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Physiology

Ca^{2+}-inhibitable adenylyl cyclase modulates pulmonary artery endothelial cell cAMP content and barrier function

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ABSTRACT **Maintenance by the endothelium of a semipermeable barrier is critically important in the exchange of oxygen and carbon dioxide in the lung. Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) and cAMP are principal determinants of endothelial cell barrier function through their mutually opposing actions on endothelial retraction. However, details of the mechanisms of this antagonism are lacking. The recent discovery that certain adenylyl cyclases (EC 4.6.1.1) could be acutely inhibited by Ca^{2+} in the intracellular concentration range provided one possible mechanism whereby elevated $[Ca^{2+}]_i$ could decrease cAMP content. This possibility was explored in pulmonary artery endothelial cells. The results indicate that a type VI Ca^{2+}-inhibitable adenylyl cyclase exists in pulmonary artery endothelial cells and is modulated by physiological changes in $[Ca^{2+}]_i$. Furthermore, the results suggest the inverse relationship between $[Ca^{2+}]_i$ and cAMP that is established by Ca^{2+}-inhibitable adenylyl cyclase plays a critical role in modulating pulmonary artery endothelial cell permeability. These data provide evidence that susceptibility to inhibition of adenylyl cyclase by Ca^{2+} can be exploited in modulating a central physiological process.**

The lung coordinates large flows of blood and gas to exchange oxygen and carbon dioxide. Efficient gas exchange requires a minimal diffusion gradient for gases and matching of ventilation with perfusion. Maintenance by the pulmonary endothelium of a semipermeable barrier is particularly important in optimizing the diffusion gradient. In humans, diseases associated with pulmonary endothelial barrier disruption include adult respiratory distress syndrome, neonatal respiratory distress syndrome, and neurogenic, high altitude, and post-transplantation pulmonary edema. Endothelial barrier disruption in these diseases is likely initiated by release of toxic oxygen radicals and proteases by activated white blood cells, ischemia–reperfusion injury, and/or neuro-humoral inflammatory and vasoactive mediators (1, 2). However, a likely final common pathway is endothelial retraction following increases in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) (3–7).

cAMP is generally thought to inhibit $[Ca^{2+}]_i$-induced endothelial retraction (8–11). Whereas in fibroblasts elevated cAMP disassembles actin (12), increased endothelial cell cAMP polymerizes the F-actin pool, promotes attachment to the basement membrane, and decreases the sensitivity of myosin light chain kinase for Ca^{2+}, all of which may contribute to protection of the endothelial barrier (8). Stelzner *et al.* (8) have shown that elevated cAMP can protect the pulmonary endothelial barrier both *in vivo* and *in vitro*. More recently, D. M. Shasby (personal communication) has shown that constitutive endothelial phosphatase activity has a similar salutary effect. Although F-actin polymerization may be a mechanism of action of cAMP, other potential mechanisms, including myosin light chain phosphorylation status, may also play an important role. Thus, in the presence of maintained cAMP kinase-dependent phosphorylation, elevated $[Ca^{2+}]_i$ itself may not be edemagenic.

A dynamic interplay, or crosstalk, between $[Ca^{2+}]_i$ and cAMP occurs at a number of levels along the respective signal transduction cascades. One site of such interaction is through the recently described Ca^{2+} sensitivity of adenylyl cyclase (EC 4.6.1.1) (13–16). Adenylyl cyclase is the transmembrane protein that cyclizes ATP to cAMP and is expressed in at least nine distinct isoforms that are either Ca^{2+} stimulated (three isoforms) (17–19), inhibited (two isoforms) (20, 21), or insensitive (four isoforms) (22, 23). Ca^{2+}-insensitive isoforms of the enzyme are ubiquitously expressed, whereas stimulatable isoforms are localized to brain and inhibitable isoforms are abundant in heart (13–16, 24, 25). In lung homogenates, adenylyl cyclases that represent all Ca^{2+} sensitivities have been found (26), but neither cell-specific expression nor functional significance has been reported. The interaction between $[Ca^{2+}]_i$ and cAMP through adenylyl cyclase was unknown, and, given the evidence in pulmonary endothelial cells that increased $[Ca^{2+}]_i$ disrupts the barrier only in the absence of increased cAMP, we sought to determine if the principal adenylyl cyclase isoform expressed in pulmonary endothelial cells was Ca^{2+} inhibitable (27, 28). We also tested if a Ca^{2+}-inhibitable isoform would promote Ca^{2+}-induced endothelial leak and may, in fact, be essential for full expression of barrier disruption by elevated $[Ca^{2+}]_i$. These ideas were tested in three ways: (*i*) measurement of cAMP formation in pulmonary artery endothelial cell (PAEC) plasma membranes and intact cells, (*ii*) reverse transcriptase PCR cloning of adenylyl cyclase isoforms from PAECs, and (*iii*) measurement of endothelial barrier function *in vitro*. Our results confirm the presence of a Ca^{2+}-inhibitable isoform of adenylyl cyclase that regulates permeability in PAECs.

