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Regulation of the Adenylyl Cyclase Signaling System in Various Types of Cultured Endothelial Cells

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Abstract We studied the effects of modulators of the adenylyl cyclase pathway on the accumulation of cAMP in endothelial cells isolated from bovine aortas, pig pulmonary arteries, human umbilical veins, and human subcutaneous adipose microvessels. In addition to quantitative differences in the basal levels, cAMP stimulation in different endothelial cell types varied in sensitivity and magnitude in response to both the direct adenylyl cyclase activator forskolin and the β-adrenergic receptor agonist isoproterenol. Furthermore, the ubiquitous phosphodiesterase inhibitor IBMX differentially enhanced both the basal and the stimulated cAMP levels in the various cell types. Histamine caused an elevation of cAMP only in bovine aortic endothelial cells and in human umbilical vein endothelial cells. Treatment of the cells with cholera and pertussis toxins, which uniquely affect G-protein subunits, resulted in divergent elevation of cAMP in the various cells. Thus, in each cell type, a distinct profile of regulation of the cAMP levels was found. Our results suggest that the adenylyl cyclase signaling system in various types of endothelial cells can be differentially regulated at the levels of receptors, G-proteins, adenylyl cyclase, and phosphodiesterase.

Key words: endothelial cells, cAMP, adenylyl cyclase, endothelial cell heterogeneity, G-proteins, phosphodiesterase

Endothelial cells (EC), which line the walls of all blood vessels, exhibit profound phenotypic and functional differences in various locations within the vascular tree [Gerritsen, 1987; Zetter, 1988; Fajardo, 1989]. The functional differences appear, for example, in the expression of extracellular matrix proteins [Sage et al., 1981; Myers et al., 1987], production of prostaglandins [Gerritsen, 1987], fibrinolytic activity [Noordhoek-Hegt, 1976; Speiser et al., 1987; van Hinsbergh, 1988], and expression of cell adhesion molecules [Kume et al., 1992; Hauser et al., 1993]. Recently, some information has emerged about differences in the way EC, derived from various locations within the vascular tree, perceive and transduce extracellular signals. For example, it has been shown that bradykinin increases $InsP_3$ production in bovine aortic endothelial cells (BAEC), but has no effect on human umbilical vein endothelial cells (HUVEC). Interestingly, thrombin has the opposite effect [Bartha et al., 1989]. Also, qualitative and quantitative differences in the effect of various agonists on the intracellular Ca^{2+} levels in HUVEC, BAEC, and bovine pulmonary artery endothelial cells have been reported [Watanabe et al., 1992]. The effects of the Ca^{2+} channel modulator, ryanodine, in both unstimulated and agonist (bradykinin, histamine)-stimulated EC derived from rat and human aorta, human umbilical vein, and bovine pulmonary artery, appear to vary with species and vascular bed of origin [Ziegelstein et al., 1994]. In addition, differences have been reported among various EC types in the resting membrane electrical potential [Himmel et al., 1993], the regulation of the plasma membrane calcium pump gene expression [Kuo et al., 1993], and the identity of the phosphodiesterase isoforms [Lugnier and Schini, 1990; Souness et al., 1990; Suttrop et al., 1993].

The possibility of differential regulation of the adenylyl cyclase signaling system in various EC types has not been explored. In response to extracellular stimuli, adenylyl cyclase is activated via G_s-protein or inhibited via G_i-protein and catalyzes the production of the second messenger cAMP from ATP. cAMP activates protein

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kinase A and is degraded by cAMP-specific phosphodiesterase. Recently, Sumpio and colleagues reported different effects of cyclic mechanical strain on intracellular cAMP concentration in BAEC and human saphenous vein endothelial cells [Iba et al., 1991, 1992]. More recently, we have shown that cyclic strain and forskolin differentially induce cAMP production in four types of endothelial cells [Manolopoulos and Lelkes, 1993].

Based on the above indications, we hypothesized that differential regulation of the adenylyl cyclase signaling cascade in EC derived from various locations within the vascular tree may be one of the commensurates of EC heterogeneity. In order to test this hypothesis and to identify at which step(s) of the cascade such differential regulation might occur, we compared the effect of pharmacologic agents that interact at specific sites of the cascade on four EC types, representing the arterial, venous, pulmonary, and microvascular circulation. These agents are: isoproterenol (β-adrenergic receptor agonist), histamine (H-receptor agonist), forskolin (activator of adenylyl cyclase), cholera toxin (activator of G_s-protein), pertussis toxin (inhibitor of G_i-protein), and IBMX (inhibitor of phosphodiesterase). Our findings suggest that the adenylyl cyclase signaling system in EC derived from various locations within the vascular tree can be differentially regulated at the level of receptors, G-proteins, adenylyl cyclase, and phosphodiesterase.

