Chloride Efflux in Cyclic AMP–Induced Configurational Change of Bovine Pulmonary Artery Endothelial Cells

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Elevation of cellular cyclic AMP by agents such as isoproterenol plus 3-isobutyl-1-methylxanthine produced rapid and reversible dendritic formation of bovine pulmonary artery endothelial cells in the monolayer. The effect did not occur with exposure of the cells to a variety of other vasoactive agents, calcium ionophore, phorbol ester, or cyclic GMP. The cyclic AMP–induced configurational change was completely inhibited by 2.5 mM N-phenylanthranilic acid or 145 mM sodium gluconate (Cl– channel inhibitors) and was partially inhibited by 2.5 mM 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS), but it was not affected by deprivation of Ca2+ or Na+ ion, 1 mM bumetanide (Cl– cotransport inhibitor), 1 mM amiloride (Na+/H+ exchange inhibitor), 0.1 mM verapamil (Ca2+ channel inhibitor), or 5 mM BaCl2 (K+ channel inhibitor), by change in cellular pH, or by pertussis toxin. Trifluoperazine (calmodulin inhibitor, 50 μM), 1 mM EGTA plus 100 μM 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8, intracellular Ca2+ antagonist), and 5 μM cytochalasin B also produced cellular retraction, but these changes were not blocked by chloride channel inhibition. In the presence of 0.1 mM ouabain plus 0.1 mM bumetanide, 36Cl– uptake was decreased by isoproterenol plus isobutylmethylxanthine while its efflux was enhanced. N-Phenylanthranilic acid inhibited the stimulated efflux. We conclude that cyclic AMP induces a configurational change of endothelial cells that is related to Cl– efflux from the cells; the cellular effects may play a role in vascular function. (Circulation Research 1990;66:957–967)

Vascular endothelial cells perform a variety of functions including regulation of permeability; transport of vasoactive substances; secretion of growth factor(s), endothelial derived relaxing and contractile factors; and production of both procoagulants and anticoagulants.1,2 It has previously been reported that several agents that may alter endothelial function, such as neurotransmitters, thrombin, and phorbol ester, induce spindle-shape changes of endothelial cells,3-5 but the mechanism of these changes is not known. These agents cause Ca2+ mobilization, inositol phosphate turnover, and production of prostaglandin I2 in endothelial cells.6,7 β-Adrenergic agonists and prostaglandins E1 and E2 increase cyclic AMP (cAMP) in endothelial cells,8,9 and cAMP blocks thrombin and transforming growth factor β–induced expression of the c-sis oncogene of these cells.10 cAMP also increases angiotensin converting enzyme.11 The β-adrenergic antagonist propranolol promotes endothelium-dependent relaxation of the thoracic aorta;12 the agonist isoproterenol induces contraction and release of angiotensin II by the mesenteric artery.13 β-Adrenergic modulation also affects growth of endothelial cells14 and atherogenesis in the rabbit aorta.15 Thus, the β-adrenergic system and cAMP of endothelial cells may play a significant role in the regulation of vascular physiology and disease. The present study demonstrates that cAMP induces reversible dendritic formation of endothelial cells and that a Cl– channel, different from Cl– cotransport or anion exchange, determines the cAMP-induced configurational change of these cells.

Materials and Methods

Endothelial Cell Culture

Bovine pulmonary artery endothelial cells were isolated both with collagenase as previously described16 and by scraping the intimal layer of the pulmonary artery. They were grown in Corning T75
flasks (Corning Glass Works, Corning, New York) containing RPMI 1640 medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml amphotericin B, and 10% fetal calf serum (HyClone Laboratories, Logan, Utah) at 37°C in 95% air-5% CO₂. All cells used in these studies were from primary and secondary cultures. Cells for secondary cultures were obtained by trypsinization (0.1% trypsin for 2–3 minutes, GIBCO Laboratories, Grand Island, New York) of confluent monolayered cells. The cells were seeded in Corning 35-mm Petri dishes at 4x10⁴ cells/dish and were cultured for 2–3 days (low density) or 6 days (confluent). Angiotensin converting enzyme and factor VIII were identified on endothelial cells by using immunofluorescent methods as previously described.16

