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Altered Expression of Cyclic Nucleotide Phosphodiesterase Isozymes during Culture of Aortic Endothelial Cells

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ABSTRACT. Primary cultures of bovine aortic endothelial cells (BAEC) express cyclic nucleotide phosphodiesterase (CN PDE) isozymes of the *PDE2*, *PDE4* and *PDE5* gene families. We report here that the isozyme profiles of CN PDE and the amounts of each vary with the passage number of BAEC cultures. Characterization by anion-exchange chromatography and pharmacological criteria were used to study CN PDE in early (4–6), intermediate (6–10), and late (>17) passages of purified BAEC. PDE2 and a minor fraction of PDE5 accounted for cyclic GMP hydrolysis in early passages, but both isozymes were lost with cell passage. Cyclic AMP was hydrolyzed by both PDE2 and PDE4 isozymes in early passage endothelial cells, but PDE4 was increased dramatically in higher passage cells. Also appearing in the higher passage cells were prominent PDE1 and minor PDE3 activities. The ratios of cytosolic to particulate activities were similar at all passages. BAEC PDE isoforms in intact cells assessed by [^{3}H]-adenine prelabeling showed that atriopeptin II decreased isoproterenol-induced cyclic AMP accumulation in early but not later passage cells, consistent with the loss of PDE2 expression. Enhancement of isoproterenol-induced cyclic AMP accumulation by rolipram, a PDE4 inhibitor, was also greatly diminished during culture passages. Changes in CN PDE isoform expression and consequent cyclic AMP turnover validate the importance of considering cell passage number when cultures of BAEC are used to study the regulation of endothelial cell cyclic nucleotide metabolism and processes mediated by cyclic nucleotides in this model system. BIOCHEM PHARMACOL **54**;10:1071–1079, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. endothelial cells; cyclic AMP; cyclic GMP; phosphodiesterases; rolipram; ANP

EC‡ are monolayered cells within the vascular wall with synthetic, metabolic, and secretory capabilities [1, 2]. Some EC processes are regulated by cAMP and cGMP [3–7], and therefore the metabolism of EC cyclic nucleotides has been studied extensively in cultured EC. Earlier reports have shown the importance of CN PDE in the control of EC cyclic nucleotide metabolism [8, 9]. Studies designed to characterize EC CN PDE, a complex of seven gene families responsible for mammalian cAMP and cGMP hydrolysis [10], have found *PDE2* and *PDE4* gene families predominant in cultured aortic and coronary EC [11–13]. However, widely variant activities and differences in isozyme profiles have been noted [13]. The purpose of the study reported here was to determine if the properties of CN PDE are maintained in EC cultures. Koga *et al.* [3] found PDE2 activation by TNFα only in early passage and not later passage BAEC, indicating the potential importance of culture passage on isoform expression. While cultures of primary EC are proven models, protein expression patterns are known to change in culture [14, 15], potentially leading to inconclusive data regarding EC functions and location differences. EC responses to physiologic and pathologic stimuli are known to decline with time in culture, some with varying rates depending upon the EC source and preparation [16–19]. The studies reported here also determined the functional significance of PDE isoforms in the intact cell by assessing [^{3}H]-cyclic AMP accumulation in [^{3}H]-adenine prelabeled cells from different culture stages in the presence of isoproterenol, ANF, and PDE inhibitors. The loss of an ANF effect on cAMP accumulation was consistent with the loss of PDE2 in higher passages. The results of these studies have been published in abstract form [20].

MATERIALS AND METHODS

Chemicals

[2,8-^{3}H]cAMP (sp. act. 27 Ci/mmol) and [8-^{3}H]cGMP (sp. act. 16 Ci/mmol) were purchased from ICN and purified as described previously [21]. Benzamidine, tosyl-lysl-chloro-

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‡ *Abbreviations:* EC, endothelial cell(s); cAMP, cyclic AMP; cGMP, cyclic GMP; CN PDE, cyclic nucleotide phosphodiesterase; BAEC, bovine aortic endothelial cell(s); ANF, atriopeptin II (rat; 8–33); TNFα, tumor necrosis factor, alpha; MIX, 1-methyl-3-isobutyl xanthine; and TCA, trichloroacetic acid.

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ketone (TLCK), aprotinin, and pepstatin A were obtained from the Sigma Chemical Co. and leupeptin from Peninsula Laboratories. DEAE-Trisacryl was supplied by IBF (Villeneuve La Garenne, France), and Dowex-1X8 (200–400) resin and MIX were from Aldrich. Other chemicals were obtained as follows: rolipram from Dr. I. Williams, Pfizer Laboratories; SQ65442 from Dr. C. Free, E.R. Squibb & Sons, Inc.; indolidan from Dr. D. W. Robertson, Eli Lilly Laboratories; M&B 22948 (zaprinast) from Dr. J. E. Souness, Rhone–Poulenc; dipyridamole from Boeringer Ingelheim Ltd.; HL-725 (trequinsin) from Hoescht–Poussel Pharmaceuticals, Inc.; xanthine derivatives from Dr. J. Wells, Vanderbilt University; and CGS-9343B from Dr. J. Norman, Ciba–Geigy Corp. The xanthine derivatives were MIX,7-propargyl- and 7-benzyl-MIX, 8-methoxymethyl- and 8-*tert*-butyl-MIX and 3-isobutyl-1-isoamyl xanthine. PDE inhibitors were dissolved in 100% ultrapure DMSO at 4 or 40 mM and stored frozen at −20°. Assay dilutions gave a final DMSO concentration of <0.25%. Antibody to PDE4 as a GalK-hPDE4a fusion protein in *Escherichia coli* was a gift from Drs. G. Livi and T. Torphy from SmithKline Beecham, Inc. [22]. Calmodulin was purified from bovine brain using W-7 affinity chromatography as described previously [23].

BAEC Preparation and Culture

Bovine thoracic aortae were obtained from a local abattoir within a few minutes of the death of the animal and packed in ice for transport. The vessels were washed with cold saline containing penicillin (300 U/mL), streptomycin (300 μg/mL), and gentamicin (50 μg/mL). Primary EC cultures were established according to the method of Loeb *et al.* [24] without the use of dispersal enzymes. Briefly, the intimal surface was scraped with a scalpel blade, and the cells were cultured in 96-well tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% (v/v) fetal bovine serum (Intergen; optimal growth selected), penicillin (100 U/mL), and streptomycin (100 μg/mL) and left undisturbed. Four passages of 1:4 cells split using trypsin harvesting were used to establish pure cultures in T75 flasks, and BAEC stocks were stored in liquid nitrogen at passage 4. Passage references are for each trypsin harvest thereafter. BAEC were characterized by their cobblestone appearance at confluence and uptake of fluorescent acetylated low density lipoprotein [25], determined in representative cells grown on chamber slides and analyzed using an ACAS 570 (Meridian Institute) confocal microscope (100X oil objective) equipped with an argon laser.

