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CHARACTERIZATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASES FROM CULTURED BOVINE AORTIC ENDOTHELIAL CELLS

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Abstract—Experiments were carried out in order to isolate and characterize the cyclic nucleotide phosphodiesterase activities in primary and low passages of cultured bovine aortic endothelial cells. The subcellular characterization of the cyclic nucleotide hydrolytic activity showed that both cAMP and cGMP hydrolytic activities were predominant in the cytosolic rather than the particulate fraction of the endothelial cell homogenate. At a low substrate concentration (0.25 μM), the major hydrolytic activity was for cAMP while at a high concentration (20 μM) it was for both cAMP and cGMP. Both cAMP and cGMP hydrolytic activities were insensitive to calmodulin. Cytosolic cyclic nucleotide phosphodiesterase activity was resolved into two distinct phosphodiesterase forms using HPLC. The first eluted form was designated cGS-PDE: it hydrolysed both cAMP and cGMP and its cAMP hydrolytic activity was markedly enhanced by the presence of cGMP. The second form was designated cAMP-PDE: it selectively hydrolysed cAMP. The cytosolic cAMP-PDE was inhibited by micromolar concentrations of cAMP-PDE inhibitors such as trequinsin, rolipram, dipyridamole or papaverine. The cGS-PDE was inhibited by micromolar concentrations of trequinsin, dipyridamole and papaverine and was insensitive to rolipram, except for the hydrolysis of cAMP which was inhibited in the micromolar range. Both the cAMP-PDE and the cGS-PDE were relatively insensitive to the selective cGMP-PDE inhibitor, zaprinast which was about 750-fold less potent on endothelial PDEs than on smooth muscle cGMP-PDE. The identification of selective and specific PDE inhibitors of the different PDE forms may allow a better understanding of the regulation and the role of cyclic nucleotides in endothelial cells.

Vascular endothelial cells separate the circulation from the surrounding vessel wall and play an important role in the regulation of many physiological processes such as haemostasis, angiogenesis and generation of vascular smooth muscle tone. Endothelial cells modulate vascular tone not only by producing potent vasorelaxing substances, such as prostacyclin [1] and endothelium-derived relaxing factor (EDRF, [2]), but also by producing vasoconstricting substances such as endothelium-derived constricting factor [3] or endothelin [4]. These substances may be generated and released in response to a number of physiological and pharmacological stimuli but their control mechanisms within these cells are not well-defined. Several reports have indicated that stimulation of cultured endothelial cells by various vasoactive agonists (prostacyclin, isoproterenol, bradykinin, acetylcholine, atriopeptins) increase either adenosine 3′,5′-cyclic monophosphate (cAMP)‡ or guanosine 3′,5′ cyclic monophosphate (cGMP) levels [5–9] or both. The metabolism and biological function of cyclic nucleotides in endothelial cells is poorly understood. In the case of human umbilical vein endothelial cells, basal and agonist (prostacyclin, PGH_2)-stimulated production of cAMP was markedly enhanced in the presence of millimolar concentrations of isobutylmethylxanthine (IBMX), a non-selective phosphodiesterase inhibitor [7, 10]. Atrial natriuretic peptide-stimulated production of cGMP in bovine aortic endothelial cells was also enhanced in the presence of a high concentration (0.5 mM) of IBMX [8]. These observations suggest that endothelial cells apparently have high rates of cyclic nucleotide phosphodiesterase activities which may participate in the regulation of the cellular levels of cyclic nucleotides and hence their responses to changes in cyclic nucleotide production. In various tissues, multiple forms of cyclic nucleotide phosphodiesterase have been identified based on their chromatographic behaviour, substrate specificity and also on their sensitivity to calmodulin and to various reference inhibitory drugs [11]. In vascular smooth muscle, three major phosphodiesterase forms have been isolated and characterized (i) a calmodulin-sensitive form which preferentially hydrolyses cGMP, named calmodulin-PDE; (ii) a form which specifically hydrolyses cGMP and is insensitive to calmodulin, designated cGMP-PDE and (iii) a form which specifically hydrolyses cAMP, called cAMP-PDE [12].

The purpose of the present study was: (1) to characterize the different cyclic nucleotides PDE activities in cultured bovine aortic endothelial cells and (2) to test the effect of various selective and non-selective PDE inhibitors. A preliminary report of some of these observations has been made [13].

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‡ Abbreviations: PDE, phosphodiesterase; CaM, calmodulin; cAMP, adenosine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; EGTA, ethylene glycol bis (β-aminoethyl ether)*N*,*N*,*N*′,*N*′tetra-acetic acid.