Configurational Change of the Endothelial Cell

The culture media were discarded, and cells were washed three times with 2 ml HEPES buffer containing (mM) NaCl 145, KCl 5, CaCl₂ 1.1, MgSO₄ 0.38, HEPES 10 (pH 7.4), and glucose 5.5 at 37°C. After a 15-minute incubation in HEPES buffer at 37°C in room air, the media were replaced with 2 ml fresh HEPES buffer and further equilibrated for 10 minutes at 37°C. All cells appeared to be normally spread out at this time, or within 15–30 minutes. After equilibration with HEPES buffer, the cells were incubated for 10 minutes with or without 0.2 mM 3-isobutyl-1-methylxanthine (IBMX). Media were then replaced with fresh HEPES buffer containing isoproterenol with or without IBMX or containing other agents without IBMX as noted in Tables 1 and 2. After a 5–60-minute incubation at 37°C, cells were observed and photographed by phase contrast microscopy. Dendritic formation is an expression of two-dimensional retraction of the cellular cytoplasm with remaining extensions of the cytoplasm that are longer than the nuclear diameter. The percent of cells with dendritic formation was determined at 30 minutes by counting 200 cells in four or more microscopic fields. The percent of cells with dendritic formation was then quantitated.

Phase Contrast and Electron Microscopic Photographs

Photographs were taken at 37°C with an inverted phase contrast microscope (Diaphot-TMD, Nikon, Garden City, New York) with Polaroid 4×5 Land film holder No. 545 and microflex UFX-II. Fixation and preparation for scanning electron microscopy were done as previously described.17,18 Endothelial cells were grown on 15-mm round plastic coverslips (Thermonex, Miles Scientific, Nuperville, Illinois). After primary fixation in 0.1 M cacodylate-buffered 3% glutaraldehyde, pH 7.3, with or without test agents for 1 hour, the specimen was fixed with 0.1 M cacodylate-buffered 1% osmium tetroxide, pH 7.3, for 20 minutes at 4°C and dehydrated through a graded series of ethanol. The coverslip was dried and coated with gold-palladium (60:40) and examined with a scanning electron microscope (Amray-1000B, Bedford, Massachusetts).

Assay of cAMP Content of Cells

Cells of eight dishes of 2–3-day cultures were collected after a 10-minute incubation with the agents tested. After extraction of cells with acetyl ether, the samples were lyophilized and resuspended in 50 mM sodium acetate buffer, pH 6.3. cAMP was assayed by an acetylation process with a [125]IcAMP radioimmunoassay kit (New England Nuclear, Boston, Massachusetts) as previously described.19

Chloride Uptake and Efflux

For ³⁵Cl⁻ uptake experiments, confluent cells (6–8 days) were washed twice with HEPES buffer and

| TABLE 1. Relation Between Cellular Configurational Changes and Cyclic AMP |
|--------------------------|------------------|------------------|
| Agent                    | Dendritic formation at 30 minutes (pmol/10⁴ cells) | cAMP (pmol/10⁴ cells) |
| Control                  | 2.0±0.1          | 10               |
| 10⁻⁴ M (-)-ISO            | ±                | 3.7              |
| 10⁻⁷ M (-)-ISO            | ±                | 6.1              |
| 10⁻⁶ M (-)-ISO            | ± 10.9±1.0       | 6                |
| 10⁻⁴ M (-)-ISO+0.2 mM IBMX| ±                | 56.3±2.9         |
| 0.2 mM IBMX               | ±                | 3.3±0.1          |
| 10⁻³ M (-)-norepinephrine | ± 10.4±0.2       | 4                |
| 10⁻³ M (-)-epinephrine    | ± 5.5            | 2                |
| 10⁻⁶ M (-)-ISO+10⁻⁴ M (+)-PROP | ± 2.1±0.1   | 3                |
| 10⁻³ M prostaglandin E₂  | ± 3.3±0.1        | 5                |
| 10⁻³ M phenylephrine      | ± 2.8±0.4        | 3                |
| 10⁻³ M arginine vasopressin | ± (2)           | 2.8±0.4          |
| 10⁻⁴ M Bay K 8644         | ± 2.2±0.3        | 4                |
| 10⁻⁶ M A23187             | ± 1.9            | 2                |
| 10⁻³ M PMA                | ± 1.1±0.8        | 4                |
| 10⁻³ M serotonin         | ± 1.8            | 2                |
| 10⁻³ M histamine          | ± 2.0            | 2                |
| 10⁻³ M acetylcholine      | ± 2.0            | 2                |
| 10⁻⁶ M PAF                | ± 1.9            | 2                |
| 10⁻³ M dibutyryl cAMP     | ±                 | ...              |
| 10⁻³ M dibutyryl cGMP     | ±                 | ...              |
| 10⁻⁴ M sodium nitroprusside| ±                 | 1.9±2            |

