Opposite effect of cAMP signaling in endothelial barriers of different origin

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Binewald, K., D. Gündüz, F. Härtel, S. C. Peters, C. Rodewald, S. Nau, M. Schäfer, J. Neumann, H. M. Piper, and T. Noll. Opposite effect of cAMP signaling in endothelial barriers of different origin. Am J Physiol Cell Physiol 287: C1246–C1255, 2004; doi: 10.1152/ajpcell.00132.2004.—cAMP-mediated signaling mechanisms may destabilize or stabilize the endothelial barrier, depending on the origin of endothelial cells. Here, microvascular coronary [coronary endothelial cells (CEC)] and macrovascular aortic endothelial cell (AEC) monolayers with opposite responses to cAMP were analyzed. Macromolecule permeability, isometric force, activation state of contractile machinery [indicated by phosphorylation of regulatory myosin light chains (MLC), activity of MLC kinase, and MLC phosphatase], and dynamic changes of adhesion complex proteins (translocation of VE-cadherin and paxillin) were determined. cAMP signaling was stimulated by the adenosine receptor agonist 5′-(ethylcarboxamido)-adenosine (NECA), the β-adrenoceptor agonist isoprotenerol (Iso), or by the adenylyl cyclase activator forskolin (FSK). Permeability was increased in CEC and decreased in AEC on stimulation with NECA, Iso, or FSK. The effects could be inhibited by the PKA inhibitor Rp-8-CPT-cAMPS and imitated by the PKA activator Sp-cAMPS. Under cAMP/PKA-dependent stimulation, isometric force and MLC phosphorylation were reduced in monolayers of either cell type, due to an activation of MLC phosphatase. In CEC but not in AEC, FSK induced delocalization of VE-cadherin and paxillin from cellular adhesion complexes as indicated by cell fractionation and immunofluorescence microscopy. In conclusion, decline in contractile activation and isometric force contribute to cAMP/PKA-mediated stabilization of barrier function in AEC. In CEC, this stabilizing effect is overruled by cAMP-induced disintegration of cell adhesion structures.

endothelial cell adhesion; endothelial permeability; isometric force; myosin light chain kinase; myosin light chain phosphatase

VASCULAR ENDOTHELIUM FORMS a dynamic barrier between plasma and interstitial space for water, solutes, and plasma proteins. A large number of studies have documented that the cAMP/PKA signaling pathway can regulate endothelial barrier function. On stimulation of this pathway, two different responses have been observed. In many studies, predominantly on endothelial monolayers of macrovascular origin (3, 19, 22, 23, 30, 37) and in isolated, perfused microvessels (1, 10, 12) stimulation of the cAMP/PKA pathway causes a stabilization of barrier property. In endothelial monolayers of coronary origin (11, 28, 41) as well as in microvessels from the coronary system (13), hamster cheek pouch (5), and adipose tissue (35), an increase in endothelial permeability was found.

From the different responses in diverse experimental systems, the question arose whether the differences could be explained mechanistically. Changes in endothelial barrier function are usually due to one of two principal mechanisms: the first is based on activation or inactivation of the actin-myosin-based contractile machinery, which is triggered by the phosphorylation state of myosin light chains (MLC). The phosphorylation state of this regulatory subunit is balanced by a Ca2+-dependent MLC kinase (MLCK) and a MLC phosphatase (MLCP), the activity of which is regulated by various signaling pathways (4, 6, 15, 42). Activation of the contractile apparatus causes changes in cell shape, opening of intercellular gaps and, thereby, a change in barrier function. Not only in cultured endothelial monolayers but also in intact vessels such as coronary venules, activation of the contractile machinery has been identified as the dominant mechanism to increase permeability (43). The second mechanism is based on the stabilization or destabilization of cell-cell or cell-matrix junctions. In endothelial cell junctions, several adhesion molecules are clustered in multiprotein complexes in which transmembrane proteins such as VE-cadherins (17) or integrins (18) bind to corresponding adhesion proteins at adjacent cells or to the extracellular matrix, respectively. These proteins are linked via intermediate proteins, such as catenins or paxillin, to the actin-based cytoskeleton at the inner leaflet of the cell membrane. Assembly and disassembly of these cell adhesion complexes are controlled by intracellular signaling mechanisms, e.g., the cAMP/PKA pathway. It has been shown that phosphorylation of cell-cell and cell-matrix junction proteins, such as catenins, vinculin, or paxillin, can provoke disintegration of these complexes (9, 17, 18, 25, 26, 38, 39). As a result, cells detach from their immediate neighbors or from extracellular matrix. Concomitant shape changes and opening of intercellular gaps are the basis for an increase in permeability (6, 20, 21, 24, 26, 34). It is as yet unclear to which extent contractile machinery and cell junctions contribute to the divergent effects of cAMP/PKA signals in endothelial barriers.

