cAMP protects endothelial barrier functions by preventing Rac-1 inhibition

J. Waschke,1 D. Drenckhahn,1 R. H. Adamson,2 H. Barth,3 and F. E. Curry2

1Institute of Anatomy and Cell Biology, University of Würzburg, D-97070 Würzburg; and 2Department of Pharmacology and Toxicology, Albert-Ludwig University, D-79104 Freiburg, Germany; and 3Department of Physiology and Membrane Biology, University of California, Davis, California 95616

Submitted 10 June 2004; accepted in final form 21 July 2004

Waschke, J., D. Drenckhahn, R. H. Adamson, H. Barth, and F. E. Curry. cAMP protects endothelial barrier functions by preventing Rac-1 inhibition. Am J Physiol Heart Circ Physiol 287: H2427–H2433, 2004. First published July 22, 2004; doi:10.1152/ajpheart.00556.2004.—cAMP enhances endothelial barrier properties and is protective against various inflammatory mediators both in vivo and in vitro. However, the mechanisms whereby cAMP stabilizes the endothelial barrier are largely unknown. Recently we demonstrated that the Rho family GTPase Rac-1 is required for maintenance of endothelial barrier functions in vivo and in vitro. Therefore, in the present study we investigated the effect of forskolin (5 μM)- and rolipram (10 μM)-induced cAMP increase on reduction of barrier functions in response to Rac-1 inhibition by Clostridium sordellii lethal toxin (LT). Forskolin and rolipram treatment blocked LT (200 ng/ml)-induced hydraulic conductivity (Lp) increase in mesenteric microvessels in vivo. Likewise, LT-induced intercellular gap formation in monolayers of cultured microvascular myocardial endothelial (MyEnd) cells and LT-induced loss of adhesion of vascular endothelial cadherin-coated microbeads were abolished. Inhibition of PKA by myristoylated inhibitor peptide (14–22) of PKA (100 μM) reduced the protective effect of cAMP on LT-induced Lp increase in vivo and gap formation in vitro, indicating that the effect of cAMP on Rac-1 inhibition was PKA dependent. Glucosylation assays demonstrated that cAMP prevents inhibitory Rac-1 glucosylation by LT, indicating that one way that cAMP enhances endothelial barrier functions may be by regulating Rac-1 signaling. Our study suggests that cAMP may provide its well-established protective effects at least in part by regulation of Rho proteins.

permeability; Rho proteins; cAMP-dependent protein kinase; vascular endothelial cadherin

THE VASCULAR ENDOTHELIUM PROVIDES a selective barrier to the surrounding tissue by limiting water and macromolecule permeability. Many life-threatening disorders, such as cancer growth and metastasis (27), atherosclerosis (8), and asthma (17), are caused in part by increased vascular leakage. It is well established that cAMP enhances endothelial barrier properties and is protective against various inflammatory mediators both in vivo (3, 4, 10, 18, 19, 44) and in vitro (7, 9, 26, 30, 33, 36, 37). Therefore, elevation of cAMP by agents such as β2-adrenergic agents is an effective therapeutic approach (39). Several signaling events, including reduction of myosin light chain phosphorylation (43), vasodilator-stimulated phosphoprotein-mediated changes in cytoskeletal tension (9), and inhibition of the GTPase Rho A (33), have been implicated in the barrier function-stabilizing effects of cAMP in cultured endothelial cells in vitro. However, the mechanisms that lead to increased barrier properties in microvessels in vivo are unknown.