Mean and mean±SEM values are recorded. cAMP, cyclic AMP (assayed as described in “Materials and Methods”); ISO, isoproterenol; IBMX, 3-isobutyl-1-methylxanthine; PROD, propranolol; PMA, phorbol 12-myristate 13-acetate; PAF, l-α-phosphatidylcholine, β-acetyl-γ-0-alkyl; cGMP, cyclic GMP.

After equilibration with HEPES buffer for 25 minutes, cells were incubated for 10 minutes with or without 0.2 mM IBMX. Media were then replaced with fresh HEPES buffer containing ISO with or without IBMX or containing other agents noted in the table without IBMX. Dendritic formation is an expression of structural change of the cellular cytoplasm with remaining extensions of the cytoplasm that are longer than the nuclear diameter. The percent of cells with dendritic formation is as follows: –, <1%; ±, 1–10%; +, 10–50%; ++, ≥50%.
TABLE 2. Effect of Media Composition on Cellular Configurational Changes Resulting From Isoproterenol and 3-Isobutyl-1-Methylxanthine

<table>
<thead>
<tr>
<th>Agent</th>
<th>Presence of dendritic formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES control</td>
<td>++</td>
</tr>
<tr>
<td>Ca²⁺-free HEPES, 0.1 mM EGTA</td>
<td>++</td>
</tr>
<tr>
<td>Na⁺-free THAM, 145 mM LiCl</td>
<td>++</td>
</tr>
<tr>
<td>Cl⁻-free HEPES, 145 mM sodium gluconate</td>
<td>-</td>
</tr>
<tr>
<td>Cl⁻-reduced HEPES, 115 mM sodium gluconate, 30 mM NaCl</td>
<td>++</td>
</tr>
<tr>
<td>2.5 mM SITS</td>
<td>±</td>
</tr>
<tr>
<td>2.5 mM N-phenylanthranilic acid</td>
<td>-</td>
</tr>
<tr>
<td>Cl⁻-free sucrose buffer</td>
<td>++</td>
</tr>
<tr>
<td>1 mM bumetanide</td>
<td>++</td>
</tr>
<tr>
<td>1 mM amiloride</td>
<td>++</td>
</tr>
<tr>
<td>1 mM ouabain</td>
<td>++</td>
</tr>
<tr>
<td>5 mM BaCl₂</td>
<td>++</td>
</tr>
<tr>
<td>20 mM TEA, 125 mM NaCl</td>
<td>++</td>
</tr>
<tr>
<td>0.1 mM verapamil</td>
<td>++</td>
</tr>
<tr>
<td>50-500 ng/ml pertussis toxin</td>
<td>++</td>
</tr>
<tr>
<td>pH 6.2 HEPES</td>
<td>++</td>
</tr>
<tr>
<td>pH 8.0 HEPES</td>
<td>++</td>
</tr>
</tbody>
</table>

THAM, tris(hydroxymethyl)aminomethane; SITS, 4-acetamidophenyl-2,2'-disulfonic acid; TEA, tetrathyamine.