MATERIALS AND METHODS

Materials

Forskolin, isoproterenol, IBMX (3-isobutyl-1-methylxanthine), histamine, N^6,2′-o-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (dibutyryl cAMP, dbcAMP), collagenase (type IA), bovine serum albumin (BSA), and endothelial cell growth supplement (ECGS) were from Sigma Chemical Co (St. Louis, MO). Cholera and pertussis toxins were from List Labs (Campbell, CA). Penicillin, streptomycin, and L-glutamine were from Fisher Scientific (Pittsburgh, PA). Heparin sodium was from Lyphomed (Deerfield, IL). Trypsin-EDTA was from Gibco (Grand Island, NY). Fungizone (amphotericin B) was obtained from the local pharmacy. Nylon meshes were from Tetco, Inc. (Elmsford, NY). Cell culture media M199 and Dulbecco's modified Eagle's medium (DMEM) were from Mediatech, Inc. (Herndon, VA). Fetal calf serum (FCS) and horse serum were from HyClone Laboratories, Inc. (Logan, UT). Human serum was prepared in the laboratory from freshly drawn blood of apparently healthy donors. [2,8-^{3}H] cAMP ammonium salt was from Amersham Corp. (Arlington Heights, IL). The cAMP-binding protein was kindly provided by Dr. Cecilia Hillard, Department of Pharmacology, Medical College of Wisconsin, Milwaukee, WI. The antibody for von Willebrand factor was from Dako Corp. (Carpenteria, CA). Acetylated low-density lipoprotein labeled with 1,1′-dioctadecyl-1,3,3,3′,3′ tetramethylindocarbo-cyanine-perchlorate (dil-ac-LDL) was from Biomedical Technologies, Inc. (Stoughton, MA). The protein assay reagent was from Pierce (Rockford, IL).

Endothelial Cell Isolation and Culture

Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase digestion from normal umbilical cords as described by Jaffe et al. [1973]. Cells were cultured in M199 medium supplemented with 10% (v/v) FCS, 10% (v/v) human serum, 30 μg/ml ECGS, 50 μg/ml heparin, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml fungizone. Studies were conducted on cells from passages 3–6.

Human adipose microvascular endothelial cells (HAMVEC) were isolated from adult subcutaneous adipose tissue with a modification of the method described by Kern et al. [1983]. Briefly, the tissue was minced, digested with 0.2% collagenase, centrifuged, and sequentially filtered through 150-μm, 70-μm, and 20-μm pore nylon meshes. Capillary fragments retained on the 70-μm and 20-μm filters were cultured in 24-well plates with M199 medium supplemented with 10% (v/v) FCS, 10% (v/v) human serum, 30 μg/ml ECGS, 50 μg/ml heparin, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml fungizone. Cells from 2–4 individuals were pooled together and used between passages 5–8.

Pig pulmonary artery endothelial cells (PPAEC) were isolated from freshly obtained vessels. After incubation for 15 min at 37°C with 0.2% collagenase, the cells were removed from the vessels by gentle rubbing, washed twice, and cultured in M199 medium supplemented with 10% (v/v) FCS, 30 μg/ml ECGS, 50 μg/ml heparin, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml fungizone. Cells used were from passages 5–8.

Bovine aortic endothelial cells (BAEC) were isolated from freshly obtained aortas following the protocol as described for PPAEC, and cultured in DMEM supplemented with 7.5% (v/v) FCS, 7.5% (v/v) horse serum, 30 μg/ml ECGS, 50 μg/ml heparin, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml fungizone. Studies were conducted on cells from passages 5–8.

Cells were maintained in a 5% CO_2–95% air (v/v), water-saturated incubator at 37°C, and passaged with trypsin–EDTA. The purity of the cultures was verified by monitoring the capacity of the cells to endocytose dil-ac-LDL and to synthesize von Willebrand factor, following protocols that we recently described elsewhere [Papadimitriou et al., 1993]. Cells retained expression of these markers throughout the passages used in this study. We also verified in preliminary experiments that (1) there were no significant variations in the basal and stimulated levels of cAMP within the passages of EC used, and (2) BAEC grew equally well and gave similar responses to the various treatments applied in this study when they were cultured in M199 medium supplemented with 10% (v/v) FCS. (data not shown).