In the present study, we used two well-defined in vitro models of endothelial barriers that exhibit opposite responses to stimulation of the cAMP/PKA signaling: first, endothelial monolayers derived from coronary microvessels [coronary endothelial cells (CEC)], a model that responds with a permeability rise on stimulation of the cAMP/PKA pathway, and, second, endothelial monolayers derived from aorta [aortic endothelial cells (AEC)], a model with a permeability-lowering response. We addressed the question on either model whether stimulation of the cAMP/PKA-signaling pathway could activate or inactivate the contractile machinery. For this purpose, the phosphorylation state of MLC, activation of MLCK and MLCP, and the generation of mechanical force were monitored.
was then studied whether the integrity of endothelial adhesion structures was affected by cAMP/PPA-dependent signaling. To elucidate this mechanism, the cellular localization of VE-cadherin, an integral protein of cell-cell junctions, and paxillin, an integral protein of cell-matrix adhesion plaques, was analyzed by cell fractionation and immunofluorescence microscopy. The cAMP/PPA-signaling pathway of the endothelial cells was activated by two different receptor agonists, the adenosine analog 5′-N-(ethylcarboxamido)adenosine (NECA) or isotopronerol (Iso), or forskolin (FSK). The first two agents activate the adenylyl cyclase coupled with endothelial adenosine or β-adrenergic receptor, respectively; the latter activates adenylyl cyclase directly.

MATERIALS AND METHODS

Materials. FITC-conjugated anti-paxillin antibody (clone 349), Falcon plastic tissue culture dishes were from Becton Dickinson (Heidelberg, Germany); FSK and SQ-22536 were from Calbiochem-Novabiochem (Bad Soden, Germany); Rp-isoform of 8-(4-chlorophenylthio)adenosine-3′,5′-cyclic monophosphorothioate (Rp-cAMPS), Sp-isoform of adenosine-3′,5′-cyclic monophosphorothioate (Sp-cAMPS) were from Biolog Life Science Institute (Bremen, Germany); MLCK pseudosubstrate (342–352) amide was from Bionol (Hamburg, Germany); 1-(5-iodonaphthalene-1-sulfonfonyl)homopiperazine (ML-7) was from Calbiochem/Merck Bioscience (Bad Soden, Germany); Transwell polycarbonate filter inserts (24-mm diameter, 0.4-μm pore size) were from Costar (Bodenheim, Germany); trypsin-EDTA was from Gibco Life Technologies (Eggenstein, Germany); fura-2 AM was from Molecular Probes (Leiden, The Netherlands); polyvinylidene difluoride (PVDF) was from Millipore (Eschborn, Germany); complete protease inhibitor cocktail and Fetalbolex were from Roche (Mannheim, Germany); dihydrothreitol, phenylmethylsulfonyl fluoride (PMSF), anti-phosphatase 1 catalytic subunit, and protein phosphatase 1 were from Roche (Mannheim, Germany); dithiothreitol, phenylmethylsulfonyl fluoride (PMSF), anti-phosphatase 1 catalytic subunit, and protein phosphatase 1 were from Sigma (Deisenhofen, Germany). All other chemicals were of the best available quality, usually analytic grade.

Cell cultures. Macrovascular AEC from porcine aortas were isolated and cultured as previously described (36). Microvascular CEC were isolated from 250-g male Wistar rats and grown in culture as previously described (32). Confluent cultures of primary endothelial cells were trypsinized in PBS (composed of (in mM) 137 NaCl, 2.7 KCl, 1.5 KH2PO4, and 8.0 Na2HPO4, at pH 7.4, supplemented with 0.05% (wt/vol) trypsin, and 0.02% (wt/vol) EDTA) and seeded at a density of 7 × 104 cells/cm2 on either 24-mm round polycarbonate filters (pore size 0.4 μm), glass coverslips, or 60-mm plastic culture dishes for determination of albumin permeability, immunohistochemistry, or protein analysis, respectively. Experiments were performed with confluent monolayers 4 days after seeding.

Experimental protocols. The basal medium used in incubations was modified Tyrode’s solution (composition in mM: NaCl 150 mM, KCl 2.7 mM, 1.2 KH2PO4, 1.2 MgSO4, 0.1 CaCl2, and 30.0 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; pH 7.4, 37°C) supplemented with 5% (vol/vol) heat-inactivated newborn calf serum (10 min, 60°C). Agents were added as indicated in the figure legends. Stock solutions of FSK, ML-7, NECA, and Rp-8-CPT-cAMPS were prepared with DMSO. Where indicated, aliquots of the stock solutions were added to the cells. Final solvent concentrations were <0.1% (vol/vol). The same final concentrations of DMSO were included in all respective controls. All other agents were dissolved in basal medium and were added where indicated.