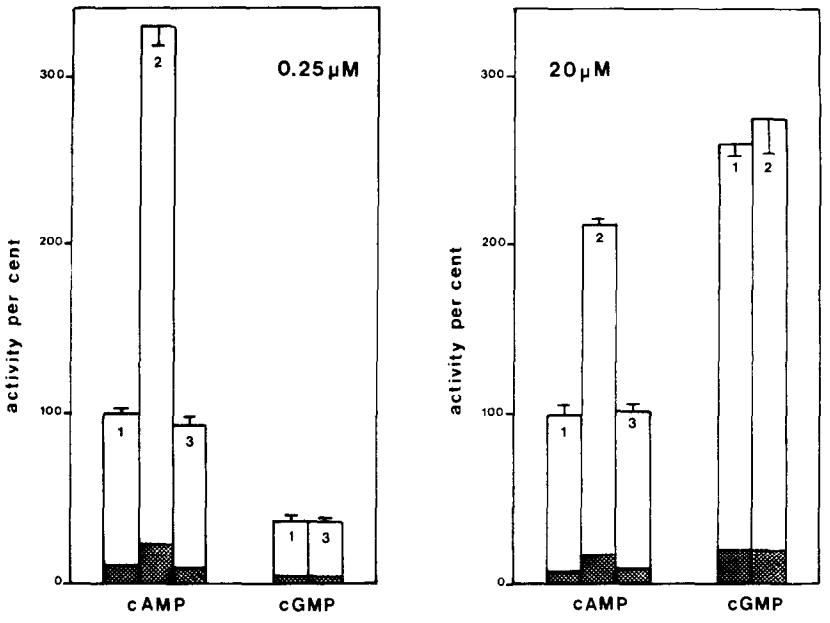

Fig. 1. Subcellular distribution of aortic endothelial phosphodiesterase activities at 0.25 μM (left panel) and 20 μM (right panel) substrate concentration. PDE activities were determined in cytosolic fraction (□) and particulate fraction (▨) for the following conditions: (1) in the presence of EGTA (1 mM) without cGMP, (2) or with 5 μM cGMP, (3) and in the presence of calcium ($CaCl_2$ 10 μM) and calmodulin (18 nM). Results were expressed as % of cAMP hydrolytic activity in the presence of EGTA and represent the mean ± SE of three determinations on different enzyme preparations. Left panel: 100% hydrolytic activity = 65 nmol/min/30 × 10^6 cells. Right panel: 100% hydrolytic activity = 1.98 μmol/min/30 × 10^6 cells.

MATERIALS AND METHODS

Cultured bovine aortic endothelial cells. Bovine aortic endothelial cells were cultured and harvested as previously described [9]. Briefly, freshly excised bovine thoracic aortae were obtained from a nearby abbatoir and placed in an ice-cold Ca^{2+}- and Mg^{2+}-free salt solution (composition in mM: NaCl, 112; $NaHCO_3$, 25; KH_2PO_4, 1; and glucose, 11.5), supplemented with an antibiotic–antimycotic mixture (1%; Gibco, Glasgow, U.K.). The aortae were opened longitudinally and pinned out flat with the luminal face being upwards and washed abundantly with Ca^{2+}- and Mg^{2+}-free salt solution. Endothelial cells were harvested by lightly scraping the intimal surface with a scalpel blade and were further dissociated by collagenase treatment (120 U/ml CLS II for 20–30 min; Cooper Biomedical, Freehold, NJ). After centrifugation (twice 120 *g* for 10 min at room temperature), the cell pellet was resuspended in culture medium [v/v Dulbecco modified Eagle's medium (Eurobio) and Ham's F 12 medium (Eurobio)], supplemented (to final concentrations) with 16% heat-inactivated fetal calf serum (IBF, Paris, France), 2.2 mM L-glutamine, 0.02 mM ascorbic acid, 0.8% non essential amino acids (Gibco), 1% antibiotic–antimycotic solution and 100 μg/ml heparin sodium salt (Choay, Paris, France) and seeded onto plastic flasks (Nunc, Copenhagen, Denmark). The culture medium was replaced after a 2 hr incubation period at 37° in a 5% CO_2 humidified incubator and thereafter every 2–3 days. Endothelial cells were subcultured by passaging confluent cells with a trypsin (1.5%)–EDTA (0.15%) mixture (Eurobio).

The endothelial cells were characterized [9] by their typical cobblestone appearance at confluence, by the selective uptake of a fluorescent acetylated-low density lipoprotein, DiI-Ac-LDL [14] and by the presence of Factor VIII-related antigen.

Phosphodiesterase preparations. Confluent cultures of endothelial cells were washed twice with salt solution (composition in mM: NaCl, 112; $NaHCO_3$, 25; KH_2PO_4, 1; glucose, 11.5; $CaCl_2$, 1.25; and $MgSO_4$, 1.2) at 37° in order to remove the cell culture medium. Endothelial cells were harvested by scraping in isotonic homogenizing buffer (composition in mM: saccharose, 250; Tris–HCl, 20 (pH 7.5); Mg acetate, 2; dithiothreitol, 1; EDTA, 5; with 2000 U/ml aprotinin, 10 μg/ml soya bean trypsin inhibitor, 10 μg/ml leupeptin and 1 mg/ml bovine serum albumin). All manipulations were conducted at 4°. Cell suspensions (approximately 10 × 10^6 cells in 1 ml) were homogenized with an ultra-turrax homogenizer (three times, 10 sec), and then centrifuged at 105,000 *g* for 60 min. The supernatant (cytosolic fraction) was fractionated into aliquots and stored at −80°. The particulate fraction was rehomogenized with 1 ml of homogenizing buffer, and then centrifuged at 1000 *g* for 20 min. The particulate fraction (1000 *g*–105,000 *g* pellet) was rehomogenized and divided into aliquots for storage at −80°. In order to separate cytosolic cyclic nucleotide PDE activity, the 105,000 *g* supernatant was injected onto an HPLC column (Mono Q HR 5/5, 8 × 75 mm, Pharmacia LKB), washed for 20 min (0.8 ml/min) with elution buffer A (20 mM Tris–HCl, 2 mM Mg acetate, 1 mM dithiothreitol, pH 7.5) and eluted by a linear NaCl (0.06–0.30 M in buffer