Experimental Protocols

Approximately 1–1.2 × 10^5 cells were seeded in each well of 12-well tissue culture plates. Experiments were performed one day after the cells had reached confluence. The agonists isoproterenol and histamine were used for the stimulation of β-adrenergic and H-histamine receptors, respectively. Forskolin was used for the direct activation of adenylyl cyclase, while cholera and pertussis toxins were used for the activation and inhibition of G_s- and G_i-proteins, respectively. The ability of the lipid-soluble cAMP analogue, dibutyryl cyclic AMP (dbcAMP), to raise the intracellular cAMP concentration was also investigated. All stimulators were added for 5 min, except for histamine, which was added for 3 min, and cholera and pertussis toxins, which were incubated with the cells for 4 h.

The phosphodiesterase that degrades cAMP is an important regulator of the intracellular cAMP concentration. In order to assess its role in the various types of EC, some of our experiments were carried out both in the absence and presence of the ubiquitous phosphodiesterase inhibitor IBMX. In the latter experiments, 1 mM IBMX was added to the incubation medium 30 min before the end of the incubation period.

Measurement of cAMP

Upon completion of an experiment, EC monolayers were washed twice with ice-cold PBS. cAMP was extracted by addition of 1 ml of 95% ethanol–5% 0.1 N HCl (v/v), pH 3.0, for 2 h at 4°C and measured by a competitive radioligand binding assay [Brooker et al., 1979], modified as described elsewhere [Manolopoulos and Lelkes, 1993]. The protein content of the wells was determined with a colorimetric protein assay, using bovine serum albumin as standard [Bradford, 1976].

Statistical Analysis

The significance of variability between results from various groups was determined by a one-way analysis of variance (ANOVA test). ANOVA was also used to calculate the minimum (threshold) concentration of forskolin required to increase cAMP levels significantly over basal levels ($P < 0.05$). Each experiment included triplicate wells for each condition tested. The data were obtained from 3–13 experiments using separate batches of cells. All results were expressed as mean ±SEM.

RESULTS

Basal Levels

We used four well-characterized, phenotypically diverse EC as representatives of the arterial (BAEC), venous (HUVEC), pulmonary (PPAEC), and microvascular (HAMVEC) circulation. The basal levels of cAMP were similar in PPAEC (12.5 ± 2.5 pmol/mg protein, n = 9), BAEC (9.4 ± 1.7, n = 13), and HUVEC (11.3 ± 1.0, n = 10), but significantly higher ($P < 0.05$) in HAMVEC (26.1 ± 4.5 pmol/mg protein, n = 10). When the various cell types were incubated for 30 min with 1 mM IBMX, the basal cAMP levels were found elevated 2- to 9-fold depending on the cell type (Fig. 1) (Absolute values: PAEC: 21.2 ± 2.5 pmol/mg protein, n = 9; BAEC: 84.7 ± 12.2, n = 13; HUVEC: 48 ± 8.5, n = 10; HAMVEC: 104 ± 29, n = 10). The order of potency by which IBMX enhanced basal cAMP levels was BAEC ≫ HUVEC ≥ HAMVEC > PPAEC and it was sustained also when forskolin- or isoproterenol-induced cAMP levels were measured, as we report below.

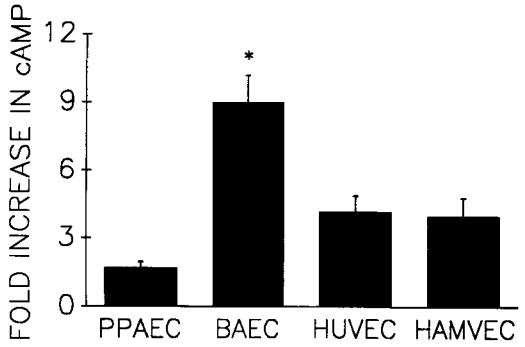

Fig. 1. Effect of IBMX on the basal levels of cAMP in various EC types. PPAEC, BAEC, HUVEC, and HAMVEC were incubated for 30 min with 1 mM IBMX. Results represent mean ±SEM from 9–13 experiments (3 wells/experiment) and are expressed as a fold increase in the cAMP content of each EC type in the presence of IBMX over identical controls from the same experiments in which IBMX was omited. *Fold increase in BAEC significantly higher than in the other 3 cell types ($P < 0.05$).