Macromolecule permeability. Endothelial cells were cultured on polycarbonate filter membranes. Permeability of the endothelial cell monolayer was determined by the flux of Trypan blue-labeled albumin across the monolayer as previously described (29).

Force measurement. Isometric force measurements were performed as previously described by Kessler et al. (14) with minor modifications. Briefly, endothelial cells were seeded at 300,000 cells/cm² on the collagen lattices that were precast in 1 × 1.5-cm molds. On these lattices, the cells were grown to confluence within 2 days. Afterward, the molds were transferred to specially designed force apparatus. The lattices were connected to force transducers (KG 7A with bridge-amplifier DUBAM 7C, Scientific Instruments, Heidelberg, Germany) and were maintained in a humidified 5% CO2 atmosphere at 37°C. After an equilibration period of 4 h, isometric force of CEC or AEC reached a plateau of 180 ± 19 or 126 ± 15 μN, respectively. These values are referred to as stable baseline forces. Only those cell-loaded lattices with a stable baseline were used for force experiments. Integrity of the endothelial monolayers was routinely checked by phase-contrast microscopy or immunofluorescence staining of the cell-cell adhesion protein VE-cadherin.

Determination of MLC phosphorylation. The phosphorylation of MLC was determined by glycerol-urea polyacrylamide gel electrophoresis and Western blot analysis as described previously (7, 31). This procedure allows separation of nonphosphorylated from phosphorylated MLC protein, the latter of which migrates more rapidly. Briefly, electrophoretically separated proteins were transblotted on PVDF membranes and incubated with an anti-MLC antibody followed by an alkaline phosphate-conjugated anti-IgG antibody. Blots were scanned densitometrically, and the stoichiometry of MLC phosphorylation (expressed as mol PO2/mol MLC) was calculated from the densitometric values of non-(MLC), mono-(MLC−P), and diphosphorylated MLC (MLC−PP) as follows

\[
\text{MLC phosphorylation} = \frac{(2 \times \text{MLC}−P) + \text{MLC}−P}{\text{total MLC}}
\]

As all MLC can become diphosphorylated, MLC phosphorylation varies between 0 and 2 mol PO2/mol MLC.

MLCK activity assay. MLCK activity was determined according to Verin et al. (40) with some modifications. Briefly, endothelial cells in 60-mm culture dishes were incubated in the presence of ML-7 (1–100 μM) and FSK (5–10 μM) or respective volumes of basal medium (controls) for 10 min at 37°C. Afterward, the dishes were rinsed twice with PBS and cells were lysed with 0.3 ml of lysis buffer (20 mM MOPS, pH 7.4, 25 mM β-glycerol phosphate, 5 mM EGTA, 10% glycerol, 10 mM NaF, 1.5 mM Na2VO3, 2 mM DTT, 1 M PMSF, and 0.1 μg/ml complete proteinase inhibitor cocktail) containing 1% NP-40 for 10 min on ice. The lysates were scraped, passed five times through a 30-gauge needle, and centrifuged for 5 min at 4°C. Kinase activity was measured with MLC from bovine muscle as substrate in NP-40-free lysis buffer containing 0.1 μCi of [γ-32P]ATP in a final volume of 50 μl for 20 min at 30°C. The reaction was terminated by transferring aliquots onto P-81 Whatman filter membranes. The membranes were immediately rinsed with ice-cold 10% TCA, washed five times with 0.75% phosphoric acid (wt/vol), and were subsequently rinsed with acetone and dried. The filter squares were counted in a scintillation counter. Specific MLCK activity was estimated by subtracting kinase activity, which was insensitive to specific inhibition with MLCK pseudosubstrate (342–352) amide or ML-7. For both inhibitors, maximum effective doses were determined to be 100 and 50 μM, respectively.