METHODS

Isolation of Lung Endothelial Cells. All studies utilized lung endothelial cells obtained from bovine pulmonary arteries using standard techniques, as described (29). Cells were studied between primary culture and sixth passage and were intermittently stained with low density lipoprotein (LDL) and factor VIII to verify the cell population. In excess of 90% of cells stain positive for LDL while ≈95% stain factor VIII positive.

Preparation of PAEC Membranes. The method for membrane isolation has been described (15). Briefly, after cell lysis in hypoosmotic buffer and centrifugation at 270 × *g* for 10 min at 4°C, the supernatant was fractionated using a discontinuous sucrose gradient (30–40%) in lysis buffer and the material at

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Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+}; PAEC, pulmonary artery endothelial cell; LDL, low density lipoprotein; IBMX, 3-isobutyl-1-methylxanthine.

the interface was collected. Membranes from the 30–40% interface were washed and resuspended in lysis buffer to a final protein concentration of 0.25–1.0 mg/ml as determined by the method of Lowry *et al.* (30). The samples were frozen and stored in liquid nitrogen.

Measurement of cAMP Accumulation from Cell Membranes. The assay of adenylyl cyclase activity measuring the conversion of [α-^{32}P]ATP [tetra(triethylammonium) salt] to [^{32}P]cAMP has been described (15).

Amplification of Adenylyl Cyclases Expressed in PAECs. Poly(A)$^+$ mRNA was directly isolated from PAECs using oligo(dT)-cellulose (Invitrogen). Degenerate antisense oligonucleotide primers to the C_{2A} region from the second cytosolic loop of adenylyl cyclase were used for amplification with PCR. The PCR products were excised from a 2% agarose gel and annealed to linearized pDIRECT vectors, and the pDIRECT vectors containing the insert were transformed into competent *Escherichia coli* (Clontech). Bacterial cultures were grown for 14–18 hr and successful inserts were selected.

cDNA Sequencing. Plasmid DNA was prepared for sequencing using standard mini-prep and alkaline denaturation techniques. Dideoxynucleotide sequencing was performed with a modified T7 DNA polymerase (Sequenase DNA Sequencing Kit, United States Biochemical).

Measurement of cAMP Accumulation from Whole Cells. cAMP was measured in cells that were grown in 35-mm six-well plates using a standard RIA technique (Biomedical Technologics, Stoughton, MA). Solutions were made with nanopure distilled water and osmolality of solutions was adjusted to 285–305 mosM and pH was adjusted to 7.35–7.40. All solutions contained the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM) and in those studies in which $LaCl_3$ (along with appropriate controls) was utilized, the salt solution was modified so that phosphate and bicarbonate were excluded. In studies of basal cAMP accumulation, either vehicle control, 0.5 mM $LaCl_3$, or Ca^{2+}-free medium (measured to be <200 nM) was added to cells and incubated at 37°C for 90 min. In studies of stimulated cAMP accumulation, bradykinin (1 μM), ionomycin (1 μM, with 1 μM external Ca^{2+}), or vehicle control was added for 2 min at 37°C, followed by a 5-min incubation with isoproterenol (25 μM). Reactions were stopped with 1 M NaOH and then neutralized to pH ≈7.0 with 1 M HCl. Solutions were acetylated, tubes were centrifuged, and the supernatant was decanted. Radioactivity of the precipitate was counted and sample cAMP was calculated from a standard curve. cAMP was standardized to protein, which was measured using the Lowry method (30).