Cells were equilibrated with HEPES buffer for 25 minutes. Media were replaced with HEPES buffer containing various agents, and cells were preincubated for 10 minutes. Media were then replaced with fresh test solutions also containing 10⁻⁴ M (-)-isoproterenol and 0.2 mM 3-isobutyl-1-methylxanthine. Dendritic formation was determined 30 minutes later. The percent of cells with dendritic formation is as follows: -, <1%; ±, 1-10%; ++, ≥50%. All agents tested were in HEPES buffer except for Na⁺-free experiments in which 8 mM THAM buffer was used.

incubated at 27°C for 2 hours with HEPES buffer containing 0.1 mM ouabain and 0.1 mM bumetanide, which were added to inhibit Na⁺,K⁺-ATPase and Na⁺/K⁺/Cl⁻ or K⁺/Cl⁻ cotransport of endothelial cells. Then, the medium was removed, and the cells were incubated with 1 ml HEPES buffer containing 0.1 mM ouabain, 0.1 mM bumetanide, and 0.25 μCi/ml ³⁶Cl⁻ (final concentration, 150 mM) for various intervals. ³⁶Cl⁻ uptake was stopped by rapidly washing the dishes twice with 2 ml ice-cold HEPES buffer; 1 ml of 0.1N NaOH was then added to the dishes, and an aliquot was measured for radioactivity by liquid scintillation counting.

For ³⁶Cl⁻ efflux measurements, confluent cell monolayers were washed twice with HEPES buffer and incubated with 1 ml HEPES buffer containing 0.5 μCi/ml ³⁶Cl⁻ (final concentration, 150 mM) at 27°C for 3 hours. The cells were then washed twice with 2 ml HEPES buffer and incubated at 27°C with 1 ml of the same buffer without ³⁶Cl⁻ in the presence of 0.1 mM ouabain and 0.1 mM bumetanide for 60-90 minutes. ³⁶Cl⁻ efflux was started by the addition of 1 ml of the HEPES buffer containing 0.1 mM ouabain and 0.1 mM bumetanide and stopped by rapidly washing the monolayers three times with ice-cold HEPES buffer. The intracellular isotope that remained was extracted with 1 ml of 0.1N NaOH. Radioactivity in each sample was measured by liquid scintillation counting.

Protein Determination

Protein concentration was determined as described by Lowry et al.²⁰

Reagents

Bumetanide and N-phenylanthranilic acid were gifts from Dr. R.D. Perrone and Dr. S. Helden, New England Medical Center Hospitals, Boston, Massachusetts, and Bay K was from Miles Laboratories, Elkhart, Indiana. ³⁶Cl⁻ (specific activity, 18.1 mCi/g chloride) was from New England Nuclear. All other agents were from Sigma Chemical, St. Louis, Missouri, and Fisher Scientific, Pittsburgh, Pennsylvania.

Statistical Analysis

The data are expressed as mean±SEM, and significance between groups was tested by Student's t test.

Results

Configurational Change of Endothelial Cells

Subconfluent endothelial cells appeared to be spread out after washing three times with HEPES buffer (Figure 1a). Dendritic formation of the cells was observed within 10-30 minutes after the addition of HEPES buffer containing 10⁻⁴ M (-)-isoproterenol and 0.2 mM IBMX (Figure 1b). The cytoplasm of the cells receded, and the cellular membrane became highly invaginated. Dendritic formation was visualized for both low density (Figures 1c and 1d) and high density (Figures 1e and 1f) cells. A similar, but less pronounced, response was noted in the presence of isoproterenol or IBMX alone. Dendritic formation was completely reversed within 2-20 minutes when the medium containing isoproterenol with or without IBMX was replaced with fresh HEPES buffer or with 10⁻⁴ M propranolol, which also blocked the change in cellular configuration (Figure 2).