CN PDE DEAE-Trisacryl Fractionation

BAEC at confluence were scraped from 150-mm tissue culture dishes after two washes with PBS. Cells harvested with trypsin or EDTA showed similar results. Cells from five 150-mm dishes were pelleted and homogenized in 5 vol. of 20 mM Tris-acetate (pH 7.4), 5 mM $MgCl_2$, 10 μM TLCK, 2000 U aprotinin/mL, 2 μM leupeptin, 2 μM pepstatin A, 10 μM benzamidine in a Duall type glass homogenizer using 20 strokes of a Teflon pestle by hand. The homogenate was centrifuged at 105,000 *g* for 30 min at 4° in a Beckman L8 ultracentrifuge, and the supernatant was applied to a DEAE-Trisacryl (4 mL bed volume) column (20 cm × 1.6 cm) pre-equilibrated with homogenization buffer. After the column was washed with 25 mL of homogenization buffer, PDE activities were eluted with two successive linear gradients of NaCl (15 mL of 0–150 mM and 20 mL of 150–500 mM) also in homogenization buffer. Forty fractions at 1.2 mL/fraction were collected at a flow rate of 0.5 mL/min and assayed immediately. For short-term storage at −20° to complete secondary analyses, ethylene glycol was added to a final concentration of 30% (v/v). The major portion of PDE activities was solubilized, but the smaller percentage of activities in the particulate fraction from the 100,000 *g* centrifugation was also analyzed after re-suspension in homogenization buffer and dispersal with a Teflon pestle as described above. DEAE-Trisacryl column recoveries of activities were greater than 70%.

Cyclic Nucleotide PDE Assay

PDE activities were determined using the two-step method of Thompson *et al.* [21]. Reaction was in 0.4 mL containing 40 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 4 mM 2-mercaptoethanol, column or cell fractions, and 0.25 μM [^{3}H]-cAMP (100,000 cpm) in the presence or absence of unlabeled 5 μM cGMP or 0.5 μM [^{3}H]-cGMP (100,000 cpm). Where calcium and calmodulin sensitivity was assayed, substrate cGMP was 25 μM, the assay mixture contained 50 ng calmodulin, and activity was determined in the presence or absence of 0.5 mM EGTA and 1 mM calcium. For the determination of V_{max} and apparent K_m values, the concentration of cAMP or cGMP was varied from 0.02 to 20 μM with constant tritiated substrate (300,000 cpm). Kinetic parameters were calculated using the program Enzfitter (V 1.05, Elsevier–Biosoft). Inhibitors were studied using twelve concentrations of drug from 0.01 to 100 μM and substrate concentrations of 0.125 μM [^{3}H]-cAMP or 0.5 μM [^{3}H]-cGMP unless indicated otherwise. The IC_{50} values were calculated from concentration–response curves using the program Prism (GraphPad; v2); 95% confidence intervals were less than 50%. Values are the averages from two or more curves. Protein was measured by the method of Bradford using BSA as standard [26].

Western Blot Analysis

Partially purified PDE4 DEAE-Trisacryl fractions were used for immunoblot analysis. PDE4 (10 pmol/min at 0.25 μM cAMP substrate) was separated on 8–15% SDS–polyacrylamide gradient gels (Jule Scientific Inc.) and transferred electrophoretically to nitrocellulose paper (0.2 μm; Schleicher & Schuell, Inc.) using a Bio-Rad mini-transfer

unit (100 V, 250 mA) in a buffer consisting of 25 mM Tris–HCl (pH 8.3), 192 mM glycine, 20% methanol. The blot was stained with Ponceau S to verify transfer, blocked for 2 hr with 100 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 3% BSA and equilibrated in 10 mM Tris–HCl (pH 6.8), 0.9% NaCl, 0.5% BSA, 0.05% Nonidet P-40, 5.0% calf serum. PDE4 immunoreactivity was detected by incubation with a 1/500 dilution of polyclonal rabbit antiserum raised against purified GalK-hPDE-1(PDE4a) fusion protein for 12 hr at 4°, washing twice with blocking buffer, incubation with HRP-linked rabbit-IgG (1/500), washing with blocking buffer without BSA (30 min), and final incubation for 30 min at room temperature with 0.05% DAB peroxidase color reagent and 0.02% hydrogen peroxide.

Intact Endothelial Cell cAMP Turnover Analysis

cAMP accumulation in intact BAEC was studied using prelabeling techniques as described by Whalin *et al.* [27]. Cells were seeded in 6-well plates at 220,000 cells/well and grown to preconfluence (approx. 800,000 cells/well); DMEM was replaced with 2 mL medium containing 2 μCi [^{3}H]-adenine/mL and incubated for 1 hr in the CO_2 incubator to label cellular ATP. The medium with unincorporated tritium was aspirated and replaced with medium containing the appropriate drugs or vehicle. Reactions were terminated by rapid aspiration of medium and immediate addition of 1 mL of 5% TCA containing [^{14}C]-cAMP tracer for column recovery calculations. Wells were scraped, and labeled cAMP was purified by Dowex-50/neutral alumina chromatography as described previously [27]. Recovery of cAMP was approximately 60–80% and intersample variability was < 5%. Double-label scintillation counting with internal standard quench curves was used to determine cross-over and recovery correction factors for each column.

RESULTS

Characteristics of CN PDE during BAEC Passages: Distribution

The specific activities of the 100,000 *g* supernatant fractions from BAEC homogenates changed from 3- to 5-fold when early passage [4–9] activities were combined from three different primary preparations and compared with those of later passages [10–17] as shown in the upper panel of Fig. 1. Total cAMP hydrolysis was constant through early passages, increased near passage 10, and fell at passage 30 (data not shown) where the cells had ceased growth and spread into large syncytial-looking cells. Total cGMP hydrolysis followed a similar course with cell passage.