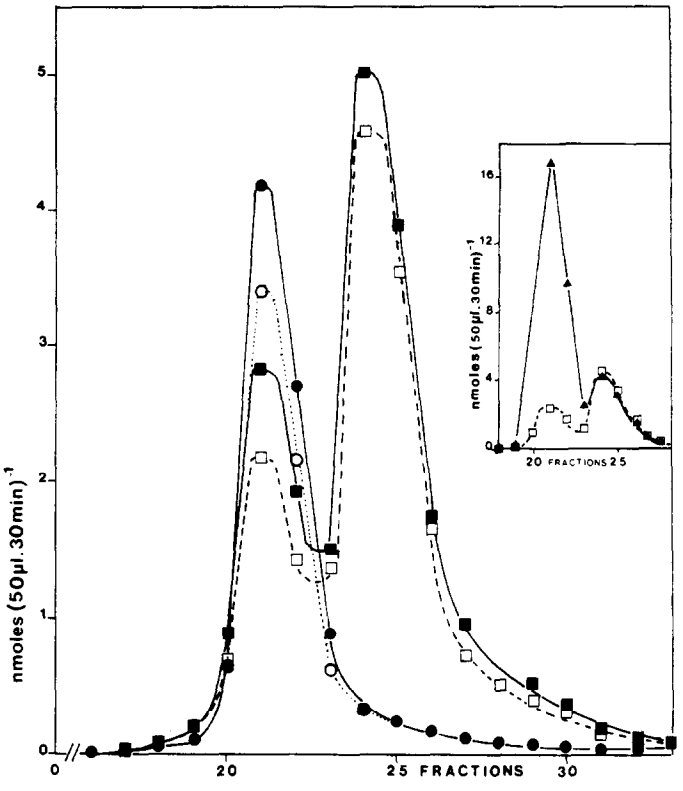

Fig. 2. Representative elution profile of cytosolic bovine aortic endothelial PDE activity separated by HPLC. PDE activity was determined using 1 μM ^{3}H-cGMP + 1 mM EGTA (○), 1 μM ^{3}H-cGMP + 18 nM calmodulin + 10 μM $CaCl_2$ (●), 1 μM ^{3}H-cAMP + 1 mM EGTA (□), 1 μM ^{3}H-cAMP + 18 nM calmodulin + 10 μM $CaCl_2$ (■) and 1 μM ^{3}H-cAMP + 5 μM cGMP + 1 mM EGTA (▲). Inset represents the stimulation of cGS-PDE by cGMP.

A) gradient. Each fraction (0.8 ml) was tested for PDE activity. Fractions under each PDE activity peak were pooled, dialysed against buffer A and stored in aliquots at −80°.

Phosphodiesterase assay. Phosphodiesterase activities were determined according to the method described by Keravis *et al.* [15] either in the presence of $CaCl_2$ (10 μM) and an excess of calmodulin (18 nM) or in the absence of calmodulin and in presence of 1 mM EGTA. Calmodulin was purified from bovine brain as described previously [16]. Kinetic studies were carried out with substrate concentrations ranging from 0.1 to 100 μM. Both incubation time and enzyme concentration in the assay medium were adjusted so that no more than 15% of the substrate was hydrolysed under the assay conditions.

Materials. Trequinsin (a gift of Hoechst, Paris, France), 1,3-isobutylmethylxanthine (IBMX) and papaverine (Sigma Chemical Co., St. Louis, MO) were dissolved in distilled water. SKF 94120 (a gift of Smith Kline & French, Welwyn, U.K.), compound AAL 05 [17] and rolipram (ZK 62711, Schering AG, F.R.G.) were dissolved in dimethyl sulfoxide. The final solvent concentration of 1% did not modify the enzymatic activities. Zaprinast (M&B 22,948, a gift of May & Baker Ltd., Dagenham, U.K.) was dissolved in 0.1 N NaOH, dipyridamole was dissolved in 0.1 N HCl and then neutralized. Aprotinin (Iniprol) was obtained from Laboratories Choay (Paris, France).

Data analysis. Values of apparent Michaelis–Menten constants (K_m) and maximal velocity (V_{max}) values were determined by computer analysis using the method of Cleland [18]. The slope of the inhibition curves and the concentrations of each drug which inhibited the enzymatic activity by 50% (IC_{50}) were determined at a substrate concentration of either 0.25 μM or 20 μM and were calculated by a non-linear regression [19]. Apparent K_i values were calculated using the method of Cheng and Prussoff [20]. Results are expressed as means ± SE of at least three determinations made in duplicate.

RESULTS

Subcellular distribution of PDE activity

The particulate and the soluble fractions of the homogenates from endothelial cells hydrolysed both cAMP and cGMP (Fig. 1). More than 80% of both the cAMP and cGMP hydrolytic activities were detected in the cytosolic (105,000 *g* supernatant) fraction. Addition of calmodulin and Ca^{2+} to the assay did not significantly affect either the cAMP or the cGMP hydrolytic activities measured in the cytosolic or in the particulate fractions, suggesting the absence of a calmodulin-sensitive PDE. The addition of cGMP (5 μM) enhanced the cAMP

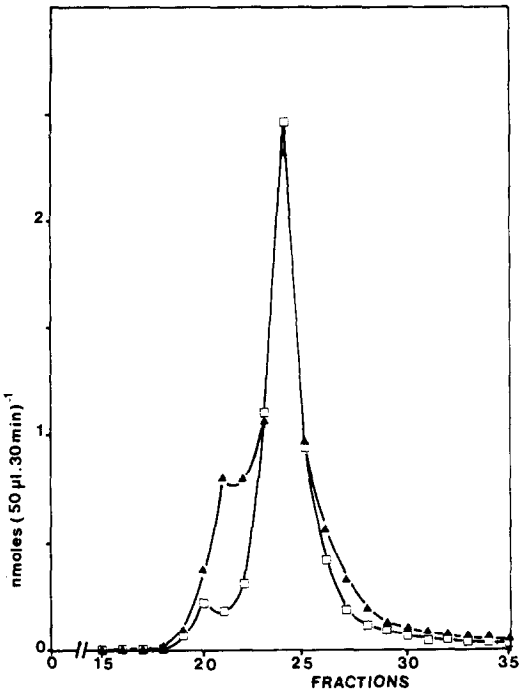

Fig. 3. Rechromatography of aortic endothelial cAMP-PDE by HPLC. PDE activity was determined using 1 μM ^{3}H-cAMP + 1 mM EGTA (□) and 1 μM ^{3}H-cAMP + 5 μM cGMP + 1 mM EGTA (▲). Fractions 23–29 from Fig. 2 were pooled, dialysed and eluted by HPLC as indicated in Materials and Methods.

hydrolytic activity of both the cytosolic and the particulate fractions at substrate concentrations assayed (by about 3-fold at 0.25 μM and 2-fold at 20 μM).