A similar configurational change occurred when cells were exposed to 10⁻⁵ M (-)-norepinephrine, 10⁻⁵ M (-)-epinephrine, or 10⁻⁵ M prostaglandin E₂. These agents produced small increases in cAMP in the absence of IBMX (Table 1) but elevated cAMP by 14-fold, 12-fold, and fivefold, respectively, and produced clear configurational changes in the presence of IBMX (60±6%, 68±6%, and 25±5%, respectively). Dibutyryl-cAMP (10⁻³ M) also produced dendritic formation of the cells. A more marked elevation of cAMP was produced by isoproterenol plus IBMX, which also caused the most pronounced dendritic formation (83±3% of endothelial cells). Both cellular dendritic formation and cAMP elevation were blocked in the presence of propranolol. A variety of other agents, including phenylephrine (α₁-agonist), histamine, serotonin, acetyl-
FIGURE 1. Scanning electron (panels a and b) and phase contrast (panels c-f) microscopic photographs of control endothelial cells and cells exposed to $10^{-6}$ M (-)-isoproterenol and 0.2 mM 3-isobutyl-1-methylxanthine (IBMX). Panel a: Control endothelial cell after 2 days in culture. Bar, 10 μm. Panel b: Endothelial cell after 2 days in culture subjected to $10^{-6}$ M (-)-isoproterenol and 0.2 mM IBMX for 30 minutes. Panel c: Control cells after 2 days in culture. Original magnification, $\times 320$. Panel d: Dendritic formation of cells in $10^{-6}$ M (-)-isoproterenol and 0.2 mM IBMX for 30 minutes after 2 days in culture. Original magnification, $\times 320$. Panel e: Control endothelial cells after 6 days in culture. Original magnification, $\times 640$. Panel f: Endothelial cells after 6 days in culture after exposure to $10^{-6}$ M (-)-isoproterenol and 0.2 mM IBMX for 30 minutes. Original magnification, $\times 640$. Arrows point to dendritic formation.
FIGURE 2.  Photomicrographs showing serial configuration change of endothelial cells (2 days in culture). Endothelial cells were treated with $10^{-6}$ M isoproterenol in the absence of 13-isobutyl-1-methylxanthine. Cells are shown at 0 minutes (panel 1) and at 20 minutes (panel 2). Then, after replacement of isoproterenol with $10^{-6}$ M propranolol, endothelial cells were photographed at 20 minutes (panel 3) and at 100 minutes (panel 4). The same cells (A,a–e) were serially observed. The photographs demonstrated the reversible nature of the change in configuration. Original magnification, $\times 320$. 
choline, L-α-Phosphatidylcholine, β-acetyl-γ-0-alkyl (PAF), vasopressin, Bay K 8644 (Ca^{2+} channel agonist), A23187 and ionomycin (Ca^{2+} ionophores), phorbol 12-myristate 13-acetate (PMA), and sodium nitroprusside (none of which elevated cAMP), did not produce a configurational change (Table 1). Dibutyryl cyclic GMP (cGMP, 10^{-3} M) also failed to produce a configurational change, but were unable to assay cAMP in the presence of 10^{-3} M cGMP. Although there was no dendritic change, PMA (10^{-5}−10^{-4} M) did produce a slight spindle-shape change in the endothelial cells, which has been previously reported. These findings indicate that configurational changes of endothelial cells are induced by cAMP but not by other second messengers tested.

Effects of Channel Blockers and Substitution of Ions in Medium

To investigate the mechanism of the cAMP-induced configurational change, we tested various transport inhibitors and ion-substituted media (Table 2). Replacement of Ca^{2+} or Na^{+} in the medium with another cation failed to inhibit the configurational change produced by isoproterenol plus IBMX. Bumetanide, an inhibitor of Na^{+}/K^{+}/Cl^{−} cotransport and K^{+}/Cl^{−} cotransport; amiloride, a Na^{+}/H^{+} exchange inhibitor; and ouabain, a Na^{+},K^{+}-ATPase inhibitor, all failed to inhibit the dendritic change produced by cAMP activation. However, 2.5 mM of 4-acetamido-4’-isothiocyanostilbene-2,2’-disulfonic acid (SITS; Cl^{−} channel blocker and Cl^{−}/HCO_{3}^{−} exchange inhibitor) and 2.5 mM N-phenylanthranilic acid (Cl^{−} channel inhibitor) inhibited the cAMP-induced structural changes of endothelial cells (Figures 3b−3d). Furthermore, replacement of 145 mM Cl^{−} with 145 mM gluconate anion totally prevented the configurational change (Figures 1d and 3a). A combination of 115 mM gluconate and 30 mM Cl^{−} failed to inhibit the cAMP-induced configurational change. The change was still observed in Cl^{−}- and gluconate-free sucrose buffer (240 mM sucrose, 1 mM MgSO_{4}, and 10 mM HEPES-Tris buffer, pH 7.4), but this effect was inhibited by N-phenylanthranilic acid.