To test if specific PDE isoforms all followed the same pattern as total activity, PDE2 (cGMP-stimulated cAMP hydrolysis) activity and its relative distribution were assayed in the same fractions. Shown in the lower panel of Fig. 1 is the increased cAMP hydrolysis at 0.25 μM substrate due to the presence of 5 μM cGMP (% control)

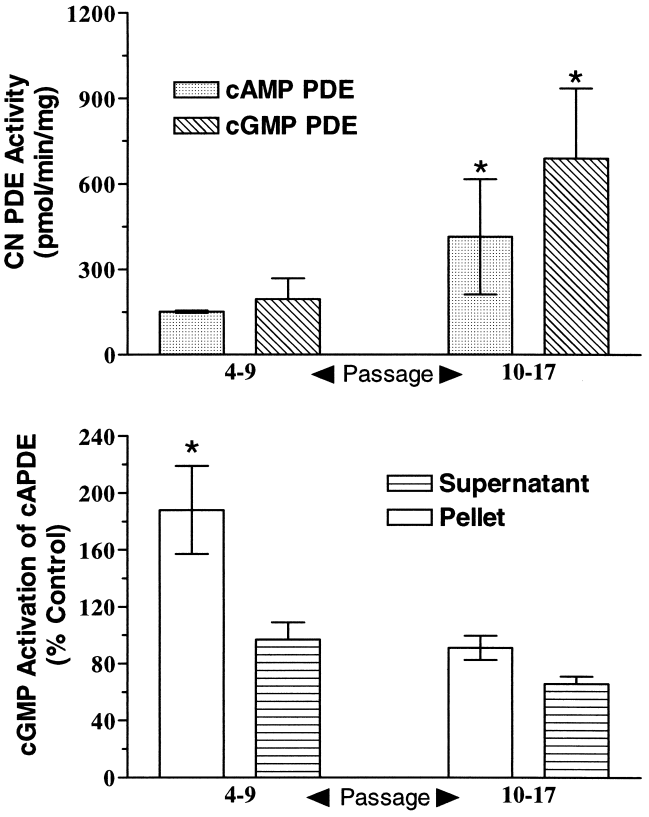

FIG. 1. BAEC CN PDE activities from cells in passages 4–17. Specific activities shown in the upper panel were supernatant fractions prepared according to Materials and Methods and measured using 0.25 μM cAMP or 0.5 μM cGMP as substrate. Data are from three different primary cultures stored frozen at passage 4. Data from passages 4–9 are combined and compared with data from passages 10–17. An asterisk indicates *P* values <0.03 and 0.04 for cAMP and cGMP hydrolysis, respectively, with N = 4 determined using Mann–Whitney tests. The lower panel shows activation of hydrolysis at 0.25 μM cAMP by 5 μM cGMP in supernatant and pellet fractions from early and late passages. Passages 4, 9, and 17 and cells 6, 10, and 17 were from continuing cultures. Values are means ± SEM (N = 3) of unstimulated or control cAMP hydrolysis (100% or 151 pmol/min/mg). An asterisk indicates *P* < 0.05 versus control assays, using Tukey–Kramer multiple comparisons test.

in the assay of supernatant and particulate fractions taken from sequential passages. A profile different from total activity emerged. PDE2 activity was prominent in pellets of early passage cells but was not detectable in later passages. At the later passages, cGMP inhibited activity slightly in the particulate fractions. The data indicate that BAEC change the expression of CN PDE isoforms during continued culture.

The subcellular distribution of PDE2 was similar in all passages. Likewise, cAMP and cGMP hydrolysis showed similar distribution in various passages (data not shown). These crude supernatant and particulate fractions showed activity distributions of 60/40% and 80/20% for cAMP and cGMP PDE, respectively. Therefore, the isozyme profile changes seen in the culture were not due to subcellular redistribution of the different isozymes.

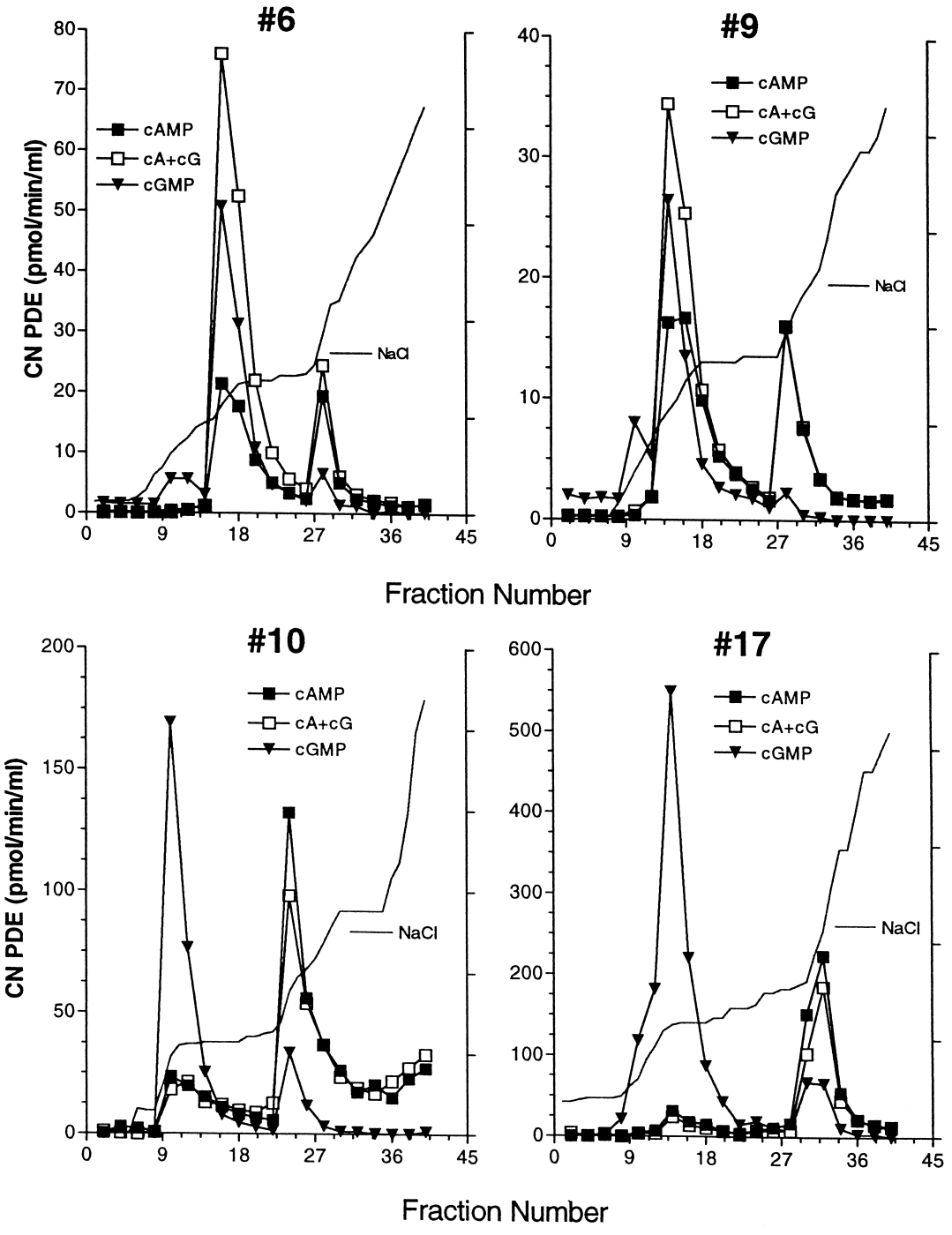

FIG. 2. DEAE-Trisacryl anion-exchange chromatography of BAEC CN PDE. CN PDE activities were measured at 0.25 μM cAMP substrate in the presence (open squares) or the absence (closed squares) of 5 μM cGMP, as well as using 0.5 μM cGMP as substrate (closed inverted triangles). Supernatants from various passages, as indicated on each panel, were prepared as described in Materials and Methods. The solid lines with no symbols show NaCl gradients from 0 to 0.5 M. Activity recoveries were greater than 75% for each column. Y-axis scales increase for passage 10 and 17 data. Column profiles are representative of 1–3 fractionations.