$[Ca^{2+}]_i$ Measurement by Fura-2 Fluorescence. $[Ca^{2+}]_i$ was estimated using the calcium-sensitive fluorophore fura-2, as we have previously described in these cells (31). After baseline $[Ca^{2+}]_i$ was assessed, PAECs were superfused with a physiological salt solution containing 2 mM $CaCl_2$ and $MgCl_2$ and the required test conditions. $[Ca^{2+}]_i$ was estimated using the technique of Grynkiewicz *et al.* (32).

Manganese (Mn^{2+}) Quench of Fura-2 Fluorescence. Mn^{2+} quenching of fura-2 fluorescence was performed to estimate the rate of divalent cation influx, using methods previously described (29). The basal rate of decline in fluorescence due to photobleaching and dye extrusion in the absence of Mn^{2+} was <0.1%/min; therefore, during superfusion with Mn^{2+}-containing Hanks' buffered salt solution, the decline in fluorescence emission in excess of this background rate was considered due to Mn^{2+} influx. Mn^{2+} quenching was calculated by measuring the slope of the fluorescence vs. time plot for the 2-min period before and after the addition of La^{3+}.

Assessment of Permeability. Cultured endothelial cells were trypsinized and resuspended at a density of 2.5×10^5 per cm^2 onto tissue-culture-treated polycarbonate filters (Costar Transwell no. 3413, 0.4-μm pore size), as described (8). In preliminary experiments we found that a radiolabeled small molecular weight tracer, sorbitol, provided superior sensitivity compared with a colorometric assay using albumin, and therefore all experiments were performed using [^{3}H]sorbitol (38 ng/ml; 24 Ci/mmol; 1 Ci = 37 GBq; DuPont/New England Nuclear). The radiolabeled tracer was added to the top or luminal chamber of the transwell plates, with the treatment protocol as indicated below. Modified essential medium alone was added to the lower or abluminal chamber. To avoid any hydrostatic gradient between the luminal and abluminal chamber, the fluid levels were carefully equalized. The transfer of [^{3}H]sorbitol from the luminal to abluminal chamber reflects permeability of the monolayer and was assessed by measuring [^{3}H]sorbitol in both chambers by a liquid scintillation counter (LS 7500, Beckman). In our studies, 2 hr was sufficient to detect [^{3}H]sorbitol in the abluminal chamber. Permeability was expressed as the [^{3}H]sorbitol transfer ratio, where the [^{3}H]sorbitol transfer ratio = abluminal divided by luminal [^{3}H]sorbitol concentration. The [^{3}H]sorbitol transfer ratio without cells was 0.190 ± 0.002 and with post-confluent endothelial cell monolayers was 0.089 ± 0.003.

Statistical Methods. Comparisons were made using either paired or unpaired Student's *t* test, one-way or two-way analysis of variance (ANOVA) with repeated measures, as appropriate. A Student–Neuman–Kuels post hoc test was applied, and differences were considered significant at $P < 0.05$.

RESULTS

Adenylyl Cyclase Activity in PAEC Membranes. To determine the Ca^{2+} sensitivity of adenylyl cyclase in PAECs, cAMP accumulation from cell membranes was measured in the presence of increasing concentrations of Ca^{2+} buffered with EGTA. Basal cAMP production in Ca^{2+}-free physiological salt solution was 5.0 ± 0.3 pmol/mg of protein per min ($n = 6$) and was increased by 25 μM isoproterenol and 15 μM forskolin to 29.5 ± 1.4 pmol/mg of protein per min ($n = 9$; $P < 0.05$) (Fig. 1*A*). Submicromolar Ca^{2+} (0–588 nM) decreased isoproterenol- and forskolin-stimulated production of cAMP in the absence of calmodulin by as much as 31% ± 2% ($n = 9$; $P < 0.05$) (Fig. 1*B*), suggesting expression of a Ca^{2+}-inhibitable isoform of adenylyl cyclase. Inclusion of calmodulin did not change the sensitivity of adenylyl cyclase to Ca^{2+} at any of the Ca^{2+} concentrations, suggesting that PAECs do not possess a Ca^{2+}-stimulated isoform of the enzyme (results not shown).