The elevation of cAMP content by isoproterenol plus IBMX was not reduced by 2.5 mM N-phenylanthranilic acid, 2.5 mM SITS, or replacement of Cl^{−} with gluconate (data not shown). These findings indicate that inhibition of cAMP-induced configurational change of endothelial cells was not due to a decrease in cellular cAMP but, rather, to an inhibition of N-phenylanthranilic acid, SITS, and gluconate-sensitive Cl^{−} transport. BaCl_{2} and tetraethylammonium, a K^{+} channel inhibitor, did not inhibit the configurational change. Also, bumetanide, an inhibitor of Na^{+}/K^{+}/Cl^{−} and K^{+}/Cl^{−} cotransport, low pH, N-ethylmaleimide, and Ca^{2+} ionophore, known inhibitors and stimulators of K^{+}/Cl^{−} cotransport, neither inhibited nor induced the configurational change. The change occurred both in buffer with HCO_{3}^{−} (RPMM 1640 medium) and without HCO_{3}^{−} (HEPES), suggesting that any Cl^{−}/HCO_{3}^{−} exchanger does not participate in the configurational change. These findings support a conclusion that the cAMP-induced configurational change is directly coupled to stimulation of a specific cellular Cl^{−} channel.

Effects of Trifluoperazine, EGTA Plus TMB-8, Cytochalasin B, and Pertussis Toxin on Configurational Changes

Next, we examined whether the cAMP-induced configurational change of endothelial cells might be related to changes in other cellular constituents such as intracellular Ca^{2+}, calmodulin, cytoskeleton, or G protein activation. In this series of experiments, we found that the block in cellular dendritic formation associated with inhibition of Cl^{−} transport was resistant to most agents that were tested. However, the dendritic formation was not blocked by Cl^{−} channel inhibition when tested in the presence of 50 μM trifluoperazine (TFF), a calmodulin inhibitor, or 1 mM EGTA plus 100 μM 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8; intracellular Ca^{2+} antagonist). TFF itself also produced a marked configurational change without dendritic formation (Figures 3e and 3f). The TFF-induced configurational change was reversed within 10−30 minutes after the exchange of medium to HEPES buffer alone (Figure 3g). EGTA alone did not produce a configurational change.
Cytochalasin B did not inhibit the cAMP-induced configurational change of the endothelial cell but, rather, produced dendritic formation itself within 10–30 minutes without an elevation of cAMP (Figures 3h and 3i), and similar to the effect of cAMP-stimulation, the configurational change induced by 5 μM cytochalasin B was partially blocked in the presence of Cl⁻ channel inhibition (Figures 3j and
3k). However, the configurational change produced by greater than 10 μM cytochalasin B was not affected by Cl- channel inhibition.

Pretreatment with pertussis toxin (50–500 ng/ml) for 60 minutes neither produced a configurational change nor inhibited the dendritic formation produced by cAMP activation. Hence, in contrast to observations with neutrophils,32 pertussis toxin-sensitive G proteins Gαi and Gq,33 were not involved in the configurational change of endothelial cells. Thus, intracellular Ca²⁺, calmodulin, cytoskeleton, and pertussis toxin-sensitive G protein do not appear to be related to cAMP-induced configurational change of endothelial cells.

36Cl⁻ Uptake and Efflux

In a separate series of experiments, 36Cl⁻ fluxes were studied in the endothelial cells. The endothelial cells rapidly accumulated 36Cl⁻, and a plateau was reached at 30 minutes (Figure 4). 36Cl⁻ uptake was decreased in the presence of ouabain and bumetanide. Isoproterenol plus IBMX, which increased cAMP (56.5±2.5 pmol/10⁶ cells), decreased 36Cl⁻ uptake by approximately 20–35% of the control value both in the presence (Figure 4) or the absence (data not shown) of bumetanide and ouabain (p<0.01). N-Phenylanthranilic acid, a putative Cl⁻ channel inhibitor in T84 colon epithelial cells,25 blocked 36Cl⁻ uptake by endothelial cells in the presence of bumetanide and ouabain with or without isoproterenol plus IBMX. These data suggest that the reduction in 36Cl⁻ uptake produced by isoproterenol plus IBMX occurs through a Cl⁻ channel.