Besides cAMP, the family of Rho GTPases has been demonstrated to be implicated in the regulation of vascular permeability (42). Recent studies from our laboratory (40, 41) demonstrated that the Rho family GTPase Rac-1 is required for maintenance of endothelial barrier functions in vivo. Inhibition of Rac-1 by Clostridium sordellii lethal toxin (LT), a bacterial toxin with glucosyltransferase function, increased hydraulic conductivity (Lp) of postcapillary venules in vivo. In cultured microvascular myocardial endothelial (MyEnd) cells, increased flux across monolayers was accompanied by formation of large intercellular gaps. In vivo, myosin-dependent contractile mechanisms did not contribute to permeability changes in response to LT. However, in vitro Rac-1 inhibition led to both increased myosin-dependent contraction and reduction of vascular endothelial (VE)-cadherin-mediated adhesion, as revealed by laser tweezer techniques with VE-cadherin-coated microbeads. The important role of both cAMP and Rac-1 in maintenance of endothelial barrier function raised the question of whether increased cAMP would also antagonize the effects of Rac-1 inhibition. Therefore, in the present study we tested the effect of increased intracellular cAMP by treatment with forskolin, an adenylyl cyclase activator, and rolipram, the inhibitor of cAMP-specific phosphodiesterase type IV, on LT-induced inhibition of Rac-1 in vivo and in vitro.

Our experiments indicate that cAMP prevents the LT-induced permeability increase in microvessels in vivo as well as gap formation and reduction of VE-cadherin-mediated adhesion in cultured endothelial cells in vitro. cAMP stabilizes endothelial barrier functions via PKA by a mechanism that prevents inhibition of Rac-1. The study suggests that regulation of Rho proteins is an important mechanism in cAMP-dependent endothelial barrier regulation.

MATERIALS AND METHODS

Cell culture. The immortalized mouse microvascular myocardial endothelial (MyEnd) cell line was grown in DMEM (Life Technologies, Karlsruhe, Germany) supplemented with 50 U/ml penicillin G, 50 μg streptomycin, and 10% FCS (Biochrom, Berlin, Germany) in a humidified atmosphere (95% air-5% CO2) at 37°C. Generation and characterization were described previously (15, 40). In brief, myocardial tissue of newborn mice was minced, digested with 0.05% trypsin (Biochrom) and 0.02% collagenase (Boehringer, Mannheim, Germany), and seeded onto gelatin-coated culture dishes. One day after being plated, adherent cells were transfected with poliovirus middle T antigen (Pym T). Pym T transfection causes a growth advantage of endothelial over nonendothelial cells, leading to a homogeneous

Address for reprint requests and other correspondence: F. E. Curry, Dept. of Physiology and Membrane Biology, School of Medicine, Univ. of California, 1 Shields Ave., Davis, CA 95616 (E-mail: fecurry@ucdavis.edu).

http://www.ajpheart.org
0363-6135/04 $5.00 Copyright © 2004 the American Physiological Society

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
monolayer of cells with endothelial morphology after 4–6 wk of culture. MyEnd cells were immunopositive for several endothelial markers like von Willebrand factor, VE-cadherin, α-, β-, and γ-catenin, platelet endothelial cell adhesion molecule 1, claudin 5, and ZO-1. Cultures were used for experiments when grown to confluent monolayers.

**Test reagents.** LT was prepared as described previously (20, 22) and used at 200 ng/ml for different time intervals under cell culture conditions as described in *Cell culture*. Activity and specificity of toxin preparations were analyzed as described previously (23). Forskolin and rolipram (both Sigma, St. Louis, MO) were used at 5 and 10 μM, respectively. Myristoylated inhibitor peptide (14-22) of PKA (Mryr-PKI; Biozol, Eching, Germany) was applied at 100 μM.

**Glucosylation assay.** Experiments were performed according to our previous study (40). MyEnd cells were incubated with LT (200 ng) in culture medium for 120 min in the presence or absence of forskolin (5 μM) and rolipram (10 μM). Afterwards, cells were harvested and lysed (20). Lysates were incubated with LT in presence of 10 μM [3H]-labeled UDP-glucose for 60 min. After precipitation with ice-cold trichloroacetic acid (20%), proteins were subjected to SDS (12.5%)-PAGE and the incorporated radioactivity of the 21-kDa band was quantified by phosphorimaging.