Adenylyl Cyclases Expressed in PAECs. A portion of the cDNA encoding the second cytosolic loop of adenylyl cyclases expressed in PAECs was amplified, subcloned, and sequenced. Three Ca^{2+}-insensitive isoforms, types II, IV, and VII, were found. These bovine isoforms showed ≈80–90% homology to the respective rat and mouse species at the nucleotide level (Table 1). A single Ca^{2+}-inhibited isoform of adenylyl cyclase, type VI, was also expressed and found to be ≈90% and ≈95% similar to other reported species at the nucleotide and amino acid levels, respectively. It therefore appears likely that the Ca^{2+} inhibition of cAMP production described in Fig. 1 results from a direct effect of Ca^{2+} on the type VI enzyme.

Basal cAMP in Intact Cells. To assess the relevance of a Ca^{2+}-inhibited adenylyl cyclase in intact cells, basal cAMP in PAECs was measured when $[Ca^{2+}]_i$ was decreased with La^{3+} (500 μM) or increased with the calcium ionophore ionomycin (1 μM). We have previously reported that La^{3+} reduces PAEC $[Ca^{2+}]_i$ to ≈50 nM by blocking Ca^{2+} entry (29). Reduced divalent cation influx with La^{3+} was verified by Mn^{2+} quenching of fura-2 fluorescence, where La^{3+} decreased divalent cation entry by 90% ± 7% ($n = 5$; $P < 0.05$). Whereas basal cAMP accumulation was 13.4 ± 0.29 pmol/ml per mg of protein, addition of 0.5 mM IBMX increased basal accumulation to 35 ± 1.7 pmol/ml per mg of protein; except where indicated, the remainder of these studies were conducted using

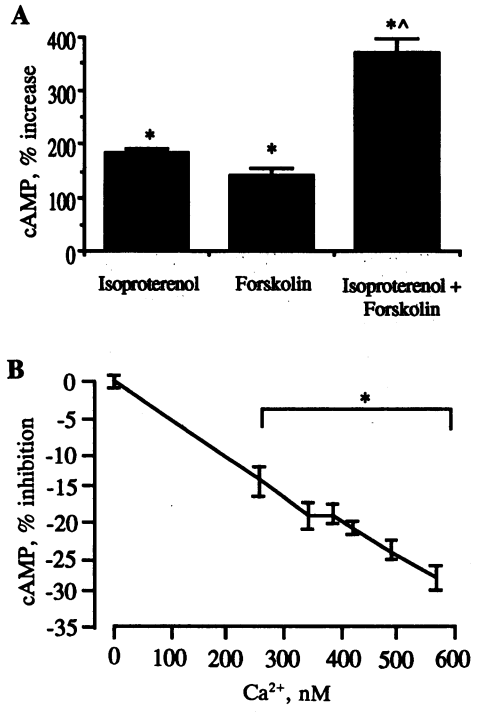

FIG. 1. (*A*) Regulation of basal PAEC membrane adenylyl cyclase activity. Basal cAMP accumulation (measured in the absence of Ca^{2+}) of 5.0 ± 0.3 pmol/mg per min (n = 6) was increased by 25 μM isoproterenol and 15 μM forskolin (n = 9; $P < 0.05$). The combination of isoproterenol and forskolin additively increased cAMP accumulation (n = 9; $P < 0.05$). *, Significantly different from control; ∧, significantly different from the individual treatments. (*B*) Regulation of isoproterenol- and forskolin-stimulated PAEC membrane adenylyl cyclase activity. These studies were conducted without calmodulin to test if adenylyl cyclase activity was directly sensitive to submicromolar Ca^{2+}. cAMP accumulation was stimulated to 29.5 ± 1.4 pmol/mg per min and was dose-dependently inhibited by Ca^{2+} (n = 9; $P < 0.05$). *, Significantly different from isoproterenol and forskolin stimulation. Data are means ± SE.