36Cl⁻ efflux from endothelial cells, calculated from 36Cl⁻ remaining in the cells, was increased by isoproterenol plus IBMX from 30% to 63% (p<0.01) at 60 minutes (Figure 5). N-Phenylanthranilic acid reversed the isoproterenol-induced increase in 36Cl⁻ efflux to control values. Thus, isoproterenol plus IBMX increased 36Cl⁻ efflux and reduced 36Cl⁻ uptake in the endothelial cells through Cl⁻ channel transport. Although N-phenylanthranilic acid also reduced the uptake of 36Cl⁻, it totally blocked the enhanced efflux of 36Cl⁻ produced by isoproterenol plus IBMX.

Discussion

The present study shows that agents that stimulate cAMP specifically induce dendritic formation of the endothelial cell in monolayer. The configurational change was rapid and reversible. The magnitude of the morphological response roughly paralleled the extent of elevation of cAMP. When cAMP returned to normal levels (for example, at 30–60 minutes after isoproterenol alone) the dendritic formation of the cells reversed to a “spread out” configuration. In the presence of IBMX, in association with isoproterenol, prostaglandin E₂, and norepinephrine, in which a higher-than-control level of cAMP persisted even at 30–60 minutes (data not shown), the dendritic formation also persisted. Hence, the configurational change appeared to be closely coupled to the cellular level of cAMP. Various other second messengers, such as intracellular Ca²⁺, cGMP, protein kinase C, and H⁺ (lower or higher pH), all failed to either produce a configurational change or inhibit the cAMP-induced dendritic formation.

The calmodulin inhibitor, TFP, and depletion of cellular Ca²⁺ with EGTA and TMB-8 also produced
a reversible cellular structural change, but the change was not prevented by Cl\(^-\) channel inhibition. Although it is tempting to speculate that reduction of intracellular Ca\(^{2+}\) or the Ca\(^{2+}\)-calmodulin complex may in itself cause a configurational change of endothelial cells, it must also be remembered that TFP and TMB-8 may have other effects on endothelial cells because of their lack of specificity. Dendritic formation in endothelial cells was also observed after exposure to cytochalasin B, which is known to sever actin filaments.\(^{34}\) In contrast to the cAMP-induced effect, the cytochalasin B–induced configurational change was only partially prevented by inhibition of the Cl\(^-\) channel. Thus, the mechanism for these effects differs from that of the cAMP-induced change. It is possible that cytochalasin B and TFP affect cellular configuration through other ion transport systems, such as Na\(^+\)/H\(^+\) or Cl\(^-\)/HCO\(_3^-\) exchange, as has been shown for cytochalasin B–induced and TFP-induced volume change in gall bladder cells.\(^{35,36}\)

Complete inhibition of the cAMP-induced configurational change was observed only in the presence of N-phenylanthranilic acid, a known Cl\(^-\) channel inhibitor for T84 colonic cells,\(^{25}\) or with replacement of Cl\(^-\) in the medium with gluconate that competes with Cl\(^-\) for the Cl\(^-\) channel in various cells.\(^{23,24}\) SITS, another Cl\(^-\) channel inhibitor, produced a more modest inhibition. These agents did not inhibit the elevation of cAMP produced by isoproterenol and IBMX; this finding indicates that Cl\(^-\) transport is critical for the configurational change.

A requirement for a specific cellular Cl\(^-\) transporter for cellular configurational change was supported by experiments showing that a variety of other inhibitors of Cl\(^-\) transport coupled to other ion movements had no effect on the cAMP-induced change. It was recently reported that cGMP activates Na\(^+\)/K\(^+\)/Cl\(^-\) cotransport in smooth muscle cells\(^{37}\) and that low pH and N-ethylmaleimide activate K\(^+\)/Cl\(^-\) cotransport in sheep red blood cells.\(^{29,30}\) These Cl\(^-\) cotransport activities are inhibited by furosemide or bumetanide. However, bumetanide (0.1–1 mM) failed to inhibit the cAMP-induced configurational change of endothelial cells, but N-phenylanthranilic acid completely inhibited the change both in the presence or absence of bumetanide. Neither the presence of HCO\(_3^-\), an inducer of Cl\(^-\)/HCO\(_3^-\) exchange, nor amiloride, a Na\(^+\)/H\(^+\) exchange inhibitor, induced a configurational change or inhibited the cAMP-induced change. The K\(^+\) channel is known to be functionally coupled to the Cl\(^-\) channel in kidney and colon cells.\(^{25,27}\) However, BaCl\(_2\) and tetraethylammonium, an inhibitor of the K\(^+\) channel, did not influence the configurational change of the endothelial cells. Thus, certain mechanisms of Cl\(^-\) movement, such as Na\(^+\)/K\(^+\)/Cl\(^-\) cotransport, other Cl\(^-\) cation cotransport, Cl\(^-\)/HCO\(_3^-\) exchange, Na\(^+\)/H\(^+\) exchange, and the K\(^+\) channel do not appear to be involved in the cAMP-induced configurational change of endothelial cells.