**Cytchemistry.** MyEnd cells were grown on coverslips coated with gelatin cross-linked with glutaraldehyde (35). LT was added to the culture medium at 200 ng/ml for up to 120 min in the presence or absence of forskolin (5 μM) and rolipram (10 μM). After incubation with toxins, the culture medium was removed and monolayers were fixed for 10 min at room temperature (RT) with 2% formaldehyde (freshly prepared from paraformaldehyde) in PBS (in mM: 137 NaCl, 2.7 KCl, 8.1 Na2HPO4, and 1.5 KH2PO4, pH 7.4). Afterwards, monolayers were treated with 0.1% Triton X-100 in PBS for 5 min. After being rinsed with PBS at RT, MyEnd cells were preincubated for 30 min with 10% normal goat serum and 1% BSA at RT and incubated for 16 h at 4°C with rat monoclonal antibody 11D4. Finally, beads were washed three times for 5 min in HBSS and stored in HBSS at 4°C for up to 8 days under permanent slow overhead rotation to avoid aggregation of beads. The concentration of beads in these stocks was ~1.6 × 10⁸ beads/ml.

**Laser tweezers.** As described previously (40), the home-built laser tweezers setup consisted of a Nd:Yag laser (1.064 nm), the beam of which was expanded to fill the back aperture of a high-numerical aperture objective (100 × 1.3 oil; Zeiss, Oberkochen, Germany), coupled through the epifluorescence illumination port of an Axiovert 135 microscope (Zeiss), and reflected to the objective by a dichroic mirror (FT 510; Zeiss). Throughout all experiments the laser intensity was 42 mW in the focal plane. Coated beads (10 μl of stock solution) were suspended in 500 μl of culture medium and allowed to interact with MyEnd monolayers for 30 min at 37°C before initiation of experiments (addition of LT alone or together with forskolin and rolipram).

**Preparation of rats for Lp measurement.** Anesthetized rats were placed on a heating pad to maintain normal body temperature. A midline surgical incision of ~1 cm was made in the abdominal wall, and the mesentery was gently taken out and spread over a pillar. The upper surface of the mesentery was continuously superfused with mammalian Ringer solution (37°C). All the experiments were carried out in straight, nonbranched segments of venular microvessels, which were typically 25–35 μm in diameter. All vessels selected had brisk blood flow and were free of white blood cells.

**Measurement of Lp of microvessel wall.** Measurements were based on the modified Landis technique, which measures the volume flux of water across the wall of a microvessel perfused via a glass micropipette after occlusion of the vessel. The assumptions and limitations of the measurement have been evaluated in detail elsewhere (28). The volume flux per unit surface area of vessel wall (J/S) was estimated during single occlusions, lasting about 10 s each, at one constant hydraulic pressure (usually 50 cmH₂O) with the assumption that the net effective pressure determining fluid flow (Pₑt) was equal to the applied hydraulic pressure minus 3.6 cmH₂O, the approximate oncotic pressure contributed by the BSA in all perfusates (10 mg/ml). Lp was estimated for each occlusion as (J/S)/Pₑt. All perfusates were mammalian Ringer solution additionally containing serum albumin at 10 mg/ml (Sigma A4378). LT was added to the perfusate at 200 ng/ml as in our previous studies (40, 41) and delivered via the micropipette for either 20 or 80 min in the presence or absence of forskolin (5 μM) and rolipram (10 μM) and Myr-PKI (100 μM). Measurements of initial J/S were made at ~10-min intervals for up to 100 min.

**Statistics.** Values throughout are expressed as means ± SE. Lp measurements (n = 5 for each group) were performed in different animals (1 experiment/animal). Baseline Lp distributions are non-Gaussian in both frog mesentery capillaries and rat mesentery venules (21, 29). Because Lp distribution under conditions of the present experiments has not been investigated, we used the nonparametric Mann-Whitney statistic to test for differences in Lp between groups.
Immunostaining and laser tweezer experiments \((n = 5\) in each set) were performed with three different passages of culture cells; for glucosylation experiments \((n = 3\) ) different cell culture dishes from one passage were used. Possible differences in bead binding between groups and Rac-1 glucosylation were assessed with unpaired Student’s \(t\)-test. Statistical significance is assumed for \(P < 0.05\).