At a low cyclic nucleotide concentration (0.25 μM), the major hydrolytic activity was for cAMP in both the cytosolic and particulate fractions: it was about 2.5-fold greater than the cGMP hydrolytic activity both in the presence or absence of calmodulin and the stimulated cAMP hydrolytic activity (determined in the presence of 5 μM cGMP and EGTA) was about 9-fold greater than the cGMP hydrolytic activity (Fig. 1, left panel). At a high cyclic nucleotide concentration (20 μM), the major hydrolytic activity was for cGMP in both the cytosolic and particulate fractions. The cytosolic cGMP hydrolytic activity was about 2.5-fold greater than the cAMP hydrolytic activity and about 1.2-fold greater than the stimulated cAMP hydrolytic activity (Fig. 1, right panel). Similarly, addition of calmodulin to the assay under these conditions did not affect the cyclic nucleotide phosphodiesterase activity.

Isolation of cytosolic PDEs

The phosphodiesterase activity of the 105,000 *g* supernatant was resolved by HPLC into two major PDE activity peaks (Fig. 2). The first eluted peak hydrolysed both cGMP and cAMP and neither activity was affected significantly by the addition of calmodulin and Ca^{2+}. The ^{3}H-cAMP hydrolytic activity was markedly enhanced, by about 6-fold, by the addition of cGMP (5 μM; Fig. 2 inset). This PDE form was designated cGS-PDE (cGMP-stimulated-

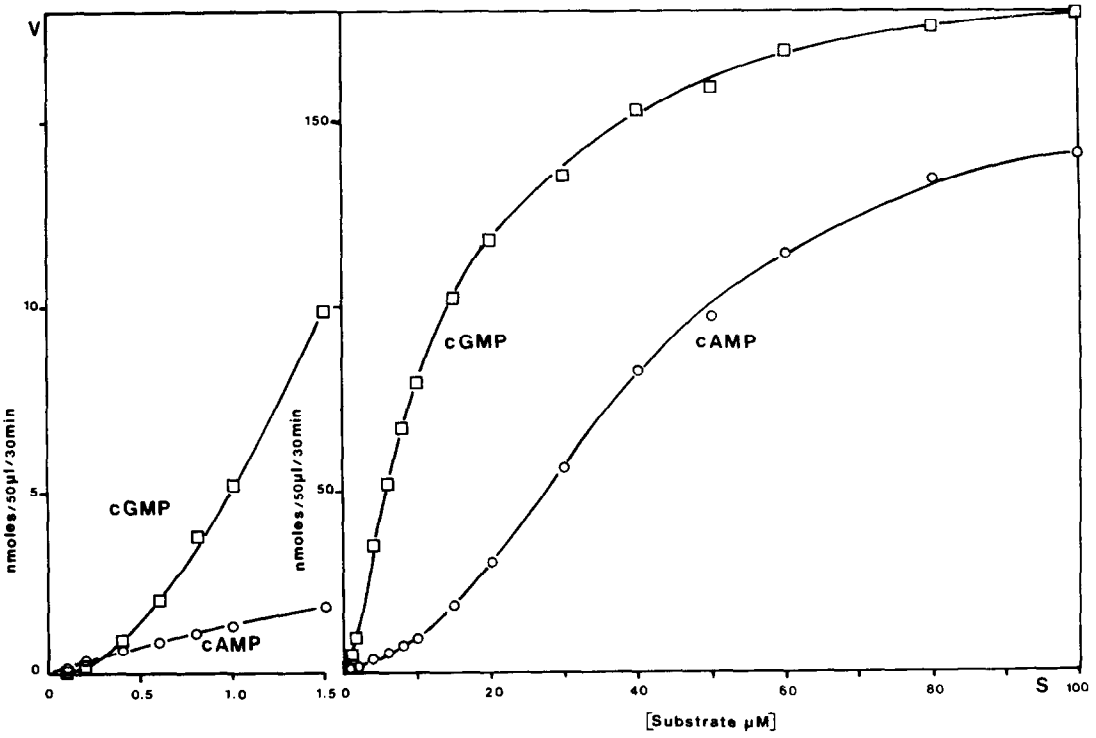

Fig. 4. The effect of substrate concentration on cyclic nucleotide hydrolysis by cGS-PDE. ^{3}H-cAMP (○) and ^{3}H-cGMP (□). Hydrolysis was measured for substrate concentrations of 0.1 to 1.5 μM (left panel) and 0.1 to 100 μM (right panel).