Characteristics of CN PDE during BAEC Passages: DEAE-Trisacryl Fractionation

Anion-exchange chromatography profiles of BAEC supernatants from passage 6, 9, 10 and 17 cells are compared in Figs. 2 and 3. The activities of cAMP PDE at 0.25 μM substrate, cGMP PDE at 0.5 μM substrate, and for cGMP-stimulated cAMP hydrolysis are shown for each profile. Using a two-tiered NaCl gradient elution, striking differences in DEAE-Trisacryl chromatographic profiles were apparent from different passages. These differences are due to changes during passage since similar quantities of BAEC were homogenized for each preparation. The CN PDE

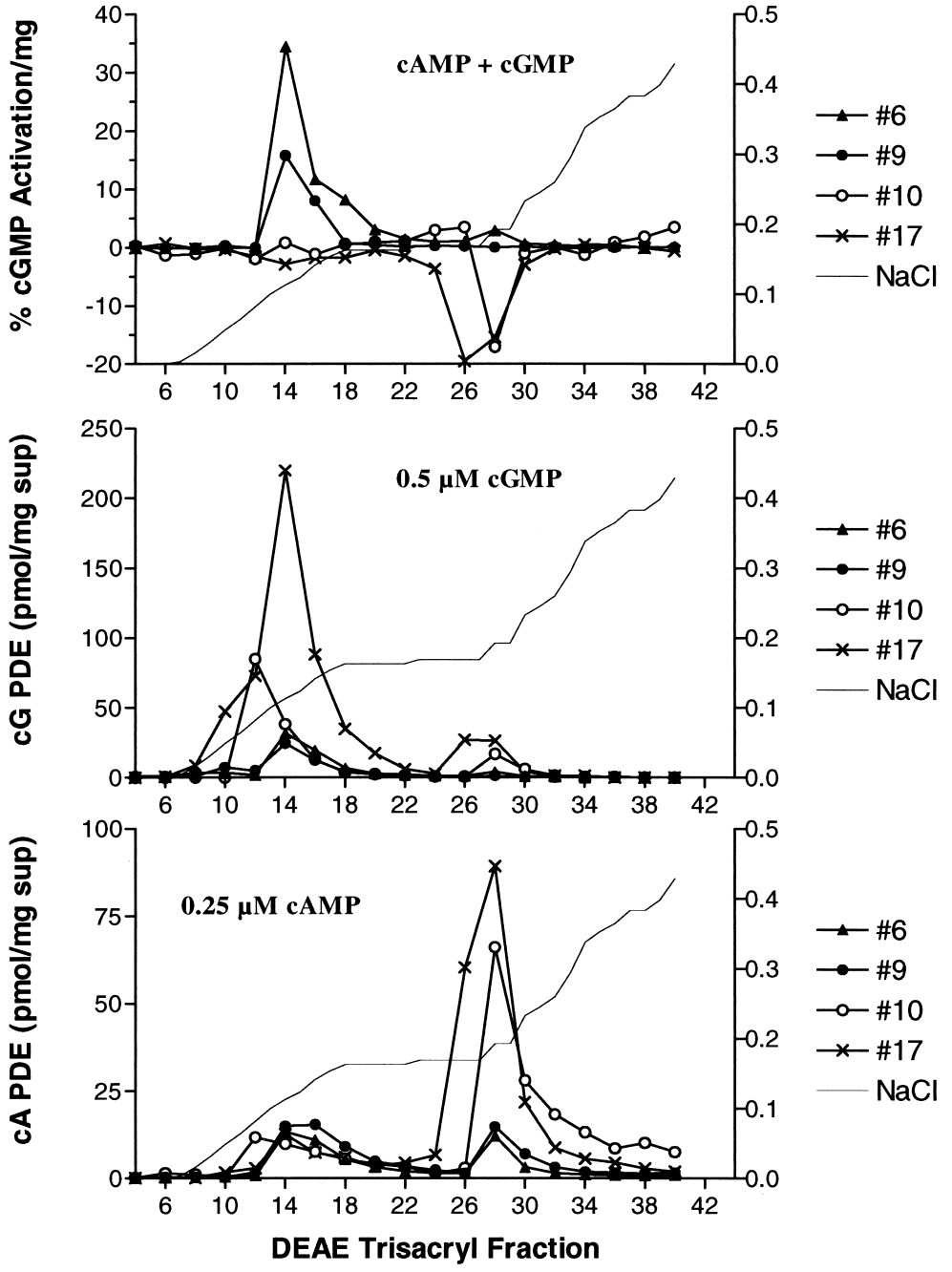

FIG. 3. Re-plot of DEAE-Trisacryl anion-exchange profiles. Data on passage 6–17 cells from Fig. 2 were replotted on the basis of mg supernatant protein applied to each column. The passage used is indicated by symbols to the right of the figure. Column fractionation procedures and CN PDE activity analyses were as described in Materials and Methods and the legend to Fig. 2.

activity changes with passage are further illustrated, using plots comparing the different preparations normalized to mg supernatant protein in Fig. 3.

PDE activities elute in three regions: 50, 110 and 170 mM NaCl. In the very low salt region, only cGMP PDE specific activity was found and this isoform was observed only in passages 6 and 9 and not in higher passages. The first peak was not affected by calcium/calmodulin (data not shown). The intermediate or second eluting fraction showed both cAMP and cGMP hydrolysis to various degrees in different passages. In passage 6, but to a lesser degree in passage 9, activation of cAMP hydrolysis by 5 μM cGMP or PDE2 activity was seen; this isoform was also not found in later passages. The EC_{50} value of cGMP for activation at 5 μM cAMP substrate was 0.5 μM (data not shown). This peak of activity was also unaffected by calcium/calmodulin in passage 6 and 9 EC.