IBMX to determine the effect of Ca^{2+} on adenylyl cyclase activity without the confounding influence of phosphodiesterases. La^{3+} increased cAMP by nearly 30%, suggesting that a small reduction in $[Ca^{2+}]_i$ was associated with a substantial increase in cAMP (n = 8; $P < 0.05$) (Fig. 2). Experiments with ionomycin were conducted in the presence of 0.25, 0.50, and 1.0 μM external Ca^{2+}. Increased $[Ca^{2+}]_i$ inhibited basal cAMP production in a dose-dependent fashion from 13% to 27% (n = 8; $P < 0.05$), demonstrating inhibition by $[Ca^{2+}]_i$ within a physiologically relevant range (Fig. 2). After a bradykinin-induced $[Ca^{2+}]_i$ transient, the inhibition of cAMP was 12% ± 2%. To exclude the possibility that phosphodiesterase inhibition amplified the bradykinin effect, these experiments were repeated in the absence of IBMX. Without IBMX, bradykinin inhibition of cAMP was ≈3-fold higher (34% ± 2.5%; n = 6; $P < 0.05$).

Stimulated cAMP in Intact Cells. We also tested if $[Ca^{2+}]_i$ affected cAMP production after β-adrenergic stimulation. To elevate $[Ca^{2+}]_i$, intact cells were pretreated with either bradykinin (1 μM) or ionomycin (1 μM with 1 μM extracellular $[Ca^{2+}]$) for 2 min and then stimulated with isoproterenol (25 μM). Bradykinin increased $[Ca^{2+}]_i$ from 83 ± 3 nM to a transient peak of 1161 ± 45 nM, which decayed to a sustained level of 283 ± 35 nM over 5 min (n = 5 experiments averaging multiple cells). Ionomycin increased $[Ca^{2+}]_i$ to a stable peak that was similar in magnitude to the bradykinin peak response (P = not significant; n = 3 experiments averaging multiple cells). Isoproterenol stimulated cAMP content to 1.7 ± 0.1 nmol/ml per mg of protein under phosphodiesterase inhibition. Bradykinin and ionomycin reduced the isoproterenol-stimulated increase in cAMP by ≈20% (n = 10 per group; $P < 0.05$) (Fig. 3). The effect of reduced $[Ca^{2+}]_i$ on isoproterenol-stimulated cAMP was also tested. To reduce $[Ca^{2+}]_i$, intact cells were pretreated with La^{3+} for 15 min and then stimulated with isoproterenol (25 μM). We found that reducing $[Ca^{2+}]_i$ significantly enhanced the production of cAMP (n = 10; $P < 0.05$) (Fig. 3).

Table 1. Comparison of amino acid homology between bovine type VI and other reported sequences within the C_{2A} region of the second cytosolic loop of adenylyl cyclase

Type VI isoform	Sequence
	1 10
Dog	ASGLNASTYD
Mouse	ASGLNASTYD
Human	ASGLNASTYD
Bovine	ASGLNASTYD
	11 20
Dog	QAGRSHITAL
Mouse	QVGRSHITAL
Human	QVGRSHITAL
Bovine	QVGRSHITAL
	21 30
Dog	ADYAMRLMEQ
Mouse	ADYAMRLMEQ
Human	ADYAMRLMEQ
Bovine	ADYAMRLM**DE**
	31 40
Dog	MKHINEHSFN
Mouse	MKHINEHSFN
Human	MKHINEHSFN
Bovine	MKHINEHSFN

The bovine sequence is ≈95% similar to the mouse and human sequences and ≈92% similar to the dog sequence within this region. The nonhomologous bovine amino acids are in boldface type. This reported bovine sequence was uniform within the four clones derived from PAECs.

cAMP and Endothelial Permeability. Since $[Ca^{2+}]_i$ and cAMP modulate barrier function and are inversely related in endothelial cells, we tested if the Ca^{2+} inhibition of adenylyl cyclase is related to endothelial cell permeability. The 2-hr