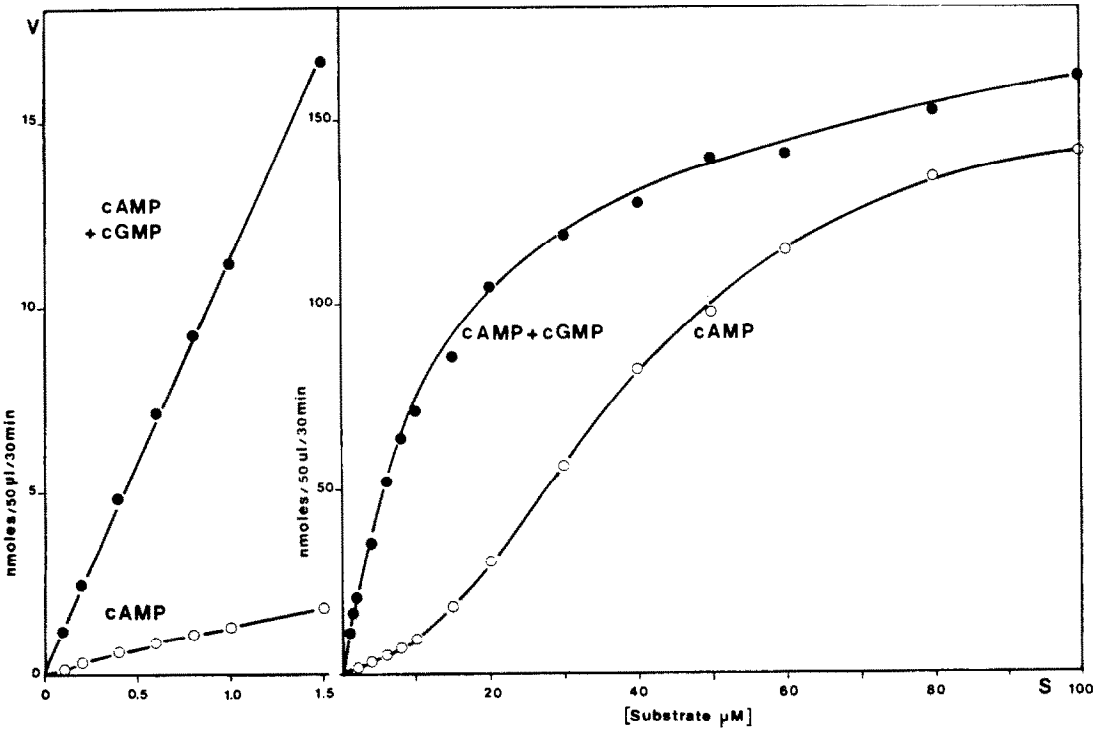

Fig. 5. The effect of substrate concentration on cAMP hydrolysis by cGS-PDE, in the absence (○) and in the presence of 5 μM cGMP (●). Hydrolysis was measured for substrate concentrations of 0.1 to 1.5 μM (left panel) and 0.1 to 100 μM (right panel).

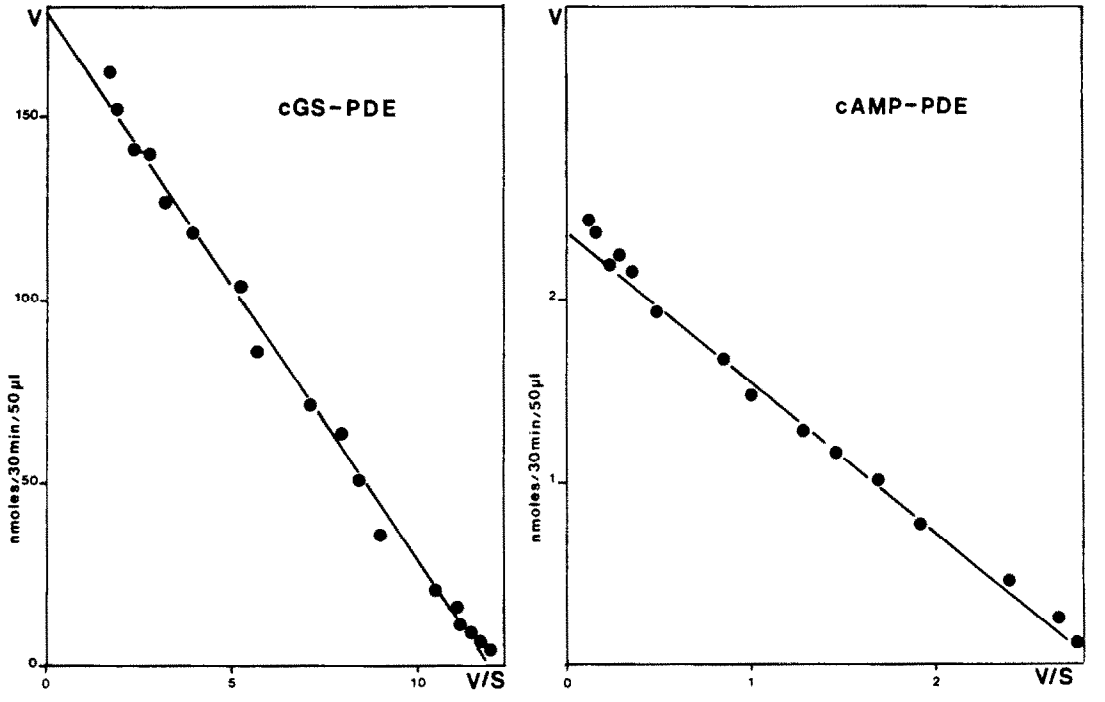

Fig. 6. Eadie-Hofstee plots of ^{3}H-cAMP hydrolysis by cGS-PDE in the presence of cGMP (left panel) and cAMP-PDE (right panel). The results are means of duplicate determinations and are representative of three experiments from three different preparations.

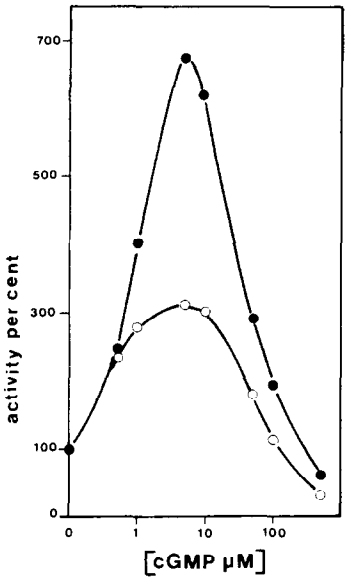

Fig. 7. The effect of increasing cGMP concentrations on the hydrolysis of cAMP by cGS-PDE. Hydrolytic activity was determined at 0.25 μM cAMP (●) with 100% = 250 pmol/min/mg and at 20 μM cAMP (○) with 100% = 17 nmol/min/mg.