**RESULTS**

**cAMP prevents LT-induced increase of microvascular \(L_p\).** When mesenteric venules were perfused with LT \((200 \text{ ng/ml})\), \(L_p\) increased after a lag phase of \(-40\) min from a baseline of \(0.9 \pm 0.3 \times 10^{-7} \text{ cm/(s·cm·H}2\text{O)}\) to a mean value of \(92.6 \pm 15.6 \times 10^{-7} \text{ cm/(s·cm·H}2\text{O)} \) within the following 40 min \((n = 5)\). To test the effect of increased cAMP, microvessels were treated with LT in presence of forskolin \((5 \text{ µM})\) and rolipram \((10 \text{ µM})\). Forskolin and rolipram completely blocked the effect of LT, as \(L_p\) averaged \(3.8 \pm 1.8 \times 10^{-7} \text{ cm/(s·cm·H}2\text{O)} \) \((n = 5)\) compared with baseline values of \(2.4 \pm 0.6 \times 10^{-7} \text{ cm/(s·cm·H}2\text{O)}\).

To see whether cAMP interfered with cellular uptake of LT, forskolin and rolipram were applied after toxin uptake. For this purpose we performed the following change of the protocol. Microvessels were perfused with LT for 20 min, followed by perfusion with 1% BSA vehicle solution. After a lag phase comparable to that above, \(L_p\) increased to \(47.8 \pm 10.5 \times 10^{-7} \text{ cm/(s·cm·H}2\text{O)}\) after 100 min of perfusion (Fig. 1A), indicating that LT uptake for 20 min is sufficient for induction of increased permeability \((n = 5)\). In a parallel set of experiments, forskolin and rolipram were added to the perfusate after withdrawal of LT at 20 min (Fig. 1B). In three of five experiments \(L_p\) was not different from controls, and in two of five experiments \(L_p\) was increased up to fivefold. The mean \(L_p\) value after 100-min perfusion was \(4.3 \pm 1.7 \times 10^{-7} \text{ cm/(s·cm·H}2\text{O)}\) for the set of five experiments. This was significantly lower than in experiments using LT alone. These experiments indicate that cAMP prevents the effects of Rac-1 inhibition on microvascular permeability and that this does not involve mechanisms blocking cellular toxin uptake.

**cAMP blocks the effect of Rac-1 inhibition by a PKA-dependent mechanism.** Many of the previous cell culture studies found in the literature showed that cAMP protects the endothelial barrier by activation of PKA \((9, 33, 43)\). To investigate whether this is also true for the inhibitory effect on LT-mediated permeability increase in vivo we used Myr-PKI \((100 \text{ µM})\) in addition to forskolin and rolipram to inhibit PKA-dependent effects. As shown in Fig. 2, after 80 min \(L_p\) values of a total of five experiments significantly increased to \(24.3 \pm 6.0 \times 10^{-7} \text{ cm/(s·cm·H}2\text{O)}\) compared with control values of \(1.2 \pm 0.3 \times 10^{-7} \text{ cm/(s·cm·H}2\text{O)}\). These results clearly demonstrate that the protective effects of cAMP against LT-induced barrier dysfunction require PKA.