Protein phosphatase assay. Protein phosphatase activity was determined according to Neumann et al. (27). To determine protein phosphatase activity, [32P]-labeled phosphorylase a, phosphorylase b (5 mg/ml) was incubated with phosphorylase kinase (200 U/ml)
in a 2-ml incubation cocktail containing 50 mM Tris, pH 7.4, 20 mM MgCl₂, 31 mM β-mercaptoethanol, 0.5 mg/ml BSA, 1 mM CaCl₂, 1 mM ATP, and 1 mM γ-[³²P]-ATP for 2 h at 30°C. The [³²P]-labeled phosphorylase a was precipitated by addition of 2 vol of ice-cold saturated ammonium sulfate solution. The cocktail was incubated for 20 min on ice and was centrifuged for 30 min at 12,000 g at 4°C. The precipitate was solubilized in 2 ml dialysis buffer (10 mM Tris·HCl, pH 7.4, 1 mM EDTA) dialyzed at room temperature against 2×2- liter dialysis buffer and finally stored at 4°C. [³²P]-labeling was estimated by measuring aliquots of the substrate in a liquid scintillation counter. For determination of protein phosphatase activity (2), diluted protein fractions from endothelial cells were preincubated in a total volume of 30 μl for 10 min at 30°C in the presence or absence of okadaic acid, the protein phosphatase 2A inhibitor. The reaction was started by addition of 20 μl of [³²P]-labeled phosphorylase a in an incubation cocktail containing 50 mM Tris·HCl, pH 7.4, 12.5 mM caffeine, 0.25 mM EDTA, 0.25% (vol/vol) β-mercaptoethanol. After 20-min incubation at 30°C, the reaction was terminated by addition of 20 μl of 50% (wt/vol) ice-cold TCA and 30 μl of 2% (wt/vol) BSA. After 15 min on ice, the suspension was centrifuged for 5 min at 12,000 g and 4°C. Aliquots of the supernatant were measured in a liquid scintillation counter. Reactions were carried out in duplicate. To ensure linear rates of phosphorylase a dephosphorylation, dephosphorylation was restricted to >25%.

Detection of protein phosphatases in the myosin-enriched and -depleted cell fraction. The content of protein phosphatases 1 and 2A (PP1 and PP2A) in the myosin-enriched cell fraction was analyzed as previously described (4). Briefly, confluent endothelial monolayers on 10-cm dishes were stimulated as indicated in the text. Afterward, the monolayers were rinsed twice with PBS to remove the incubation medium, 200 μl of homogenization buffer (0.1 mM EDTA, 28 mM mercaptoethanol, 1 μg/ml Pefabloc, Tris·HCl, pH 7.4) were added, and dishes were cooled immediately to −80°C. Afterward, the cells were scraped and homogenized. Homogenates were incubated with a high-salt buffer [0.6 M NaCl, 0.1% (vol/vol) Tween 20, 1 μg/ml Pefabloc] for 1 h at 4°C and centrifuged at 4,500 g for 30 min at 4°C. Supernatants were diluted 10-fold with assay buffer (0.1 mM EDTA, 28 mM mercaptoethanol, Tris·HCl, pH 7.0) and centrifuged again at 8,200 g for 40 min at 4°C. The pellet (myosin-enriched fraction) and the supernatant (myosin-depleted fraction) were eluted in Laemmli sample buffer (16) and analyzed by Western blot using antibodies against the catalytic subunits of PP1 and PP2A. In accordance with previous reports (7), the myosin-enriched fractions contained PP1 but not PP2A.

Immunofluorescence microscopy. Confluent endothelial monolayers were rinsed three times with PBS (10 mM phosphate buffer, 2.7 mM KCl, and 137 mM NaCl, pH 7.4), then fixed with 100% methanol for 10 min at −20°C, and washed again three times with PBS. The cells were covered with 100 μl of an anti-pan-cadherin (diluted 1:100 in PBS) or a FITC-conjugated anti-paxillin antibody (diluted 1:100 in PBS) and incubated for 1 h at 4°C and centrifuged at 4,500 g for 30 min at 4°C. Supernatants were diluted 10-fold with assay buffer (0.1 mM EDTA, 28 mM mercaptoethanol, Tris·HCl, pH 7.4) and centrifuged again at 8,200 g for 40 min at 4°C. The pellet (myosin-enriched fraction) and the supernatant (myosin-depleted fraction) were eluted in Laemmli sample buffer (16) and analyzed by Western blot using antibodies against the catalytic subunits of PP1 and PP2A. In accordance with previous reports (7), the myosin-enriched fractions contained PP1 but not PP2A.

