and which may not allow the estimation of the production of cyclic nucleotide when experiments are performed without the presence of a phosphodiesterase inhibitor (Hopkins & Gorman, 1981; Makarski, 1981; Brotherton, 1986).

The content of cyclic GMP in unstimulated porcine endothelial cells was augmented by treatment of the cells with superoxide dismutase and reduced following incubation of the cells with either haemoglobin or methylene blue. These observations indicate the existence of a tonic release of endothelium-derived relaxing factor(s) from unstimulated endothelial cells. The fact that superoxide dismutase increases the accumulation of cyclic GMP in unstimulated but not that in agonist-stimulated cells may be due to the presence of a small amount of superoxide anions which are able to reduce significantly the limited basal production of endothelium-derived relaxing factor without affecting that evoked by the agonists. Treatment of endothelial cells with L-NMMA, a specific inhibitor of the formation of NO from L-arginine (Rees *et al.*, 1989), did not affect the content of cyclic GMP of unstimulated endothelial cells. A possible explanation for the lack of effect of the antagonist is that, under basal conditions, the enzyme which synthesizes NO from L-arginine preferentially utilizes endogenous L-arginine rather than the L-

arginine analogue. A basal release of endothelium-derived relaxation factor(s) from cultured endothelial cells has also been obtained under bioassay conditions (Gryglewski *et al.*, 1986; De Nucci *et al.*, 1988; Boulanger *et al.*, 1989).

The production of cyclic GMP evoked by bradykinin, ADP and A23187 is impaired by L-NMMA but not by its D-enantiomer, D-NMMA. This inhibition of the production of cyclic GMP does not seem to be due to a non-specific effect of the L-NMMA as the production of the cyclic nucleotide detected in response to exogenous NO (this study) or to an atrial natriuretic peptide (unpublished observations) were not affected. These observations suggest that bradykinin, ADP and A23187 stimulate the production and/or release of NO or of NO-containing substances from endothelial cells which then enhances the activity of soluble guanylate cyclase and hence induces the accumulation of cyclic GMP.

D-NMMA increased the basal production of cyclic GMP and that evoked by nitric oxide. One possible explanation would be that the compound prolongs the half-life of endothelium-derived relaxing factor NO, possibly by scavenging superoxide anions; the fact that both basal and NO-stimulated production are augmented, whereas only the former is augmented by superoxide dismutase, argues against this hypothesis. Alternatively, D-NMMA may have a weak stimulating effect on guanylate cyclase, or possess some inhibitory action on cyclic GMP phosphodiesterases; these interpretations fail to explain why the compound did not affect the response to bradykinin, ADP and A23187. The present observations do not allow further speculation on the mechanism underlying this surprising effect.

Nitric oxide, a direct activator of soluble guanylate cyclase (Katsuki *et al.*, 1977; Arnold *et al.*, 1977), also enhanced markedly the content of cyclic GMP, further indicating that after a limited number of passages, porcine aortic endothelial cells in culture retain a functional soluble guanylate cyclase. In the case of NO, the production of cyclic GMP was short lasting, a response which may be explained by the oxidation of nitric oxide into the biologically inactive NO₂. Bradykinin and ADP also enhanced the production of cyclic GMP in a transient manner, suggesting a short-lasting release of NO-containing factor(s) and hence activation of soluble guanylate cyclase. In the case of bradykinin, this may be due to the desensitization of the kinin-receptors on the cell membrane (Luckhoff *et al.*, 1988). Alternatively, bradykinin and ADP may be degraded by ectoenzymes such as carboxypeptidases, angiotensin-converting enzymes and nucleoside diphosphatases into inactive or less active metabolites with regard to their ability to enhance the activity of soluble guanylate cyclase. However, when the release of endothelium-derived relaxing factor(s) is stimulated by a non-receptor operating agent such as A23187, the content of cyclic GMP is increased maximally after 30 s of stimulation and declined only slightly thereafter. Thus, the calcium ionophore, in contrast to bradykinin and ADP, may induce a long lasting production of NO-containing factor(s) by endothelial cells.

Neither bradykinin, A23187 nor NO affect the content of cyclic AMP in endothelial cells, indicating that cyclic AMP does not play an important role in the biological responses induced by these substances in cultured endothelial cells. However, ADP slightly but significantly increased the production of cyclic AMP. Such a response may possibly be related to the metabolism of adenosine diphosphate into adenosine, a substance which could stimulate the production of cyclic AMP in cultured endothelial cells from swine aorta (Goldman *et al.*, 1983).

In conclusion, substances that increase the production of endothelium-derived relaxing factor(s) markedly augment the content of cyclic GMP in porcine cultured aortic endothelial cells. The production of cyclic GMP probably is mediated by an enhanced production of NO or of NO-containing substance(s), which increases the activity of soluble guanylate cyclase and hence the production of cyclic GMP. Cyclic GMP may play an important second messenger role in the bio-

logical responses induced by various hormones and pharmacological agents in endothelial cells.

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