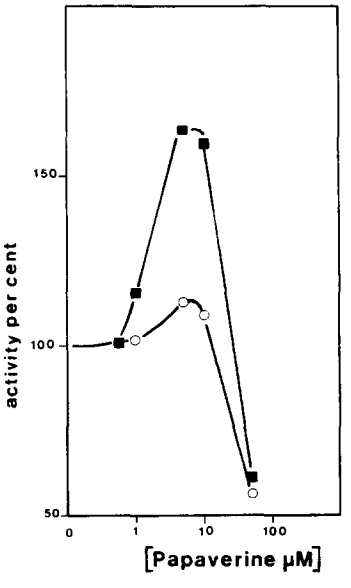

Fig. 8. The effect of increasing concentrations of papaverine on cGS-PDE. Hydrolytic activity was determined at 0.25 μM cGMP (■) with 100% = 194 pmol/min/mg and at 20 μM cAMP (○) with 100% = 17 nmol/min/mg.

PDE). The second eluted peak hydrolysed specifically cAMP and was insensitive to calmodulin and Ca^{2+} and to cGMP (Fig. 2 inset and Fig. 3). This form of PDE was called cAMP-PDE. A further purification of this form clearly showed its insensitivity to cGMP and its homogeneity (Fig. 3).

The representation of the hydrolysis velocity versus the substrate concentration for cGS-PDE for both ^{3}H-cAMP and ^{3}H-cGMP is illustrated in Fig. 4. In a concentration range below 0.25 μM, the cAMP hydrolytic activity was slightly but significantly greater than the cGMP hydrolytic activity (Fig. 4, left panel) while at higher substrate concentrations, the cGMP hydrolytic activity was markedly greater than the cAMP hydrolytic activity. The maximal velocity of cAMP hydrolysis represented about 80% of the maximal cGMP hydrolysis velocity (110 nmol/min/mg). The hydrolysis of cGMP was characterized by a positive homotropic cooperativity with a Hill coefficient of 1.2. The cAMP hydrolytic activity of cGS-PDE was markedly increased by the addition of cGMP (5 μM); the increase was about 9-fold at 1.5 μM of ^{3}H-cAMP (Fig. 5). At concentrations of cAMP greater than 40 μM, the cAMP hydrolytic activity was in the same range in the presence and absence of cGMP (5 μM) and the maximal velocity for the hydrolysis of cAMP was about 90% of that measured in the presence of cGMP. This enzymatic behaviour of cGS-PDE demonstrated that cGS-PDE is allosterically regulated by cGMP. The Eadie-Hofstee plot of the cAMP hydrolytic activity by the cGS-PDE in the presence of cGMP showed a Michaelis–Menten behaviour with an apparent K_m value of 15 ± 2 μM and a V_{max} of 100 nmol/min/mg (correlation coefficient = 0.997; Fig. 6, left panel).

The Eadie–Hofstee plot of the kinetics of the cAMP-PDE form was fitted with an apparent K_m value of 0.91 ± 0.7 μM and a V_{max} of 7 nmol/min/mg (correlation coefficient = 0.992), indicating that this enzyme also has a Michaelis–Menten behaviour (Fig. 6, right panel).

Stimulation of cGS-PDE

To characterize the stimulatory effect of cGMP on the hydrolysis of cAMP by cGS-PDE, the effect of increasing concentrations of cGMP was investigated at a low (0.25 μM) and high (20 μM) concentrations of ^{3}H-cAMP (Fig. 7). The maximal stimulatory effect of cGMP was obtained at 5 μM; it was about 7-fold at a ^{3}H-cAMP concentration of 0.25 μM and about 3-fold at 20 μM. Concentrations of cGMP greater than 100 μM inhibited the cAMP hydrolytic activity (IC_{50} = 600 μM at a ^{3}H-cAMP concentration of 0.25 μM, and 300 μM at 20 μM).

As PDE inhibitors can compete with cyclic nucleotides at the enzyme site, the possibility that these compounds may interact with the allosteric site was investigated by testing the effects of increasing concentrations of reference inhibitory drugs on the hydrolytic activities of cGS-PDE. At a low concentration of ^{3}H-cGMP (0.25 μM), phosphodiesterase inhibitors such as papaverine (5–10 μM) stimulated the cGMP hydrolytic activity by more than 1.5-fold (Fig. 8). Similar stimulations were obtained with other PDE inhibitors: trequinsin 1.2-fold at 1 μM; dipyridamole 1.3-fold at 1 μM; AAL 05 1.2-fold at 10 μM; IBMX 1.4-fold at 10 μM; zaprinast 1.5-fold at 100 μM (data not shown). At high concentrations of ^{3}H-cGMP (20 μM), all tested compounds exerted an inhibitory effect (see later). In the case of the hydrolysis of cAMP at a low substrate concentration (0.25 μM), the PDE inhibitors exerted an inhibitory effect (see below). However, at a high ^{3}H-cAMP concentration (20 μM), the PDE inhibitors had a slight stimulatory effect. For example, papaverine increased the hydrolytic activity by about

Table 1. The effects of PDE inhibitors on the cGS-PDE form isolated from endothelial cells. IC_{50} values were obtained at different substrate concentrations with and without 5 μM cGMP

Compounds	cGS-PDE (IC_{50}, μM)			
^{3}H-Substrate 5 μM cGMP	0.25 μM cAMP −	0.25 μM cAMP +	20 μM cAMP +	20 μM cGMP −
Trequinsin	1.7 ± 0.2	1.4 ± 0.2	0.3 ± 0.1	0.2 ± 0.04
Rolipram	3.0 ± 0.3	NS	NS	NS
Dipyridamole	2.8 ± 0.3	1.9 ± 0.2	4.5 ± 0.3	3.7 ± 0.4
Papaverine	9.1 ± 0.5	5.6 ± 0.4	12 ± 0.6	7.9 ± 0.5
IBMX	17 ± 1	8 ± 0.5	17 ± 2	13 ± 1
AAL 05	83 ± 7	17 ± 2	28 ± 3	20 ± 2
Zaprinast	135 ± 12	75 ± 6	174 ± 19	90 ± 7

Results are the mean ± SE of three determinations made on different enzyme preparations. NS, not significant.