Effect of Forskolin

Forskolin, which directly activates adenylyl cyclase, increased cAMP levels in a dose-dependent fashion in all four cell types (Fig. 2A), but the sensitivity and responsiveness varied considerably from one cell type to another. The threshold concentration of forskolin required to increase cAMP levels significantly ($P < 0.05$) in each cell type was 0.32 μM in HAMVEC, 2.32 μM in BAEC, 4.52 μM in HUVEC, and 18.77 μM in PPAEC (see also Fig. 2A, insert). At the highest concentration of forskolin used (100 μM), HAMVEC responded with a 85 ± 17-fold increase in cAMP levels over unstimulated controls (n = 4), HUVEC responded with a 27.7 ± 5 fold increase (n = 4), while the response in BAEC and PPAEC was limited to 3.8 ± 1 (n = 6) and 3.7 ± 0.5 fold increase (n = 4), respectively. Inclusion of IBMX increased the responsiveness and decreased the threshold of forskolin concentration in all cell types (Fig. 2B). The order of potency by which IBMX enhanced forskolin-stimulated cAMP levels followed a similar pattern as in the case of basal levels. The threshold concentration values in this case were 0.014 μM in HAMVEC, 0.09 μM in BAEC, 1.65 μM in HUVEC, and 5.76 μM in PPAEC (see also Fig. 2B, insert), while the responses to stimulation with 100 μM forskolin were 70 ± 5-fold increase in cAMP levels in HAMVEC over unstimulated controls (with IBMX), 28 ± 5 and 22 ± 6-fold increase in HUVEC and BAEC, respectively, and only 6 ± 1-fold increase in PPAEC (Fig. 2B).

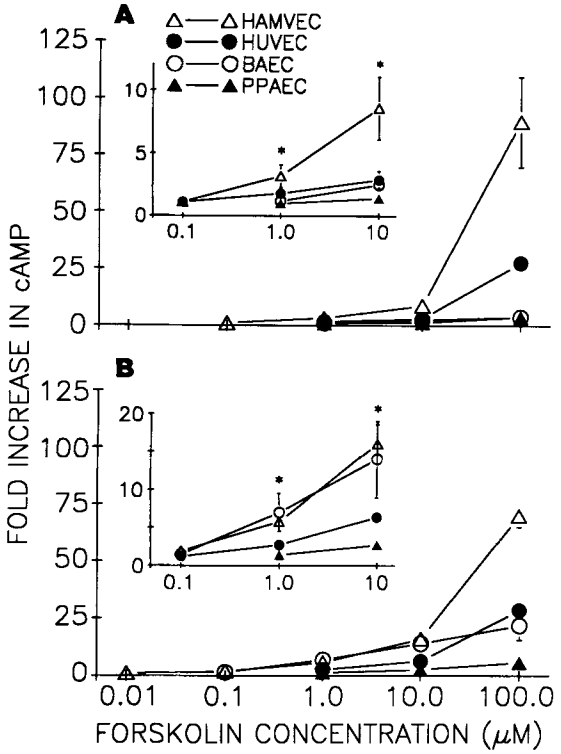

Fig. 2. Effect of forskolin on cAMP accumulation in various EC types, in the presence and absence of IBMX. BAEC (blank circle), HUVEC (filled circle), PPAEC (filled triangle) and HAMVEC (blank triangle) were stimulated for 5 min with increasing concentrations of forskolin (0.01–100 μM): **(A)** in the absence of IBMX, and **(B)** in the presence of 1 mM IBMX. Results represent mean ±SEM from 4–6 experiments (3 wells/experiment) and are expressed as a fold increase in cAMP content over unstimulated controls from the same experiments. *Cyclic AMP levels significantly higher than the levels in the other cell types ($P < 0.05$).