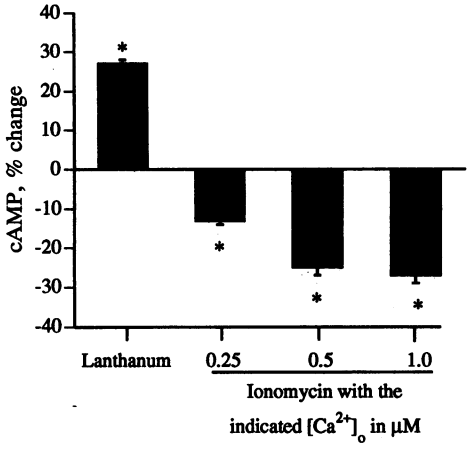

FIG. 2. Effect of $[Ca^{2+}]_i$ on basal cAMP content in intact PAECs. cAMP accumulation was measured over 90 min in intact cells in the presence of 500 μM IBMX (to eliminate the potentially confounding effects of phosphodiesterases). Basal cAMP accumulation was 35 ± 1.7 pmol/ml per mg of protein (n = 8). La^{3+} (500 μM), which decreases $[Ca^{2+}]_i$, increased cAMP content (n = 8; $P < 0.05$). The Ca^{2+} ionophore ionomycin (1 μM) administered in the presence of 0.25, 0.50, or 1.0 μM extracellular Ca^{2+} reduced cAMP content (n = 8; $P < 0.05$). *, Significantly different from control cAMP. Data are means ± SE.

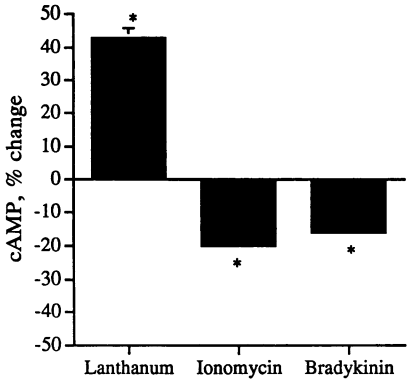

FIG. 3. Effect of $[Ca^{2+}]_i$ on β-agonist-induced cAMP content in intact PAECs. $[Ca^{2+}]_i$ was either reduced by a 15-min incubation with La^{3+} or increased by a 2-min incubation with ionomycin or bradykinin, and then cells were stimulated for 5 min with 25 μM isoproterenol. Isoproterenol stimulated cAMP accumulation to 1.7 ± 0.1 nmol/ml per mg of protein. Whereas La^{3+} (500 μM) increased, ionomycin (1 μM) and bradykinin (1 μM) decreased, cAMP content (n = 10 per group; $P < 0.05$). *, Significantly different from isoproterenol-stimulated cAMP. Data are means ± SE. SE not shown are <1.5 and are not visible above the bars.

transfer of the small molecular weight tracer [^{3}H]sorbitol was measured across postconfluent monolayers of endothelial cells as an *in vitro* bioassay of permeability. La^{3+} (500 μM), which decreased $[Ca^{2+}]_i$ and increased cAMP, reduced the transfer rate of [^{3}H]sorbitol by nearly 40%, whereas IBMX (500 μM), which increases cAMP, decreased permeability by 16% (Fig. 4). Ionomycin (1 μM), which elevated $[Ca^{2+}]_i$ and decreased cAMP, elicited a 25% increase in permeability. Inhibition of the cAMP-dependent protein kinase with R_pcAMPS (Biolog Life Science Institute, La Jolla, CA) also increased leak, but to a lesser extent (≈15%). These increases in permeability were less than that evoked by the thrombin-positive control. However, the combination of ionomycin plus R_pcAMPS increased permeability by 32%, equal to the positive control.

DISCUSSION

This study examined the potential role of a Ca^{2+}-inhibitable adenylyl cyclase in modulating the antagonism between