**LT-induced intercellular gap formation in cultured endothelial cells was blocked by cAMP in PKA-dependent manner.** To investigate the mechanisms whereby cAMP protected endothelial barrier function against the effects of LT, we treated cultured MyEnd cells with LT \((200 \text{ ng/ml})\) in the presence and absence of forskolin and rolipram \((5 \text{ experiments for each condition})\). As we described previously \((40, 41)\), when applied alone LT \((200 \text{ ng/ml}, 120 \text{ min})\) induced formation of stress fibers (Fig. 3f) and large intercellular gaps (Fig. 3b) in MyEnd monolayers compared with controls (Fig. 3, a and e). Immunostaining of VE-cadherin, which was distributed along intercellular junctions in control cells (Fig. 3a), was lost at margins of intercellular gaps (arrows in Fig. 3b). In the absence of LT, treatment with forskolin \((5 \text{ µM})\) and rolipram \((10 \text{ µM})\) did not affect monolayer integrity and VE-cadherin distribution (Fig. 3c) but reduced stress fibers (Fig. 3g). When forskolin and rolipram were applied together with LT \((120 \text{ min})\), gap formation and VE-cadherin redistribution were abolished (Fig. 3d) and stress fibers were less numerous than in experiments with LT alone (Fig. 3h). To compare the experimental situation of the in vivo experiments to the in vitro situation, we treated MyEnd cells with LT for 20 min, followed by incubation with culture medium for an additional 100 min. As shown in Fig. 4a, LT treatment for 20 min is sufficient to induce gap formation. When forskolin and rolipram \((5 \text{ and } 10 \text{ µM, respectively})\) were applied after toxin withdrawal, cAMP still completely inhibited LT-induced gap formation (Fig. 4b), demonstrating that cAMP in this context does not inhibit toxin uptake. When Myr-PKI was applied together with forskolin and rolipram, large LT-induced gaps were present in the monolayers (Fig. 4c). Together, these experiments indicate that in cultured endothelial cells intercellular gap formation in response to Rac-1 inhibition is blocked by cAMP in a PKA-dependent fashion.
**cAMP inhibits LT-induced reduction of VE-cadherin-mediated adhesion.** To test whether cAMP would exert its protective effects on endothelial monolayer integrity by directly affecting VE-cadherin-mediated adhesion, VE-cadherin-coated beads were allowed to settle on the cell surface for 30 min to promote formation of cell-to-bead contacts (40). In this control group 54 ± 4% of VE-cadherin-coated beads were bound to the surface of MyEnd cells (n = 10). With the bound beads as the control (100%) value, after LT treatment (200 ng/ml, 120 min; n = 5) the number of bound beads dropped to 52 ± 4% of control (Fig. 5). In experiments in which monolayers were treated with forskolin (5 μM) and rolipram (10 μM) alone, bound beads remained at 94 ± 4% of control, indicating that cAMP did not directly affect bead binding (n = 5). However, when MyEnd cells were incubated with LT in the presence of forskolin and rolipram, bound beads were also at 94 ± 4% of control (n = 5). This suggests that cAMP has no direct effect on VE-cadherin-mediated adhesion but antagonizes the effect of Rac-1 inhibition on VE-cadherin-mediated adhesion.

**cAMP inhibits inactivation of Rac-1 by LT.** The results described above clearly demonstrate that cAMP protects the endothelial barrier against dysfunction induced by inhibition of Rac-1. To investigate whether cAMP would exert these effects on VE-cadherin-mediated adhesion in culture and on microvessel $L_p$ in vivo independently of inactivation of Rac-1 or by directly regulating Rac-1, we carried out glucosylation assays comparable to our previous study (40) in the presence of increased cAMP. LT is a glucosyltransferase, and it inhibits the function of Rac-1 by glucosylation (22, 23). For control experiments MyEnd cell lysates were subjected to postglucosylation with [14C]UDP-glucose in the presence of LT (Fig. 6). Strong incorporation of [14C]UDP-glucose into lysate Rac-1 indicated that Rac-1 was not glucosylated in control MyEnd cells (n = 3). Although forskolin (5 μM) and rolipram (10 μM) alone seemed to decrease postglucosylation after 120 min, the effect was not significantly different from control (n = 3). When MyEnd cells were treated with LT (200 ng/ml) for 60 or 120 min, 67% and 88% of Rac-1 was glucosylated by LT, respectively (n = 3 for each group). When treatment of MyEnd cells with LT was carried out in the presence of forskolin and rolipram, glucosylation of Rac-1 by LT was significantly reduced. After 60 min, only 19% and after 120 min, only 36% of cellular Rac-1 was preglucosylated under conditions of increased cAMP. This corresponds to a 72% reduction of Rac-1 inhibition after 60 min and a 60% reduction after 120 min (n = 3 for each group). These experiments demonstrate that one of the mechanisms by which cAMP protects endothelial barrier functions against LT is prevention of Rac-1 glucosylation.