Effect of Isoproterenol

The β-adrenergic agonist, isoproterenol, induced a dose-dependent increase in cAMP in all EC types (Fig. 3A). The stimulatory action of isoproterenol was maximal at 1 μM and declined thereafter. All cell types exhibited a similar level of maximal response to isoproterenol stimulation, but the threshold concentration in BAEC was one order of magnitude lower than in the other cell types (Fig. 3A). Inclusion of IBMX in the medium resulted in a modest potentiation of cAMP response in HUVEC, HAMVEC, and PPAEC (Fig. 3B). However, in BAEC, the potentiating effect of IBMX was much more pronounced, reaching a 10 fold increase of cAMP levels in the presence of 1 μM isoproterenol (Fig. 3B).

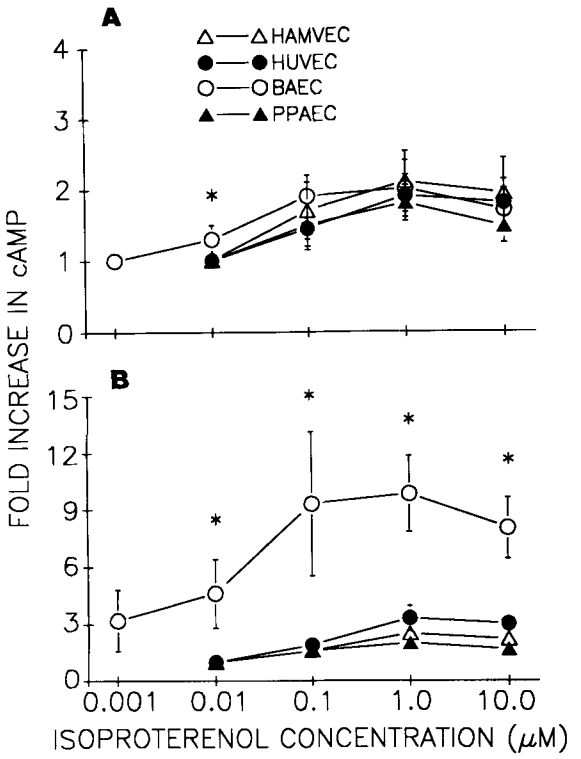

Fig. 3. Effect of isoproterenol on cAMP accumulation in various EC types, in the presence and absence of IBMX. BAEC (blank circle), HUVEC (filled circle), PPAEC (filled triangle) and HAMVEC (blank triangle) were stimulated for 5 min with increasing concentrations of isoproterenol (0.001–10 μM): **(A)** in the absence of IBMX, and **(B)** in the presence of 1 mM IBMX. Results represent mean ±SEM from 4–6 experiments (3 wells/experiment) and are expressed as a fold increase in cAMP content over unstimulated controls from the same experiments. *Cyclic AMP levels in BAEC significantly higher than the levels in the other 3 cell types ($P < 0.05$).

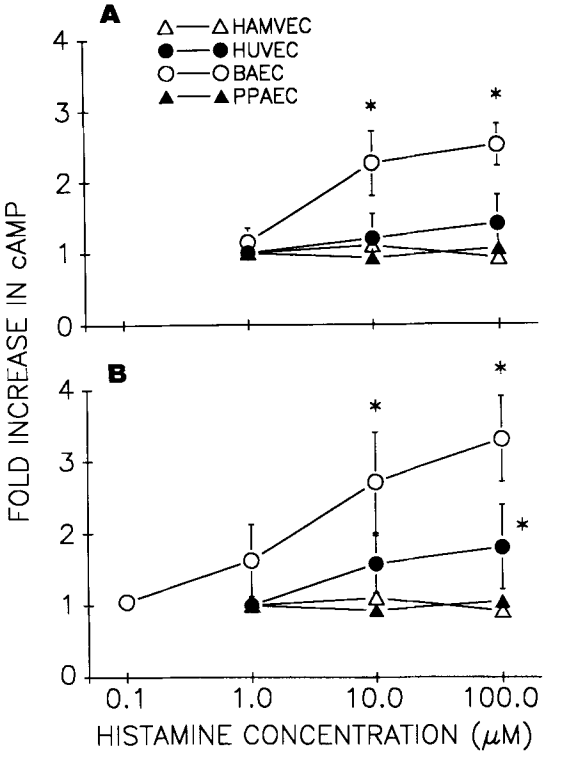

Fig. 4. Effect of histamine on cAMP accumulation in various EC types, in the presence and absence of IBMX. BAEC (blank circle), HUVEC (filled circle), PPAEC (filled triangle) and HAMVEC (blank triangle) were stimulated for 3 min with increasing concentrations of histamine (0.1–100 μM): **(A)** in the absence of IBMX, and **(B)** in the presence of 1 mM IBMX. Results represent mean ±SEM from 3 experiments (3 wells/experiment) and are expressed as a fold increase in cAMP content over unstimulated controls from the same experiments. *cAMP levels significantly higher than the basal levels ($P < 0.05$).