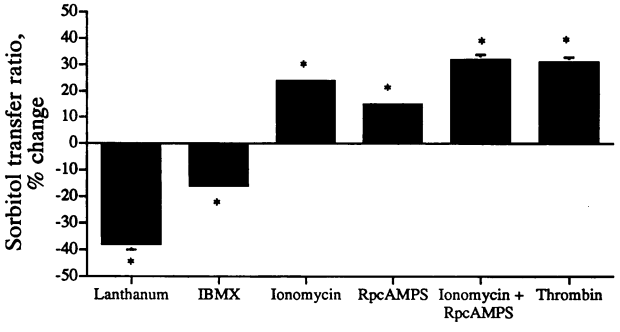

FIG. 4. Role of the type VI adenylyl cyclase on endothelial permeability. The potential role of a Ca^{2+}-inhibitable adenylyl cyclase in endothelial permeability was tested by assessing the transfer ratio of [^{3}H]sorbitol across 12-day postconfluent monolayers of PAECs under conditions of altered $[Ca^{2+}]_i$ and cAMP. The basal 2-hr transfer ratio of [^{3}H]sorbitol was 0.089 ± 0.003. La^{3+} (500 μM) and IBMX (500 μM) reduced the transfer ratio (n = 6 per group; $P < 0.05$). Ionomycin (1 μM), the cAMP-dependent protein kinase inhibitor R_pcAMPS (3 mM), and their combination increased monolayer leak to [^{3}H]sorbitol (n = 6 per group; $P < 0.05$). *, Significantly different from basal [^{3}H]sorbitol transfer. Data are means ± SE. SE not shown are <1.5 and are not visible above the bars.

$[Ca^{2+}]_i$ and cAMP in the control of endothelial permeability. Our first experiments demonstrated Ca^{2+} inhibition of adenylyl cyclase in the submicromolar range in isolated plasma membranes from PAECs, both in the presence and absence of calmodulin. In general, multiple isoforms of adenylyl cyclase are expressed in cells and tissue, and although studies with isolated membranes address the enzyme's Ca^{2+} sensitivity, the isoforms of enzymes expressed can only be identified by knowing RNA or protein sequence. We therefore amplified poly(A)$^+$ RNA using degenerate oligonucleotide primers directed against the second cytosolic loop of adenylyl cyclase, in the C_{2A} region, and sequenced the PCR products. These primers have been successfully used to isolate all known mammalian isoforms. Sequence analysis of the PCR products demonstrated expression of adenylyl cyclase isoforms II, IV, and VII (Ca^{2+} insensitive) and VI (Ca^{2+} inhibited). A concern with this PCR approach is that poly(A)$^+$ RNA may be amplified from cells in primary culture that are not endothelial in origin. Though this is a possibility in our experiments, the only Ca^{2+}-inhibitable isoform of adenylyl cyclase found was type VI. Thus, if a Ca^{2+}-inhibitable isoform of adenylyl cyclase is expressed in PAECs, as our physiological data suggest, it is most likely the type VI isoform.

To determine the relevance of isolated membrane and sequence studies to *in vivo* conditions, we measured basal cAMP production in intact cells. Other investigators have reported substantial basal Ca^{2+} influx in endothelial cells, which is equivalent to maximal agonist-induced Ca^{2+} entry (33). However, the significance of this inward Ca^{2+} "leak" in regulating PAEC function has not been previously addressed. In our experiments, inhibition of basal Ca^{2+} influx by La^{3+} increased cAMP accumulation, demonstrating regulation of adenylyl cyclase by the inward Ca^{2+} leak. Our studies did not determine if Ca^{2+} influx *per se* regulated the enzyme; however, Cooper *et al.* (34) have shown that Ca^{2+} entry rather than intracellular Ca^{2+} release regulates type I and type VI adenylyl cyclases transfected in human embryonic kidney 293 cells and have recently shown that Ca^{2+} entry is, in fact, mandatory for regulation of the type VI enzyme (D.M.F.C., unpublished data). Further, our Mn^{2+} quenching studies demonstrated that La^{3+} reduced divalent cation influx by ≥90%, while reducing $[Ca^{2+}]_i$ by only 40%, suggesting that changes in influx rather than free cytosolic Ca^{2+} may be more important in PAECs as well.

In addition to basal regulation of adenylyl cyclase by Ca^{2+}, we found β-adrenergic-stimulated cAMP accumulation was inhibited by Ca^{2+}, though to a lesser extent than was basal accumulation. Bradykinin, which transiently elevated $[Ca^{2+}]_i$ to ≈1 μM, decreased isoproterenol-induced cAMP by 12%, whereas a more sustained elevation in $[Ca^{2+}]_i$ and Ca^{2+} influx with ionomycin decreased agonist-induced cAMP by nearly 20%. A reduction in Ca^{2+} influx and $[Ca^{2+}]_i$ with La^{3+} potentiated the isoproterenol induction of cAMP by ≈45%. Collectively, sequence analysis and membrane and intact cell studies support the idea that Ca^{2+} and cAMP are inversely related in PAECs through the activity of a Ca^{2+}-inhibitable adenylyl cyclase and suggest that Ca^{2+} inhibition of adenylyl cyclase is due to a direct effect of Ca^{2+} on the type VI enzyme.