Detergent fractionation and Western blot. Paxillin was determined in detergent-soluble- and insoluble fractions of cell proteins to estimate its translocation between the cytoskeleton and cytosolic compartment. Endothelial monolayers were rinsed three times with PBS and incubated with 0.3 ml of NP-40 solubilization buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM dithiothreitol, 10 mM PMSF, 50 mM Tris·HCl, pH 8) for 10 min at 4°C. The supernatant (detergent-soluble, cytosolic fraction) was removed, the culture dish was rinsed three times with ice-cold PBS, and the remaining detergent-insoluble proteins (detergent-insoluble, cytoskeleton-rich fraction) were solubilized into 0.3 ml of Laemmli SDS buffer (16). To determine the total paxillin concentration, endothelial monolayers were rinsed three times with PBS and solubilized into 0.3 ml Laemmli SDS buffer (total cell lysate). Equal volumes of either detergent-soluble, detergent-insoluble fractions, or total protein lysate were applied for SDS-PAGE [12% (wt/vol) total acrylamide concentration] and transferred onto PVDF membranes. The membranes were blocked with 3% (wt/vol) bovine serum albumin and probed with an anti-paxillin antibody (0.2 μg/ml). The signal was visualized with an alkaline phosphatase-conjugated anti-rabbit IgG antibody (0.1 μg/ml).

Fig. 1. Effects of 5′-N-(ethylcarboxamido)-adenosine (NECA; 100 nM), isoprotrenol (Iso; 10 μM), and forskolin (FSK; 5 μM) on cAMP concentration of coronary (CEC; A) and aortic (AEC; B) endothelial monolayers. In the presence of SQ-22536 (SQ; 50 μM), an adenylyl cyclase inhibitor, the FSK-induced increase in the cAMP concentration is attenuated. Data are means ± SD after 10-min incubations of 5 separate experiments with independent cell preparations. *P < 0.05 vs. control (C), #P < 0.05.
Statistical analysis. Data are given as means ± SD of n experiments of independent cell preparations. The comparison of means between groups was performed by one-way ANOVA followed by a Bonferroni post hoc test. Changes of parameters within the same group were assessed by multiple ANOVA analysis. Probability (P) values of <0.05 were considered significant.

RESULTS

cAMP-induced responses in CEC. In CEC, cellular cAMP concentration (Fig. 1A) and macromolecule permeability (Fig. 2A) were markedly increased in the presence of receptor-mediated activators of adenylyl cyclase, NECA (100 nM) or Iso (10 μM), or the receptor-independent activator FSK (5 μM). At the concentrations applied, the agents increased cAMP concentrations and macromolecule permeability to similar levels, respectively. In endothelial monolayers derived from hearts of guinea pig and swine, a comparable increase in cellular cAMP levels and macromolecule permeability could be induced by NECA and Iso (data not shown). The presence of SQ-22536 (50 μM), an inhibitor of adenylyl cyclase, blunted the effect of the receptor agonists (not shown) or of FSK on cAMP concentration (Fig. 1A). SQ-22536 also attenuated the FSK effect on permeability (Fig. 2A). Application of the direct activator of PKA, Sp-cAMPS (200 μM), caused an increase in permeability (Fig. 3A). This effect could be abolished in the presence of the specific, cell membrane-permeable PKA inhibitor Rp-8-CPT-cAMPS (200 μM).

Fig. 2. Effects of NECA (100 nM), Iso (10 μM), and FSK (5 μM) on albumin permeability of CEC (A) and AEC (B) endothelial monolayers. SQ (50 μM), an adenylyl cyclase inhibitor, attenuated the FSK effects on permeability. Data are means ± SD after 10-min incubations of 5 separate experiments with independent cell preparations. *P < 0.05 vs. C. #P < 0.05.

Fig. 3. Effects of Sp-cAMPS (Sp; 200 μM), a direct PKA activator, on albumin permeability of CEC (A) and AEC (B) endothelial monolayers. Rp-8-CPT-cAMPS (Rp; 200 μM), a PKA inhibitor, attenuated the Sp effects on permeability. Data are means ± SD after 10-min incubations of 5 separate experiments with independent cell preparations. *P < 0.05 vs. C. #P < 0.05. n.s., Corresponding data of Rp vs. Rp plus Sp were not significantly different.
We analyzed whether the activation of cAMP/PKA signaling could alter isometric force generated by endothelial monolayers. As shown in Fig. 4A, FSK (5 μM) reduced isometric force from basal value of 180 ± 19 to 24 ± 19 μN (P < 0.05 vs. time 0; n = 5) within 10 min. The receptor agonists NECA (100 nM) or Iso (10 μM) exerted similar effects and lowered isometric force to 32 ± 25 or 39 ± 17 μN (P < 0.05 vs. time 0; n = 5), respectively. These results show that in CEC monolayers adenylyl cyclase and PKA mediate a pathway that increases barrier permeability and reduces mechanical tension within the monolayer.