The DEAE profiles showed major changes in the cAMP/cGMP activity ratios with increasing passage; the first activity eluted in the intermediate salt gradient fractions and contained an overlapping mixture of PDE1 and PDE2 isoforms. In the earliest fractions of passage 10, slight

calcium/calmodulin activation was apparent, and PDE2 was seen only by measuring activity in the presence of 7-benzyl-MIX, a PDE1 inhibitor (data not shown). In contrast, the same peak of activity from passage 17 BAEC showed calcium/calmodulin sensitivity and no PDE2 activity.

The predominant activities eluted in the higher salt regions of the DEAE columns from all passage preparations showed a substrate preference for cAMP. cAMP hydrolysis at 0.25 μM substrate was not changed by 5 μM cGMP, suggesting a PDE4 activity. cAMP hydrolysis in the high salt peak was increased significantly in passage 10 and 17 profiles (Fig. 2).

Characteristics of CN PDE during BAEC Passages: Inhibitor Studies

Concentration–response curves were determined for selective inhibitors of CN PDE activities of the early (cGMP specific, PDE5), intermediate (cGMP stimulated, PDE2) and high salt (cAMP specific, PDE4) eluting fractions from passage 6. The rank order of potency for inhibitors of Peak 1 (fractions 10–13) was dipyridamole and zaprinast (IC_{50} = 0.2 μM) > trequinsin (IC_{50} = 0.8 μM) > MIX, SQ 65442 (IC_{50} = 4 μM) ⩾ indolidan, CGS 9343B, rolipram (IC_{50} > 50 μM) when determined using 0.5 μM cGMP substrate. DEAE-Trisacryl Peak 2 cGMP hydrolysis (fractions 16–18) showed a preference for trequinsin (IC_{50} = 0.4 μM) over all other inhibitors tested, with an order of potency as follows: trequinsin ⩾ dipyridamole, MIX (IC_{50} = 7,13 μM) > zaprinast, SQ 65442 (IC_{50} = 28,37 μM) > rolipram, CGS 9343B, indolidan (IC_{50} = > 50 μM) also determined with 0.5 μM cGMP substrate. cAMP hydrolysis by the high salt eluting fraction showed the following rank order of potency: rolipram, SQ 65442, trequinsin (IC_{50} = 0.2 μM) ⩾ dipyridamole, MIX (IC_{50} = 2–4 μM) ⩾ GCS 9343B, indolidan, zaprinast (IC_{50} > 50 μM) with 0.125 μM cAMP substrate. Inhibitor sensitivities indicated that early passage BAEC express PDE2 and PDE4 family activities with minor amounts of PDE5 [13, 28]. Passage 9 preparations fractionated by DEAE-Trisacryl showed inhibition patterns similar to those of passage 6 cells.

At later passages, the rank order of potency of the lower salt fractions was different and consistent with the loss of PDE2 and the appearance of PDE1 isoforms. The rank order of potency of inhibitors for the low salt eluting PDE from the passage 10 DEAE-Trisacryl column was trequinsin, MIX, zaprinast, dipyridamole (IC_{50} values = 2–4 μM) > SQ 65442 (8 μM) ⩾ rolipram, indolidan, CGS 9343B (>50 μM). Thus, the inhibitor data also indicated that the early fractions contain an unresolved mixture of PDE2 and PDE1 [28]. However, by passage 17, expression in the low salt DEAE fractions (approximately 8–20) was almost exclusively PDE1 as shown by a greater than 2-fold increased activity in the presence of calcium/calmodulin using 25 μM cGMP substrate. The apparent K_m for cAMP was 12 μM and for cGMP 2 μM. The dominant calcium/calmodulin sensitivity occurred in these fractions and showed inhibitor profiles for cGMP hydrolysis consistent with PDE1 activity. The activated enzyme was inhibited by MIX, trequinsin, and zaprinast with IC_{50} values of 3.3, 4.1, and 9.1 μM, respectively, and showed IC_{50} values > 50 μM for dipyridamole, indolidan, rolipram, and SQ 65442. Calcium/calmodulin activated cGMP PDE also showed sensitivity to xanthine derivatives with potency ratios of 7-benzyl- and 8-methoxy-methyl-MIX derivatives (IC_{50} = 1–2 μM) > 7-propargyl- and 8-*tert*-butyl-MIX derivatives (IC_{50} = 10–20 μM) and no inhibition by the 1-isoamyl-3-isobutyl xanthine derivative at 100 μM.

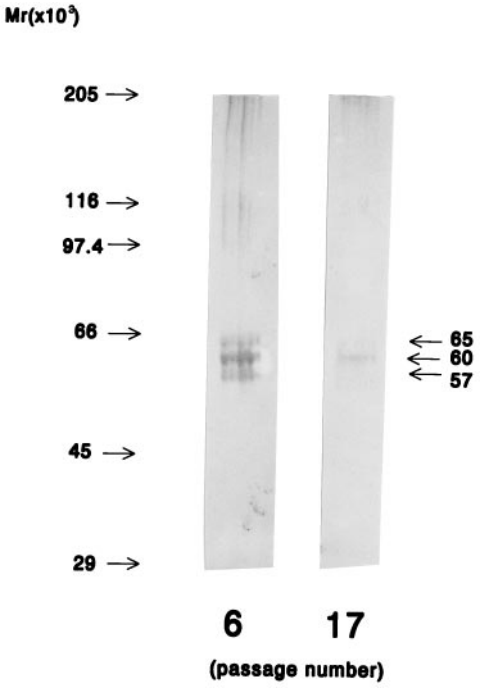

FIG. 4. Immunoreactivity of passage 6 and 17 PDE4 isozymes. Partially purified PDE4 DEAE-Trisacryl fractions were used for immunoblot analysis performed as described in Materials and Methods. PDE4 (10 pmol/min at 0.25 μM cAMP substrate from each passage) was separated on 8–15% SDS–polyacrylamide gradient gels and electrophoretically transferred to 0.2 μm nitrocellulose paper. The blot was stained with Ponceau S to verify equivalent protein transfers and mark molecular weight standards, blocked, and PDE4 immunoreactivities detected with polyclonal rabbit antiserum raised against purified GalK-hPDE-1(4a) fusion protein followed by incubation with HRP-linked rabbit-IgG and 0.05% DAB peroxidase color reagent.

The high salt eluting cAMP PDE activities of passages 10 and 17 maintained the same rank order of inhibitor potency as the early passages with IC_{50} values for rolipram ranging from 0.15 to 0.35 μM. Rolipram inhibited these activities up to 100%. The apparent K_m was 0.3 ± 0.07 μM for cAMP, indicating a high affinity for both fractions. The increased specific activity of the higher passage fractions PDE4 was studied further by western blotting (Fig. 4). The same amount of PDE4 activity from passage 6 and 17 high salt DEAE fractions was applied to SDS–PAGE. Less immunoreactivity was seen in the passage 17 than in the passage 6 peak, suggesting an increased specific activity of the same PDE protein in later passages.