While 145 mM gluconate in the absence of Cl\(^-\) inhibited the cAMP-induced configurational change, a combination of 115 mM gluconate and 30 mM Cl\(^-\) failed to inhibit the change. We observed that 20–30 mM Cl\(^-\) was critical for the expression of the cAMP-induced change as long as gluconate was also present in the medium. Total replacement of Cl\(^-\) with sucrose rather than another anion failed to inhibit the cAMP-induced change, but the cAMP-induced configurational change in the presence of sucrose was inhibited by N-phenylanthranilic acid. These findings suggest that Cl\(^-\) transport is critical for the structural change and that, in the presence of sucrose, Cl\(^-\) is derived from inside the cell. Furthermore, gluconate ion appears to inhibit the Cl\(^-\) channel of the endothelial cells.

In the presence of bumetanide and ouabain, 36Cl\(^-\) efflux from endothelial cells was increased while 36Cl\(^-\) uptake was inhibited by isoproterenol plus IBMX (Figures 4 and 5). These effects were blocked by N-phenylanthranilic acid, a Cl\(^-\) channel transport inhibitor. TFP, which itself caused a configurational change of endothelial cells, actually increased 36Cl\(^-\) uptake in the endothelial cells, supporting a different mechanism for its effect from that of isoproterenol plus IBMX (data not shown). The findings support a concept that the cAMP-induced changes in 36Cl\(^-\) flux were not passively brought about by the configurational change in endothelial cells but, rather, by activation of 36Cl\(^-\) efflux through Cl\(^-\) channel transport. Thus, the cAMP-induced Cl\(^-\) extrusion is related to and essential for the induction of the configurational change in the endothelial cells.

It has been previously reported that endothelial cells change their configuration in response to thrombin,\(^3\) histamine,\(^4,5\) serotonin,\(^4\) phorbol ester, and forskolin.\(^3\) Norepinephrine and serotonin, but not histamine, increase the number of stress fibers and surface area of bovine aortic endothelial cells.\(^4\) The dramatic change in cell shape observed in our studies was not reported in studies of umbilical vein and aortic endothelial cells treated with histamine, phorbol ester, and forskolin.\(^3\) However, the differences between these studies and ours could be explained by the inclusion of experiments with subconfluent cells in our studies. The subconfluent cells showed the most dramatic morphological responses. Also, the most pronounced morphological changes in our studies were observed when cAMP was elevated the most, that is, in the presence of isoproterenol plus IBMX, a condition that was not evaluated in the other studies. It remains to be determined whether cAMP is a common factor for configurational changes produced by the various agents noted above, but thrombin, histamine, and phorbol ester are able to produce or potentiate elevation of cAMP in endothelial cells.\(^38,39\)

Many studies have now shown either directly or indirectly that cAMP alterations play a role in vascular physiological and pathological processes. For example, propranolol promotes endothelium-
dependent relaxation of the thoracic aorta; isoproterenol induces contraction and release of angiotensin II of the mesenteric artery. CAMP blocks the expression of c-sis oncogene and stimulates angiotensin converting enzyme of endothelial cells. β-Adrenergic modulation also affects growth of endothelial cells and atherogenesis in rabbit aorta. Further exploration is needed to determine whether or not CAMP-activated Cl" transport and endothelial cell configurational changes participate in physiological and pathological processes.

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References


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