In the next step, we analyzed whether the changes observed in monolayer permeability might be related to corresponding changes in MLC phosphorylation, the regulatory subunit of the contractile elements. Under basal conditions, MLC phosphorylation of endothelial cells grown on collagen lattices or culture dishes amounted to 0.76 ± 0.1 or 0.71 ± 0.06 mol PO₄/mol MLC, respectively, indicating that basal MLC phosphorylation was not significantly different under both culture conditions (P > 0.05; n = 5). Addition of FSK caused an approximately complete reduction of MLC phosphorylation within 15 min in cells seeded on either collagen lattices (data not shown) or culture dishes (Fig. 5A). The FSK-induced MLC dephosphorylation was completely abolished if endothelial cells were incubated in the presence of the PKA inhibitor Rp-8-CPT-cAMPS. These results indicate that the signaling via adenylyl cyclase and PKA inactivates indeed the contractile machinery in these cells.

Fig. 4. Effect of FSK (5 μM) on isometric force developed by CEC (A) and AEC (B) endothelial monolayers. Data are means ± SD of 5 separate experiments with independent cell preparations. *P < 0.05 vs. C.

Fig. 5. Effects of FSK (5 μM) on myosin light chain (MLC) phosphorylation of CEC (A) and AEC (B) endothelial cells. Rp (200 μM), a PKA inhibitor, prevents FSK-induced dephosphorylation of MLC. Data are means ± SD of 5 separate experiments with independent cell preparations. *P < 0.05 vs. C. Corresponding data of Rp + FSK vs. Rp were not significantly different.
The next question was whether activation of cAMP/PKA signaling would affect the cell adhesion structures in microvascular CEC. Therefore, changes of the localization of VE-cadherin and paxillin in cell adhesion complexes were analyzed by immunohistochemistry. On addition of FSK, immunostaining of VE-cadherin at cell-cell junctions and paxillin at focal adhesion plaques slowly vanished within the first 15 min (Fig. 6A). Fading of paxillin staining slightly preceded that of VE-cadherin. Cell fractionation showed that paxillin is shifted from the insoluble to the soluble cell fraction, consistent with its delocalization from membrane-associated adhesion complexes (Fig. 6B).

*cAMP-induced responses in AEC.* Under control conditions, the basal cAMP concentration and macromolecule permeability of AEC were of the same magnitude as that found in CEC. NECA (100 nM), Iso (10 μM), or FSK (5 μM) increased the cAMP concentration to similar levels in AEC (Fig. 1B). The adenylyl cyclase inhibitor SQ-22536 (50 μM) could inhibit the FSK effect. In contrast to CEC, AEC monolayers responded with a reduction of macromolecule permeability when exposed to NECA, Iso, or FSK (Fig. 2B). Again, SQ-22536 (50 μM) could block the FSK effect. The effect of adenylyl cyclase activators on permeability could be mimicked by the PKA activator Sp-cAMPS (200 μM; Fig. 3B), and this effect on permeability could be inhibited by Rp-8-CPT-cAMPS (3 μM). These data show that compared with CEC, monolayers of aortic origin exhibit an opposite barrier response to the tested stimuli.

In the next step, we analyzed whether stimulation of this pathway also influenced the contractile apparatus of AEC. On addition of NECA (100 nM), Iso (10 μM), or FSK (5 μM), isometric force significantly declined within 10 min from a control level of 126 ± 15 to 25 ± 16, 32 ± 17, or 24 ± 19 μN (P < 0.05 vs. time 0 min, n = 5), respectively. As shown for FSK in Fig. 4B, the FSK-induced contractile response was identical to that observed in microvascular coronary monolay-

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A

B

**Fig. 6.** Effect of FSK on the cellular localization of VE-cadherin and paxillin in coronary endothelial monolayers. FSK provokes delocalization of VE-cadherin from cell boarders and paxillin from focal adhesion sites into cytoplasm and a shift of paxillin from detergent-insoluble into detergent-soluble fraction in a time-dependent manner. A: VE-cadherin and paxillin immunohistochemistry; endothelial cells were exposed to 5 μM FSK for the time indicated. B: Western blot analysis of paxillin in total cell lysates and in detergent-insoluble (is) and detergent-soluble (s) fractions; endothelial monolayers were exposed to 5 μM FSK for 5 and 15 min, respectively. Bar = 50 μm.
ers. The FSK-stimulated decline in isometric force was paralleled by a reduction of MLC phosphorylation (Fig. 5B). This effect was inhibited by the PKA inhibitor Rp-8-CPT-cAMPS. The response of MLC phosphorylation in AEC was also comparable to that of CEC.