Characteristics of Particulate BAEC CN PDE during Passage

Particulate cAMP and cGMP PDE activities showed total activity changes similar to those of the supernatant fractions. Passages 4, 6, and 9 were equal, and later passages were increased. Inhibitor concentration–response curves against particulate PDE using 0.125 μM cAMP substrate showed rolipram, SQ 65442, and trequinsin to be the most effective inhibitors (IC_{50} = 1–2 μM), and indolidan, CGS 9343B, and zaprinast the least effective inhibitors (IC_{50} > 50 μM). The data suggest a predominant PDE4 in this preparation [28]. As was shown above for supernatant PDE4, a change in the regulation of the particulate fraction also occurred between passages 9 and 17. In the presence of 50 μM rolipram, the remaining passage 9 particulate activity showed cGMP stimulation and no inhibition by indolidan, a selective PDE3 inhibitor. In contrast, passage 17 particulate activity was inhibited partially by cGMP and indolidan, properties indicative of some PDE3 expression (data not shown).

Functional Significance of PDE2 and PDE4 Regulation of Intact BAEC Assessed by cAMP Accumulation

Potential PDE2 regulation of intact BAEC cAMP accumulation was studied using isoproterenol-induced cAMP accumulation after ANF pretreatment. BAEC were monitored for an ANP effect on most passages from 5 to 28. ANF showed an EC_{50} = 5 ± 2 nM at passage 14 with cGMP accumulation as measured by radioimmunoassay at 25 min.

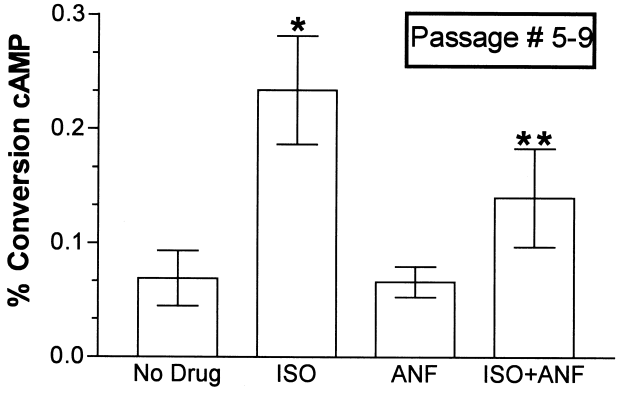

FIG. 5. ANF inhibition of isoproterenol-induced cAMP accumulation. Intact passage 5–9 BAEC were studied using prelabeling techniques as described in Materials and Methods. Cells at near confluence in 6-well plates were prelabeled with [^{3}H]-adenine for 1 hr, washed, and treated with 10 μM isoproterenol in the presence and absence of 100 nM ANF for 15 min. Reactions were terminated by rapid aspiration of medium and immediate addition of 1 mL of 5% TCA containing [^{14}C]-cAMP tracer for column recovery calculations. Labeled cAMP was purified by Dowex-50/neutral alumina chromatography with 60–80% recovery and < 5% intersample variability. Data (± SD) from all five passages were compiled with triplicate determinations in each experiment. Key: (*)$P < 0.001$ vs no drug or ANF alone, and (**)$P < 0.01$ vs isoproterenol, calculated using the Tukey–Kramer multiple comparisons test.

TABLE 1. Cyclic nucleotide phosphodiesterase inhibitor potentiation of isoproterenol-induced cAMP accumulation in intact BAEC cells

Drug	Basal (%)	Isoproterenol potentiation (%)
No drug control	100 ± 54	
+Isoproterenol (10 μM)	260 ± 59	
+Iso + CN PDE inhibitor		
Rolipram		1730 ± 453
SQ65442		533 ± 70
Trequinsin		743 ± 96
Ro 20-1724		462 ± 70
Dipyridamole		103 ± 35
MIX		106 ± 17
Indolidan		87 ± 11
CGS-9343B		140 ± 31
Zaprinast		134 ± 28
t-Butyl-xanthine		114 ± 23
Isoamyl-xanthine		486 ± 57

cAMP accumulation was measured in BAEC cells using ^{3}H-prelabeling as described in Materials and Methods. Preincubation with [^{3}H]-adenine was for 60 min followed by washing and medium changes. CN PDE inhibitors were added 15 min before isoproterenol (10 μM), which induced cAMP synthesis for 15 min. Data were compiled from 2–5 different experiments with triplicate determinations in each. Values show stimulation above vehicle controls (ISO) or potentiation above isoproterenol stimulation (ISO + PDE inhibitor) with SEM. None of inhibitors except for rolipram showed any stimulation or inhibition of basal cAMP accumulation. Rolipram alone showed stimulation equivalent to isoproterenol (220%).

In the early passage cells (No. 5–9), ANF (100 nM) reduced isoproterenol (10 μM) stimulated cAMP accumulation (Fig. 5), whereas ANF alone had no effect on cAMP accumulation. The effect of ANF on isoproterenol-induced cAMP accumulation was lost in cells from passages 10 through 28 obtained from the same primary culture (data not shown). The intact cell turnover data are consistent with the loss of PDE2 in higher passage cells. ANF receptors, as judged by the ability of ANP to enhance cGMP accumulation, were lost at approximately passage 24.

Inhibitors of PDE4 activity showed marked potentiation of isoproterenol-induced cAMP accumulation in intact BAEC (Table 1), as measured with prelabeling techniques. The rank order of potency (IC_{50}) from the intact cell accumulation data was similar to that of the isolated DEAE fractions. Although the resultant magnitude of potentiation was different, the inhibitors studied showed similar actions on the isoproterenol effect in early, middle, or late passage cells, indicating the critical role of PDE4 in cAMP metabolism in BAEC. Rolipram showed an EC_{50} of 2.6 μM for potentiation of the isoproterenol effect (data not shown) or about 10-fold higher than PDE inhibition and cAMP release in the intact rat lung model [6]. Rolipram was the only PDE inhibitor that enhanced even small amounts of cAMP accumulation in the absence of isoproterenol in BAEC. Even though the PDE4 isoform was expressed at all passages and was a major regulator of cAMP accumulation in BAEC, when studied systematically through many cell passages, rolipram potentiation of isoproterenol-induced cAMP diminished during culture (Fig.