Effect of Histamine

Histamine, in addition to its interaction with H_1-receptors which results in the activation of phospholipase C, also interacts with H_2-receptors to activate adenylyl cyclase [Takeda et al., 1992]. We assessed the effect of histamine on cAMP accumulation in the four EC types and found that, in the absence of IBMX, only BAEC responded with a substantial dose-dependent increase in cAMP contents (Fig. 4A). In HUVEC, the histamine-induced cAMP elevation was statistically insignificant, even at the highest concentration of the agonist used (100 μM). In the presence of IBMX, histamine induced a dose-dependent increase in cAMP levels in BAEC and HUVEC, but again, had no effect on the cAMP levels in HAMVEC and PPAEC (Fig. 4B).

Effect of Cholera and Pertussis Toxins

Both the activator of G_s-protein cholera toxin (2.5 μg/ml) and the inhibitor of G_i-protein pertussis toxin (500 ng/ml) significantly increased cAMP levels in all EC types studied. No significant differences were found in the cholera toxin-induced cAMP accumulation among BAEC, HUVEC, and PPAEC. However, cAMP accumulation in HAMVEC was significantly higher than in the other cell types (Fig. 5). By contrast, when the cells were treated with pertussis toxin, it was the cAMP response of PPAEC that was found significantly higher than in the other EC types (Fig. 6).

Effect of Dibutyryl Cyclic AMP

Dibutyryl cyclic AMP (dbcAMP), a lipid-soluble analog of cAMP, has been used extensively in studies on the role of cAMP in the regulation of

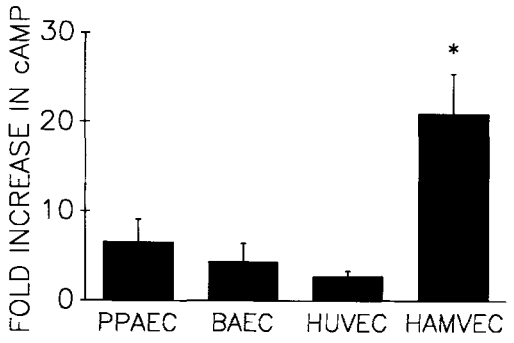

Fig. 5. Effect of cholera toxin on cAMP accumulation in various EC types. BAEC, HUVEC, PPAEC, and HAMVEC were incubated for 4 h with 2.5 μg/ml cholera toxin. IBMX (1 mM) was present during the last 30 min of the incubation period. Results represent mean ±SEM from 3 experiments (3 wells/experiment) and are expressed as a fold increase in cAMP content over unstimulated controls from the same experiments. *cAMP levels in HAMVEC significantly higher than the levels in the other 3 cell types ($P < 0.05$).

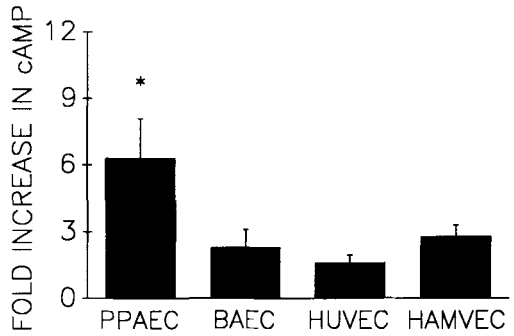

Fig. 6. Effect of pertussis toxin on cAMP accumulation in various EC types. BAEC, HUVEC, PPAEC, and HAMVEC were incubated for 4 h with 500 ng/ml pertussis toxin. IBMX (1 mM) was present during the last 30 min of the incubation period. Results represent mean ±SEM from 3 experiments (3 wells/experiment) and are expressed as a fold increase in cAMP content over unstimulated controls from the same experiments. *Cyclic AMP levels in PPAEC significantly higher than the levels in the other 3 cell types ($P < 0.05$).