We then analyzed whether the activation of cAMP/PKA signaling by FSK caused a decline of MLC phosphorylation by inactivation of MLCK kinase. Endothelial cells were exposed to FSK and MLCK activity was determined in cell homogenates of treated cells. Exposure of endothelial cells to 5 μM FSK caused a reduction of MLCK activity by 25 ± 12% compared with control (P < 0.05; n = 5), indicating that MLCK activity is modulated by the cAMP pathway in endothelial cells. However, this reduction of MLCK activity is too moderate to account for the cAMP-induced MLC dephosphorylation determined in intact cells. Therefore, in the first step, the role of MLC phosphatase was analyzed in intact endothelial cells using a pharmacological approach. For that reason, FSK-stimulated MLC phosphorylation was determined on one hand under inhibition of MLCK by ML-7, and on the other hand under inhibition of MLC phosphatase by calyculin A. If endothelial cells were exposed to increasing concentrations of the inhibitor of MLCK, ML-7, MLC phosphorylation declined concentration dependently (Fig. 7A), indicating that the basal activity of MLCK kinase contributes to the basal state of MLC phosphorylation. FSK (5 μM) in copresence of 50 μM ML-7, a dose that completely blocked MLCK activity in endothelial cells, caused a further drop of the MLC phosphorylation level. These data indicate that a cAMP-dependent decrease in barrier permeability in CEC monolayers.

As recently shown (4, 7), dephosphorylation of MLC in endothelial cells requires translocation and binding of the catalytic subunit of protein phosphatase to myosin. Therefore, in a second approach, translocation of the protein phosphatase 1 subunit into the myosin-enriched cell fraction and the phosphatase activity were analyzed in FSK-stimulated endothelial cells. As shown in Fig. 8A, FSK increased phosphatase content in the myosin fraction. The increase of the phosphatase content in the myosin-enriched cell fraction went along with a reduction of the phosphatase content in the corresponding myosin-depleted cell fraction. The analysis of the phosphatase activity (Fig. 8B) showed that the FSK-stimulated signaling mechanism not only shifted protein phosphatase 1 into myosin-enriched cell fraction but also caused a distinct increase in phosphatase activity in that fraction.

Finally, the influence of FSK on the cellular localization of VE-cadherin and paxillin was analyzed. As shown by immunostaining in Fig. 9, the distribution of VE-cadherin and paxillin was unaffected by FSK, demonstrating that in AEC stimulation of cAMP/PKA signaling does not cause disintegration of cell adhesion junctions.

**DISCUSSION**

In this study, we analyzed the mechanisms by which signals activating the cAMP/PKA pathway can cause opposite effects on the barrier function of endothelial monolayers. We found that in CEC as well as in AEC monolayers, cAMP/PKA signaling causes a decrease in isometric tension due to an inactivation of the endothelial contractile elements. In CEC, however, cAMP/PKA signaling also provokes a disintegration of cellular adhesion complexes. It is the predominance of this latter effect that explains why cAMP/PKA signaling leads to an increase in barrier permeability in CEC monolayers.
In the past, it was documented in a number of studies that stimulation of the cAMP/PKA pathway causes opposing effects on the control of endothelial barrier function. However, the divergent responses in endothelial cells of different origins have remained unexplained. In the present study, we observed that stimulation of cAMP production provokes an increase in macromolecule permeability not only in CEC from rat but also in CEC derived from guinea pig and swine. This suggests that the increase in permeability is a unique cAMP effect in microvascular CEC and rules out the possibility that this cAMP effect on barrier function is due to heterogeneity of species.

We used agonists for receptors coupled in a stimulating manner to adenylyl cyclase in endothelial cells as well as FSK, which activates adenylyl cyclase directly. We showed that the investigated effects on endothelial barrier function and contractile activation were comparable at similar elevations of cellular cAMP contents. These effects were blocked by the highly specific inhibitor of PKA, Rp-8-CPT-cAMPS. With these maneuvers, we analyzed the response of endothelial monolayers to activation of cAMP/PKA signaling. Although effects of cAMP/PKA signaling on endothelial barrier function have been studied before, it has remained unclear which mechanisms are responsible for the divergent effects of cAMP on endothelial barrier function in different experimental models. Patterson et al. (30) demonstrated that stimulation of the cAMP/PKA pathway reduces phosphorylation of MLC in endothelial cells. The effect was attributed to inhibition of MLCK, which was shown to be directly phosphorylated by PKA. These data and findings from other groups (3, 10, 22) have led to the assumption that stabilization of endothelial barriers via cAMP/PKA signaling is due to an inactivation of the contractile machinery based on inactivation of MLCK.