A principal physiological role for $[Ca^{2+}]_i$ and cAMP in endothelial cells is modulation of barrier function. We therefore sought to determine if a Ca^{2+}-inhibitable adenylyl cyclase could promote Ca^{2+}-induced barrier dysfunction using an *in vitro* assay of endothelial permeability. This assay has been used by multiple investigators as a tool to study pulmonary endothelial barrier properties and has shown generally good correlation with studies of lung edema in whole animals (8). We found inhibition of the effects mediated by basal cAMP by R_pcAMPS resulted in a 15% increase in permeability. Stelzner *et al.* (8) and others (9–12) have previously shown that the opposite is also true—i.e., addition of exogenous cAMP

improves the basal barrier property of PAECs. Also consistent with this idea, a reduction in basal $[Ca^{2+}]_i$ and Ca^{2+} influx by La^{3+} both increased cAMP and improved barrier function. Finally, an increase in $[Ca^{2+}]_i$ and Ca^{2+} influx by ionomycin reduced cAMP and caused leak. Taken together, these experiments support the idea that alterations in PAEC $[Ca^{2+}]_i$ result in interrelated changes in cAMP and permeability. However, two of our experiments suggest that suppression of adenylyl cyclase may not entirely explain the permeability increase to elevated $[Ca^{2+}]_i$. The permeability increase to ionomycin alone exceeded that to R_pcAMPS alone. In addition, after pretreatment with R_pcAMPS, ionomycin was able to elicit a further increase in permeability. These results suggest that increased $[Ca^{2+}]_i$ has cAMP-independent effects on permeability as well. Our experiments did not address the nature of these additional effects.

Numerous investigators have demonstrated that a rise in endothelial $[Ca^{2+}]_i$ in response to various inflammatory stimuli initiates fluid leak (3–7). Increased $[Ca^{2+}]_i$ is generally thought to activate the cell's contractile apparatus and thereby cause endothelial retraction and increased intercellular passage of water, solutes, macromolecules, and cells. Whereas increased $[Ca^{2+}]_i$ promotes endothelial retraction, cAMP is protective against the development of leak. Elevating endothelial cell cAMP with β-adrenergic agonists, phosphodiesterase inhibitors, prostacyclin mimetics, and cAMP analogues reduces or prevents inflammatory mediator-induced permeability both *in vitro* and *in vivo* (35–38). Although the independent effects of $[Ca^{2+}]_i$ and cAMP on barrier function have been previously reported, the endogenous relationship between these intracellular second messengers in regulating permeability has not been extensively investigated. He and Curry (28) recently tested the hypothesis that cAMP reduced $[Ca^{2+}]_i$ in endothelial cells and was therefore protective against ATP-induced permeability. However, although exogenous cAMP protected systemic microvessels from development of leak, the rise in $[Ca^{2+}]_i$ was not reduced. This result is consistent with the hypothesis that changes in $[Ca^{2+}]_i$ alone may not be sufficient to fully explain agonist-induced permeability increases and that Ca^{2+} regulation of adenylyl cyclase may also play an important role.

We conclude from our experiments that PAECs express multiple isoforms of adenylyl cyclase but that the type VI isoform likely accounts for the cell's Ca^{2+} sensitivity. The Ca^{2+} inhibitability of adenylyl cyclase occurs within a physiological range of $[Ca^{2+}]_i$ and likely contributes to permeability induced by bradykinin and possibly other inflammatory mediators. In the future it will be important to determine if the Ca^{2+} inhibition of adenylyl cyclase is *required* for development of permeability or if the enzyme's Ca^{2+} sensitivity is a *modulator* of the degree of vascular leak that develops during inflammation. Also not addressed by these studies is the role of other second messenger systems, such as protein kinase C, which may regulate adenylyl cyclase activity and endothelial barrier properties. However, the present studies suggest that an important step in the genesis of lung edema may be inhibition of endogenous cAMP production through an interaction between endothelial Ca^{2+} and the type VI adenylyl cyclase.

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