cell growth and morphology. We measured the accumulation of cAMP in the various EC types following incubation with increasing concentrations of dbcAMP (0.5–500 μM) and found that BAEC, PPAEC, and HUVEC responded similarly to this compound: An increase in cAMP levels was detectable at a dbcAMP concentration of 5 μM and did not seem to approach a plateau even at a concentration of 500 μM (Fig. 7). By contrast, in HAMVEC, the dose-response curve of dbcAMP was shifted to the left by one order of magnitude, and the absolute amounts of accumulated cAMP were significantly higher than in the other cell types, at all the doses of

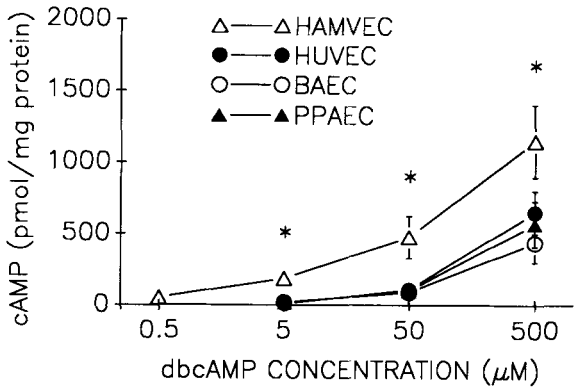

Fig. 7. Effect of dbcAMP on cAMP accumulation in various EC types. BAEC (blank circle), HUVEC (filled circle), PPAEC (filled triangle), and HAMVEC (blank triangle) were stimulated for 5 min with increasing concentrations of dbcAMP (0.5–500 μM). Results represent mean ±SEM from 4 experiments (3 wells/experiment) and are expressed as pmol cAMP/mg protein. *Cyclic AMP levels in HAMVEC significantly higher than the levels in the other 3 cell types ($P < 0.05$).

dbcAMP assayed (Fig. 7). Phosphodiesterase inhibition with IBMX did not alter the accumulation of cAMP in any of the EC studied (data not shown).

One way to explain our results with dbcAMP is that there are differences in the lipid/membrane permeability among various EC types. Although this finding is not directly relevant to the adenylyl cyclase signaling system, it provides evidence for the differential ability of EC to handle dbcAMP. Therefore, caution should be exercised in interpretating results obtained with this experimental agent.

DISCUSSION

The basic mechanism of adenylyl cyclase signaling is well understood. However, in EC, most of the studies on receptors, mediators, and effectors linked to an increase in the intracellular cAMP levels were done without addressing possible differences resulting from EC heterogeneity. Based on our initial findings that forskolin and cyclic strain differentially affect cAMP levels in cultured EC isolated from aortic, pulmonary arterial, venous, and microvascular vessels [Manolopoulos and Lelkes, 1993], we hypothesized that the type of the vessel and the location within the vascular tree from which EC are derived may be related to differential regulation of the adenylyl cyclase signaling cascade. Our results show a significant heterogeneity in the responses of the various EC types to agents that specifically interact with the components of the

adenylyl cyclase cascade, e.g., receptors (β-adrenergic, H-histamine), mediators (G_s- and G_i-proteins), effectors (adenylyl cyclase), and regulators (phosphodiesterase).

All the cell types tested in our study responded to forskolin, which directly activates adenylyl cyclase, but their responses varied considerably in both sensitivity and responsiveness (Fig. 2A,B). These results suggest that the capacity of adenylyl cyclase to produce cAMP differs among cultured EC derived from various locations in the vascular tree. However, in evaluating these results it should be kept in mind that forskolin, while acting on adenylyl cyclase, might be influenced in its potency by the interactions of the enzyme with G_s [Darfler et al., 1982]. Therefore, some of the differences in the potency of forskolin may be due to cell-specific variations in the ability of adenylyl cyclase to form linkages with G_s.

In view of the recent findings that several isoforms of adenylyl cyclase exist in mammalian cells [Tang and Gilman, 1992], an attractive hypothesis for explaining our observations with forskolin is that the adenylyl cyclase isoform content of various types of EC might be qualitatively and/or quantitatively different. Indeed, our preliminary studies, using reverse transcription-PCR analysis, suggest differences in the pattern of adenylyl cyclase isoform expression in cultured EC derived from several rat tissues [Manolopoulos et al., 1994].