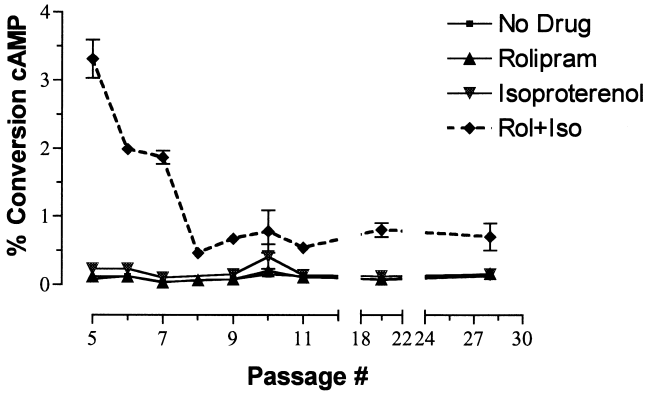

FIG. 6. Modified rolipram potentiation of isoproterenol-induced cAMP accumulation with passage number. Cells from consecutive passages were plated and prelabeled as indicated in Materials and Methods and in the legend to Fig. 5. Isoproterenol (10 μM), rolipram (20 μM), or both drugs were used to induce cAMP accumulation for 12 min. Each data point is the mean (± SD) of 3–5 wells of cells. Error bars that are not visible were smaller than the symbol used. The no drug line is under the rolipram line (▲).

6). Since PDE4 showed similar properties, the reduced cAMP accumulation occurring during passage likely occurred from modified regulation of synthesis by the β-adrenergic receptor and adenylate cyclase.

DISCUSSION

cAMP and cGMP regulation of endothelial cell functions has been studied extensively in cells cultured from a variety of sources, including large vessels such as bovine aorta. Primary cultures are passed several times to achieve both purity and quantity of cells for biochemical analyses, and it is sometimes assumed that higher passage cells have maintained early passage parameters as models of EC functions. PDE activity has been reported to vary in ECs from different anatomic locations based on MIX-enhanced cAMP accumulation [18]. Also, forskolin and isoproterenol enhanced cAMP accumulation, and adenylate cyclase activity varied by mechanisms independent of species arguments. We have shown previously that TNFα incubation with BAEC results in PDE2 activation but only in lower passage cells [3]. The results of the studies reported here show that CN PDE activities of BAEC change during culture. The amount and type of *PDE* gene family expression were modified as passage number increased. The reasons for variant enzyme or receptor expression are sometimes difficult to define. Even with a widely used EC preparation such as that isolated from human umbilical veins, variable effects are seen [15]. Changes in enzyme expression during primary cultures of many types of cells are widely observed, and our finding that CN PDE isoforms change in culture is potentially a confounding factor in interpreting and comparing signal transduction data from previous studies. Manolopoulos *et al.* [29] and Stevens *et al.* [30] found that EC from different locations expressed different adenylate cyclase isoforms. However, possible culture passage changes similar to CN PDE activity changes were not determined.

The findings reported here confirm and extend earlier studies on EC cyclic nucleotide phosphodiesterase analysis. Early passage BAEC showed a minor peak of PDE5 as reported by Kishi *et al.* [13] along with the main isoforms of PDE2 and PDE4 as described by Lugnier and Schini [12] and Souness *et al.* [11]. The current data provide an explanation for the differences among these previous studies with significant PDE isoform and activity variances.

Later passage BAEC lost both PDE5 and PDE2 isoforms. This was accompanied by the appearance of PDE1 along with a small amount of PDE3 activity and markedly increased PDE4 activity. PDE4 activity was also expressed in early passage cells with both fractions showing high affinity for cAMP substrate and 100% inhibition by rolipram. Western blots of PDE4 immunoreactivities suggested an increased specific activity of the same or a catalytically similar isoform expressed in higher passage cells. Increased BAEC PDE4 specific activity was similar to TNFα activation of PDE4 also involving higher specific activity rather than more enzyme protein [3]. The small amount of PDE3 isoform found was confined to particulate fractions but is confirmatory of earlier studies of Suttorp *et al.* [31] on porcine pulmonary artery EC. Seid *et al.* [32] detected calmodulin-stimulated activity in lysates of cultured porcine aortic endothelial cells. Data suggest that EC from different locations may express different PDE isoforms. Further studies will be required to determine the role of PDE5 in cGMP metabolism and regulation of EC PDE3 and PDE1 isoforms.

cAMP accumulation studies in intact EC showed that PDE2 can function in a crossover role to provide a mechanism for agonists that increase cGMP levels to regulate cAMP content in EC. ANF decreased cAMP accumulation induced by isoproterenol in early passage BAEC expressing PDE2, but not in higher passage cells lacking PDE2 in supernatant, particulate, or anion-exchange fractionated samples. These studies confirm findings of Kishi *et al.* [9] that ANF decreased BAEC cAMP with or without isoproterenol by a mechanism involving PDE2 activation. PDE2 has also been shown to have a similar role in ANF modulation of adenosine-induced cAMP accumulation in PC12 cells [27] and in adrenal tissue [33].

PDE4 activity of early and late passage cells showed similar sensitivity to inhibitors but had a much higher specific activity in higher passages. Inhibition by rolipram, SQ 65442, and trequinsin in the submicromolar range was very similar to data obtained by Lugnier and Schini [12] and Souness *et al.* [11]. Close correlation to effects of inhibitors in intact cell cAMP accumulation experiments indicate that regardless of other isoform expression, high affinity PDE4 isoform is a primary regulator of cAMP accumulation in BAEC. These findings suggest a rationale why Manolopoulos *et al.* [29] found even 1 mM MIX relatively ineffective at potentiation of isoproterenol-induced total cAMP levels in cultured EC from several

sources. Rolipram and trequinsin were superior to other inhibitors at potentiation of forskolin-induced cAMP accumulation in porcine aortic EC [8]. Almost two decades ago, Pledger *et al.* [34] demonstrated that CN PDE activities are subject to many factors in fibroblast cultures including cell density, growth conditions, and serum content. In EC tissue culture, these factors include expression of specific families of PDE isozymes at different passages.