1.1-fold at 10 μM (Fig. 8). Similarly, small stimulatory effects were obtained with: trequinsin 1.1-fold at 1 μM; dipyridamole 1.2-fold at 1 μM; AAL 05 1.04-fold at 10 μM; IBMX 1.07-fold at 10 μM; zaprinast 1.07-fold at 100 μM (data not shown).

Inhibition of cytosolic PDEs by selective and non-selective PDE inhibitors

To characterize further the different molecular forms of cytosolic PDEs present in the supernatant of endothelial cell homogenates, the inhibitory effects of various phosphodiesterase inhibitors on each isolated enzyme were determined.

The inhibition of cGS-PDE by PDE inhibitors was studied for the hydrolysis of ^{3}H-cAMP or ^{3}H-cGMP alone and for ^{3}H-cAMP in the presence of cGMP (5 μM). The IC_{50} values found were shown in Table 1.

Due to the allosteric behaviour of cGS-PDE, the inhibitory effects were determined at a low (0.25 μM for ^{3}H-cAMP) or high (20 μM for ^{3}H-cGMP) substrate concentration and at both 0.25 and 20 μM for the hydrolysis of cAMP in the presence of cGMP (5 μM). The order of potency for inhibition of cGS-PDE in each condition was trequinsin > dipyridamole > papaverine > IBMX > AAL 05 > zaprinast ⩾ rolipram. Note that rolipram only inhibited significantly the cGS-PDE at a substrate concentration of 0.25 μM ^{3}H-cAMP (the IC_{50} value was about 3 μM). The addition of cGMP (5 μM) significantly decreased the IC_{50} values of dipyridamole, papaverine, IBMX, AAL 05 and zaprinast. For example, AAL 05 was about four-fold more potent in the presence of cGMP than in its absence. At a high substrate concentration (20 μM), the PDE inhibitors similarly inhibited the hydrolysis of both cAMP and cGMP. Apparent K_i values for dipyridamole (1.9 μM), papaverine (5.3 μM), IBMX (7.6 μM), AAL 05 (14 μM) and zaprinast (75 μM) were calculated since the compounds competitively inhibited the hydrolysis of cAMP by cGS-PDE in the presence of cGMP (5 μM).

The apparent K_i values of PDE inhibitors on endothelial cell cAMP-PDE (Table 2) were compared to those obtained previously for the low K_m cAMP-PDE enzyme from bovine aortic smooth muscle [12] as shown in Table 2. For the endothelial cAMP-PDE, the order of potency for inhibition of cAMP hydrolysis was trequinsin > rolipram > dipyridamole > papaverine > IBMX > AAL 05 > zaprinast while the compound SKF 94120 was relatively inactive (inhibition of about 25% at a concentration of 100 μM; data not shown). In the case of the smooth muscle tissue, the order of potency was trequinsin > papaverine > rolipram > IBMX > AAL 05 > dipyridamole > zaprinast.

Table 2. Apparent K_i values obtained for cAMP-PDE form isolated from endothelial cells and vascular smooth muscle from bovine aorta

Compounds	Apparent K_i on isolated cAMP-PDE (μM)	
	Endothelial cells	Smooth muscle*
Trequinsin	0.3 ± 0.1	0.1 ± 0.03
Rolipram	0.8 ± 0.3	1.5 ± 0.3
Dipyridamole	1.3 ± 0.2	3.9 ± 0.9
Papaverine	1.4 ± 0.2	0.9 ± 0.3
IBMX	6.8 ± 1	2.7 ± 0.5
AAL 05	24 ± 3	3.6 ± 0.6
Zaprinast	82 ± 5	74 ± 3

Results are expressed as mean ± SE of three determinations made on different enzyme preparations.
* Results are taken from Ref. 12 (K_m = 0.4 μM).

A significant correlation ($r = 0.966$, $P < 0.005$) between the log K_i values of cAMP-PDE from both endothelial cells and smooth muscle tissue was obtained, suggesting that the two enzyme forms were similar. However, in the case of AAL 05, the K_i value for inhibition of the cAMP-PDE from vascular smooth muscle was about seven-fold less than in endothelial cells.

DISCUSSION

The present investigations demonstrate that cultured bovine aortic endothelial cells hydrolyse both cAMP and cGMP. The subcellular characterization of the cyclic nucleotide phosphodiesterase activities indicated that more than 80% of the hydrolytic activity was associated with the cytosolic fraction of the endothelial cell homogenates. The characterization of the cytosolic hydrolytic activity by HPLC indicated the presence of a cAMP-PDE and

of a cGS-PDE (which hydrolysed both cAMP and cGMP and whose cAMP hydrolytic activity was markedly enhanced in the presence of cGMP).

For low concentrations of cyclic nucleotides (0.25 μM), the subcellular distribution of PDE activity showed that cAMP was preferentially hydrolysed compared to cGMP and this hydrolytic activity was associated with the cytosol. A preferential hydrolysis of cAMP compared to cGMP has also been observed in a crude homogenate of freshly isolated endothelial cells from rabbit aorta (the cAMP hydrolytic activity was about 2.7-fold greater than for cGMP at a substrate concentration of 0.4 μM; [21]). These observations suggest that at low cyclic nucleotide concentrations, endothelial cells apparently have a high cAMP hydrolytic activity which may participate in the regulation of cAMP levels in these cells. At a high concentration of cyclic nucleotide (20 μM), both cAMP and cGMP were hydrolysed similarly by the cytosolic fraction of the endothelial cells homogenate. The hydrolysis of cAMP was markedly enhanced by the presence of cGMP. These observations are in accordance with the fact that the production of cAMP in human umbilical vein endothelial cells evoked by prostacyclin is detected only in the presence of IBMX, a non-selective cyclic nucleotide PDE inhibitor [7, 10]. Forskolin induced the production of cAMP and an inhibition of endothelial cell proliferation, with both effects being markedly enhanced in the presence of PDE inhibitors such as IBMX, rolipram and dipyridamole [22]. The basal production of cGMP in porcine aortic endothelial cells and that simulated by atrial natriuretic factor (8–33) in bovine aortic endothelial cells were greatly increased by treatment of the cells with zaprinast and IBMX, respectively [23, 8]. These observations indicate that the phosphodiesterases participate actively in the regulation of cAMP and cGMP levels and hence are of importance in the biological responses to changes in cyclic nucleotide production in endothelial cells.