In some endothelial models, it has also been observed that stimulation of the cAMP/PKA signaling can cause a reorganization of the F-actin-based cytoskeleton and stabilizes cell adhesion structures (19). Stimulation of cAMP synthesis can increase junctional strands between endothelial cells of intact microvessels (1) and reduces gap formation in venules induced by inflammatory stimuli (21). An interesting observation was also made by Huxley and colleagues (12, 13), who showed that in intact microvessels the glycocalyx of endothelial cells can substantially contribute to the cAMP-mediated modulation of endothelial barrier function. None of these studies could explain, however, why cAMP/PKA signaling leads to a loss of barrier function in other endothelial models and microvessel preparations.

In the present study, the effects of cAMP/PKA-signaling mechanisms on MLCK and MLCP were studied using two different approaches. Direct determination of the MLCK activity showed that these signals cause only a moderate inhibition of MLCK activity, too small to account for the pronounced

![Fig. 8. FSK-induced translocation of protein phosphatase 1 (PP1) to myosin of AEC. A: Western blots of myosin-enriched and myosin-depleted cell fractions with use of an anti-PP1 antibody (top) or an anti-MLC antibody (bottom) after 10-min exposure of endothelial cells to FSK (5 μM). Similar blots were obtained in 3 other experiments with independent cell preparations. B: increase in PP1 activity of the myosin-enriched fraction after 10-min exposure in the presence of 5 μM FSK. PP1 activity of the myosin-enriched fraction in nontreated cells is set to 100%. *P < 0.05.](https://www.ajpcell.org/content/287/5/C1253/F1)

![Fig. 9. Cellular localization of VE-cadherin and paxillin in FSK-treated aortic endothelial monolayers. In these cells, FSK (5 μM) does not provoke delocalization of VE-cadherin from cell borders or paxillin from focal adhesion sites. VE-cadherin and paxillin immunohistochemistry of endothelial cells exposed to 5 μM FSK for 5 and 15 min, respectively. Bar = 20 μm.](https://www.ajpcell.org/content/287/5/C1253/F2)
dephosphorylation of MLC found in cAMP-stimulated cells. Analysis of the MLC phosphorylation level in endothelial cells in which MLC was completely inhibited revealed that stimulation of cAMP/PKA signaling causes a drop in MLC phosphorylation even under these conditions. This observation indicated that dephosphorylation of MLC is largely due to a cAMP-induced increase in MLCP activity. Indeed, we found that translocation of the catalytic subunit of protein phosphatase 1 into the myosin-enriched fraction, indicative of site-specific phosphatase activation, is increased under these conditions.

In the case of AEC, MLC dephosphorylation and decline in force were closely correlated with the decrease in barrier permeability when the cAMP/PKA-signaling pathway was activated. Comparable elevations of the cellular cAMP levels by the different stimuli were accompanied by similar effects on force, MLC phosphorylation, and monolayer permeability. This indicates a causal relationship between isometric tension and permeability and a tight coupling of the cAMP-signaling mechanism to these cellular responses, as has been suggested by several studies before (8, 15, 30, 31, 34).

In monolayers of CEC, however, permeability was increased despite contractile inactivation when cAMP/PKA signaling was activated. This occurred even though the two experimental models were very similar in terms of culture conditions. In contrast to AEC, cAMP/PKA signaling caused a rapid disintegration of cellular adhesion complexes in CEC. The changes in VE-cadherin and paxillin delocalization occurred almost simultaneously with the changes in permeability. Previous studies have shown that endothelial barrier function is disturbed when adhesion complexes disintegrate (8, 17, 18, 25). One possible explanation is that if the tethering forces keeping cells spread on the extracellular matrix are lost and intercellular adhesion is weakened, cellular shape changes lead to the opening of gaps between cells. Because delocalization of paxillin proceeded faster than that of VE-cadherins in cAMP/PKA-stimulated CEC, one may speculate that the loss of cell adhesion starts at the cell-matrix adhesion sites rather than at cell-cell junctions. The molecular mechanisms by which cAMP/PKA signaling can cause destabilization of cell adhesion structures in endothelial cells are largely unknown. It has been shown previously in adrenal cortex-derived cells (9, 33) that PKA can stimulate dephosphorylation of paxillin due to activation of a protein tyrosine phosphatase possessing a Src homology (SH)2 domain (SHP-2). Dephosphorylation of paxillin went along with translocation of the phosphorylated protein to cytoplasm, reorganization of focal adhesion plaques, and detachment of the cells from the extracellular matrix. Recently, it has been shown that SHP-2 is a component of cell adhesion also in endothelial cells (38, 39).

In conclusion, the cAMP/PKA-mediated control of contractile elements in macro- and microvascular endothelial cells is identical. Tension is reduced, which, per se, stabilizes barrier function. In coronary microvascular cells, there is an additional cAMP-mediated effect, i.e., cell detachment, that predominates in the control of barrier function.

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