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References

1. Vane JR, Glyglewski RJ and Botting RM, Endothelial cell as a metabolic and endocrine organ. *Trends Pharmacol Sci* **8:** 491–496, 1987.
2. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K and Masaki T, A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **323:** 411–415, 1988.
3. Koga S, Morris S, Ogawa S, Liao H, Bilezikian JP, Chen G, Thompson WJ, Ashikaga T, Brett J, Stern DM and Pinsky DJ, TNF modulates endothelial properties by decreasing cAMP. *Am J Physiol* **268:** C1104–C1113, 1995.
4. Stout RW, Cyclic AMP: A potent inhibitor of DNA synthesis in cultured arterial endothelial and smooth muscle cells. *Diabetologia* **22:** 51–55, 1982.
5. Schafer AL, Gimbrone MA and Handin RI, Regulation of endothelial cell function by cyclic nucleotides. In: *Biology of Endothelial Cells* (Ed. Jaffe EA), pp. 248–258. Martinus Nijhoff, Boston, MA, 1984.
6. Barnard JW, Seibert AF, Prasad VR, Smart DA, Strada SJ, Taylor AE and Thompson WJ, Reversal of pulmonary capillary ischemia–reperfusion injury by rolipram, a cAMP phosphodiesterase inhibitor. *J Appl Physiol* **77:** 774–781, 1994.
7. Marczin N, Ryan US and Catravas JD, Endothelial cGMP does not regulate basal release of endothelium-derived relaxing factor in culture. *Am J Physiol* **263:** L113–L121, 1992.
8. Martin W, White DG and Henderson AH, Endothelin-derived relaxing factor and atriopeptin II elevate cyclic GMP levels in pig aortic endothelial cells. *Br J Pharmacol* **93:** 229–239, 1988.
9. Kishi Y, Ashikaga T, Watanabe R and Numano F, Atrial natriuretic peptide reduces cyclic AMP by activating cyclic GMP-stimulated phosphodiesterase in vascular endothelial cells. *J Cardiovasc Pharmacol* **24:** 351–357, 1994.
10. Conti M, Nemoz G, Sette S and Vicini E, Recent progress in understanding hormonal regulation of phosphodiesterases. *Endocr Rev* **16:** 1–20, 1995.
11. Souness JE, Diocee BK, Martin W and Moodie SA, Pig aortic endothelial-cell cyclic nucleotide phosphodiesterases. *Biochem J* **266:** 127–132, 1990.
12. Lugnier C and Schini VB, Characterization of cyclic nucleotide phosphodiesterases from cultured bovine aortic endothelial cells. *Biochem Pharmacol* **39:** 75–84, 1990.
13. Kishi Y, Ashikaga T and Numano F, Phosphodiesterases in vascular endothelial cells. *Adv Second Messenger Phosphoprotein Res* **25:** 201–213, 1992.
14. Betz E, Cell culture systems to study progression and inhibition of intimal proliferations. *Basic Res Cardiol* **86:** 79–86, 1991.
15. Watson CA, Camera-Bensen L, Palmer-Crocker R and Pober JS, Variability among human umbilical vein endothelial cultures. *Science* **268:** 447–448, 1995.
16. Pearson JD, Carleton JS and Hutchings A, Prostacyclin release stimulated by thrombin or bradykinin in porcine endothelial cells cultured from aorta and umbilical vein. *Thromb Res* **29:** 115–124, 1983.
17. Agar A and Martin W, Loss of receptor-mediated ^{86}Rb efflux from pig aortic endothelial cells in culture. *Br J Pharmacol* **80:** 5–6, 1983.
18. Manolopoulos VG, Samet MM and Lelkes PI, Regulation of adenylyl cyclase signaling system in various types of cultured endothelial cells. *J Cell Biochem* **57:** 590–598, 1995.
19. Tracey WR and Peach MJ, Differential muscarinic receptor mRNA expression by freshly isolated and cultured bovine aortic endothelial cells. *Circ Res* **70:** 234–240, 1992.
20. Ashikaga T, Strada SJ and Thompson WJ, Altered expression of bovine aortic endothelial cell (BAEC) cyclic nucleotide (CN) phosphodiesterase (PDE) isozymes during culture. *FASEB J* **7:** A759, 1993.
21. Thompson WJ, Terasaki WL, Epstein PM and Strada SJ, Assay of cyclic nucleotide phosphodiesterase and resolution of multiple molecular forms of enzyme. *Adv Cyclic Nucleotide Res* **10:** 69–92, 1979.
22. Livi GP, Kmetz P, McHale MM, Cieslinski LB, Sathe GM, Taylor DP, Davis RL, Torphy TJ and Balcarek JM, Cloning and expression of cDNA for a human low-K_m, rolipram-sensitive cyclic AMP phosphodiesterase. *Mol Cell Biol* **10:** 2678–2686, 1990.
23. Black EW, Strada SJ, Garrett RL, Kvietys PR, Thompson WJ and Norman JA, Inhibition of gastric acid secretion *in vivo* and *in vitro* by a new calmodulin antagonist, CGS 9343B. *J Pharmacol Exp Ther* **248:** 208–214, 1989.
24. Loeb AL, Johns RA, Milner P and Peach MJ, Endothelium relaxing factor in cultured cells. *Hypertension* **9:** (Suppl III), 182–186, 1987.
25. Voyta JC, Via DP, Butterfield CE and Zetter BR, Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. *J Cell Biol* **90:** 2034–2040, 1984.
26. Bradford MM, A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
27. Whalin ME, Scammell JG, Strada SJ and Thompson WJ, Phosphodiesterase II, the cGMP-activatable cyclic nucleotide phosphodiesterase, regulates cyclic AMP metabolism in PC12 cells. *Mol Pharmacol* **39:** 711–717, 1991.
28. Thompson WJ, Cyclic nucleotide phosphodiesterases: Pharmacology, biochemistry and function. In: *International Encyclopedia of Pharmacology and Therapeutics* (Ed. Taylor CW), Chap. 12, pp. 287–313. Pergamon Press, Oxford, 1993.
29. Manolopoulos VG, Liu J, Unsworth BR and Lelkes PI, Adenylyl cyclase isoforms are differentially expressed in primary cultures of endothelial cells and whole tissue homogenates from various rat tissues. *Biochem Biophys Res Commun* **208:** 323–331, 1995.
30. Stevens T, Nakahashi Y, Cornfield DN, McMurtry IF, Cooper DMF and Rodman DM, Ca^{2+}-inhibitable adenylyl cyclase modulates pulmonary artery endothelial cell cAMP content and barrier function. *Proc Natl Acad Sci USA* **92:** 2696–2700, 1995.
31. Suttorp N, Weber U, Welsch T and Schudt C, Role of phosphodiesterases in regulation of endothelial permeability *in vitro*. *J Clin Invest* **9:** 1421–1428, 1993.
32. Seid JM, MacNeil S and Tomlinson S, Calcium, calmodulin, and the production of prostacyclin by cultured vascular endothelial cells. *Bio Sci Rep* **3:** 1007–1015, 1983.
33. MacFarland RT, Zelus BD and Beavo JA, High concentrations of a cGMP-stimulated phosphodiesterase mediate ANP-induced decreases in cAMP and steroidogenesis in adrenal glomerulosa cells. *J Biol Chem* **266:** 136–142, 1991.
34. Pledger WJ, Thompson WJ, Epstein PM and Strada SJ, Regulation of cyclic nucleotide phosphodiesterase forms by serum and insulin in cultured fibroblasts. *J Cell Physiol* **100:** 497–508, 1979.