The cyclic nucleotide hydrolytic activity of both the cAMP-PDE and the cGS-PDE were insensitive to calmodulin. This observation is surprising as a calmodulin activity has been detected in lysates of cultured porcine aortic endothelial cells [24] and a calmodulin-stimulated cyclic nucleotide hydrolytic activity was reported in several vascular tissues such as vascular smooth muscle and heart [11]. The lack of a calmodulin-stimulated PDE in endothelial cells is not due to the isolation procedure used since under similar conditions, a calmodulin-stimulated PDE has been identified in vascular smooth muscle and in brain tissue [25]. Nevertheless, cyclic nucleotide PDE activities which are insensitive to calmodulin have also been identified in human lymphocytes [26].

The characterization of the cytosolic cAMP-PDE indicated an apparent Michaelis–Menten behaviour with a K_m value of 0.9 μM, suggesting a high affinity enzyme with only one hydrolytic site. The K_m value of this cAMP-PDE is similar to the low K_m value reported for bovine aortic smooth muscle cAMP-PDE (K_m = 0.4 μM, [12]). Various PDE inhibitors had similar effects on the cAMP-PDE from cultured bovine aortic endothelial cells and on that from bovine aortic smooth muscle [12] suggesting that the cAMP-PDE forms are similar in these two cell types. Furthermore, SKF 94120 (up to a concentration of 100 μM) and cGMP (5 μM) were ineffective as inhibitors of endothelial cell cAMP-PDE, while in cardiac ventricle, SKF 94120 and cGMP specifically inhibited the cAMP-PDE (K_i = 0.8 μM and 0.1 μM, respectively [27, 28]. These observations suggest that endothelial cells and cardiac ventricular tissues have different forms of cAMP-PDE.

The characterization of cGS-PDE showed that the hydrolysis of cyclic AMP was enhanced by the presence of cGMP, indicating that the enzyme is allosterically regulated by cGMP. A similar cGS-PDE was purified to homogeneity from bovine heart and adrenal gland [28, 29] and from calf liver [30], but was absent from human, bovine and rat aortic smooth muscles [12]. In the presence of cGMP, the cGS-PDE was characterized by a single K_m for cAMP of 15 μM. An increased hydrolysis of cAMP by cGS-PDE in response to production of cGMP may participate in the regulation of cAMP levels. This may be one of the physiological effects of increases in cGMP in endothelial cells. An indirect effect of cGMP via the regulation of cAMP levels has also been proposed in adrenal gland and in cardiac tissue [28].

The cGS-PDE hydrolytic activities studied at a substrate concentration of 0.25 μM for ^{3}H-cAMP and of 20 μM for ^{3}H-cGMP were inhibited similarly by various PDE inhibitors. It is worthwhile to mention that zaprinast only inhibited the hydrolysis of cGMP by cGS-PDE at high concentrations (IC_{50} = 90 μM). In contrast, at a substrate concentration of 20 μM for ^{3}H-cAMP and of 0.25 μM for ^{3}H-cGMP, the hydrolytic activities were stimulated by low concentrations of PDE inhibitors, suggesting an interaction of these drugs with the allosteric site of cGS-PDE. Similar observations and conclusions have been reported in calf liver [31]. These observations emphasize the complexity of the effect of PDE inhibitors on cGS-PDE activity. Under certain substrate conditions, low concentrations of PDE inhibitors activate the enzyme while at high concentrations they have an inhibitory effect, which is probably due to interaction with the catalytic site. The cAMP hydrolytic activity of cGS-PDE in the presence of cGMP was competitively inhibited by micromolar concentrations of dipyridamole and papaverine and also by higher concentrations of IBMX, AAL 05 and zaprinast. The comparison of the cyclic nucleotide hydrolytic activities between endothelial cells and smooth muscle tissue indicated that: (1) the cAMP hydrolytic activity of cGS-PDE from endothelial cells and the cAMP-PDE of the smooth muscle tissue are similarly inhibited by PDE inhibitors at a low substrate concentration; (2) the cAMP hydrolytic activity of cGS-PDE in the presence of cGMP differed from the smooth muscle cAMP-PDE by its insensitivity to rolipram; and (3) the cGMP hydrolytic activity of cGS-PDE differed from the cGMP-PDE in smooth muscle by its relative insensitivity to zaprinast.

Previous observations have shown that compounds such as forskolin, prostacyclin and atrial natriuretic peptides stimulated the production of cAMP and

cGMP and that this response is potentiated by various PDE inhibitors [8, 9, 22, 23]. The present investigation demonstrates a cAMP and cGMP hydrolytic activities in cultured bovine aortic endothelial cells resulting from the presence of two distinct PDE forms, a cAMP-PDE and a cGS-PDE. The enzymatic characterization of these PDE forms, as well as their sensitivity to selective and non-selective PDE inhibitors, indicate a major cAMP hydrolytic system in endothelial cells, while a predominant cGMP hydrolytic system has been identified in smooth muscle [12]. The predominant cAMP hydrolytic activity in endothelial cells is due in part, to the presence of a cGS-PDE, which has not been identified in vascular smooth muscle. Another major difference between the PDE activities from endothelial cells and smooth muscle is the absence in endothelial cells of both a calmodulin-dependent PDE and a specific cGMP-PDE. Further investigations are needed to identify selective and specific inhibitors of the different PDE forms from endothelial cells. This may improve our understanding of the regulation and physiological role of cyclic nucleotides in endothelial cells, as well as their possible influence on the interactions between endothelial cells and neighbouring cells such as smooth muscle or blood-formed elements.

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