All EC types tested in this study contain β-adrenergic receptors, as shown by the ability of isoproterenol to increase their cAMP levels (Fig. 3A,B). This result essentially confirms previous findings by several investigators [Schafer et al., 1980; Hopkins and Gorman, 1981; Bacic et al., 1992]. Interestingly, the response effect of isoproterenol in BAEC was different than in the other EC types, and seems to be associated with higher phosphodiesterase activity in this cell type (Fig. 3A,B). The responses of the various EC types to histamine were more diverse, as an increase in cAMP levels was found in BAEC and HUVEC, while no change could be detected in PPAEC and HAMVEC, even in the presence of IBMX (Fig. 4A,B). These results are in agreement with the findings of other investigators suggesting the presence of H_2 receptors in some EC types (BAEC and HUVEC) [Hekimian et al., 1992; Takeda et al., 1992] but not in others (human pulmonary arterial and human dermal microvascular EC) [Ortiz et al., 1992; Bull et al., 1991]. Therefore, it appears that heterogeneity of the adenylyl cyclase signaling in various types of EC occurs also at the receptor level.

A spectrum of substrates for bacterial toxin-mediated ADP-ribosylation has been found in several EC types [Garcia et al., 1992; Gil-Longo et al., 1993]. As reported in the present study, both cholera and pertussis toxins cause an increase in cAMP levels in all EC types studied (Figs. 5, 6). Yet, we found significant heterogeneity in the ability of both toxins to increase cAMP levels in different types of endothelial cells, strengthening our notion that the G-proteins which control the activity of adenylyl cyclase may be a site of differential regulation of the adenylyl cyclase signaling system in various EC types. Detailed comparative studies on the presence and abundance of the various isoforms of G_s and G_i proteins are necessary in order to determine the molecular nature of this heterogeneity. It should also be kept in mind that both cholera and pertussis toxins, in their actions on G proteins, can produce additional effects on the regulation of ion channels, such that alterations in ion fluxes and concentrations in cells might affect the signaling expressed by adenylyl cyclase [Reisine, 1990; Dolphin et al., 1991].

We found that the ability of the potent phosphodiesterase inhibitor, IBMX, to enhance both the basal and stimulated cAMP levels varies between various EC types. This result indicates different degrees of involvement of phosphodiesterase in the regulation of intracellular cAMP levels in the various cell types. Recently, the presence of two phosphodiesterase isoforms (type II and type IV), was reported in BAEC and in pig aortic endothelial cells [Lugnier and Schini, 1990; Souness et al., 1990]. However, in PPAEC, an additional isoform (type III) was detected [Suttrop et al., 1993]. Our results, taken together with these observations, suggest that the phosphodiesterase enzyme family which degrades cAMP may be another site of differential regulation of the intracellular cAMP concentration in EC derived from various locations within the vascular tree. The availability of isoform-specific pharmacologic inhibitors of phosphodiesterase provides an excellent tool for the characterization of the role of individual phosphodiesterase isoforms in the regulation of cAMP concentration in EC types, and such studies are currently ongoing in our laboratory.

As our results were obtained with cells derived from 3 different species, one could argue

that conclusions from our experiments might be biased by species-related differences [Zetter, 1988]. However, it has been shown that some functional and morphological characteristics of EC are conserved in different species while they vary greatly among different organs of the same species [van Hinsbergh, 1988; Mizrachi et al., 1989; Auerbach et al., 1992]. Indeed, in this and a previous study [Manolopoulos and Lelkes, 1993], we found striking differences in the regulation of intracellular cAMP concentration between cell types derived from the same species (human). Therefore, it appears that at least some of the differences reported here are dependent upon the distinct anatomical origin of EC, rather than upon their species origin.

We conclude that the adenylyl cyclase signaling system in EC derived from various locations within the vascular tree can be differentially regulated at several possible sites and that the regulation of intracellular cAMP levels exhibits a distinct profile in each EC type. Given the important role of cAMP in growth, proliferation, and differentiation, as well as in several EC-specific functions including tissue plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI-1) expression [Santell and Levin, 1988], permeability [Takeda et al., 1992; Suttrop et al., 1993], as well as thrombomodulin and tissue factor activity [Archipoff et al., 1993], a better understanding of the fine regulation of the adenylyl cyclase signaling system in different types of EC may provide useful insight in the phenotypic and functional diversity of these cells. Although a causal relationship between differences in the adenylyl cyclase signaling and EC heterogeneity has yet to be established, we propose that these differences might contribute to, or be caused by, EC phenotypic and functional diversity.

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