**DISCUSSION**

cAMP prevents endothelial cell dysfunction induced by Rac-1 inhibition. It is well established that cAMP enhances endothelial barrier function in most microvessels and most vascular endothelial (VE)-cadherin (a–d) and F-actin (e–h) (n = 5 for each condition) is shown. a and e: Distribution of VE-cadherin and F-actin in control cells. LT (200 ng/ml, 120 min) induced interruptions of VE-cadherin staining along margins (arrows) of large intercellular gaps (b, f), F/R (5 and 10 μM, respectively; 120 min) reduced the number of stress fibers (g) without affecting VE-cadherin distribution (c). F/R completely prevented LT-induced gap formation when applied together for 120 min (d and h). Scale bar is 20 μM for all images.
cAMP regulates Rac-1

and are given with SE.

When F/R and LT were applied together, the number of bound beads was not significantly different from controls. Values were normalized to control values and are given with SE.

cultured endothelial cell monolayers. This study provides further insight into the mechanisms by which cAMP enhances endothelial barrier properties in vivo. At least with respect to the action of LT, the novel result is that cAMP protects endothelial barrier functions by preventing Rac-1 inhibition in intact microvessels and in cultured microvascular endothelial cells in vitro. In the first part of the discussion we evaluate the mechanistic activity of cAMP in Rac-1 regulation. We then evaluate the significance of this result to the regulation of endothelial barrier permeability and examine other proposed mechanisms of action of cAMP to modulate increased permeability in intact microvessels and in cultured cells.

We previously demonstrated that Rac-1 inhibition by LT-induced glucosylation causes large increases in the permeability of rat mesenteric microvessels and cultured mouse myocardial endothelial cells (40, 41). A key finding of our previous investigation was that inhibition of Rac-1 by LT reduces the binding of beads coated with the extracellular domain of mouse VE-cadherin to mouse myocardial endothelial cells. Part of the mechanism included glucosylation of Rac-1 by LT, which is a glucosyltransferase. Our new results demonstrate that increased levels of endothelial cell cAMP, produced by exposure to rolipram and forskolin, almost completely block the reduction in bead binding induced by LT exposure, and this is associated with reduced Rac-1 glucosylation. Both our new and our previous results conform to the hypothesis that Rac-1 regulates barrier properties by mechanisms involving VE-cadherin-mediated adhesion. VE-cadherin is thought to be one of the main adhesive proteins required for maintenance of intercellular junctions (5), but our results do not exclude a role for other adhesion proteins such as components of the tight junction. An important control experiment was to test that cAMP did not act by blocking the uptake of the toxin into endothelial cells. This was confirmed by showing that when forskolin and rolipram were applied subsequent to LT uptake, the LT increase in response to Rac-1 inhibition was abolished, just as when LT and cAMP were applied together. The protective effect of increased cAMP, stimulated by forskolin and rolipram, was significantly attenuated by the cell-permeant form of the PKA inhibitory peptide Myr-PKI. However, a PKA-dependent mechanism may not be the only mechanism of action of cAMP. We previously demonstrated (2, 3) that experimental conditions that increase intracellular cAMP decrease the baseline permeability in both frog and rat mesenteric microvessels. Ultrastructural investigation in the frog vessels showed that there was an increase in the number of tight junction strands within the clefts of frog microvessels, but corresponding studies in rat microvessels have not been done. It is possible that PKA-dependent mechanisms are less important for endothelial barrier regulation in vivo than they are in cultured endothelium. This might be reflected in our results, which show that Myr-PKI only partially inhibited the $L_p$-reducing effect of forskolin and rolipram on LT-induced $L_p$ increase in vivo whereas it seemed to completely abolish the effect of increased cAMP on LT-induced gap formation in intact microvessels.

![Fig. 5. cAMP increased by F/R inhibited LT-induced reduction of VE-cadherin bead binding. Summary data of laser tweezer experiments (n = 5 for each condition) are shown. After settlement of beads for 30 min on MyEnd cell surface (control values), cells were treated for 120 min with either LT (200 ng/ml) or F/R (5 and 10 μM, respectively) alone or in combination. Whereas LT reduced VE-cadherin-mediated bead binding by ≈ 50%, F/R had no effect. When F/R and LT were applied together, the number of bound beads was not significantly different from controls. Values were normalized to control values and are given with SE.](image)

![Fig. 6. cAMP blocked LT-induced glucosylation of Rac-1 in MyEnd cells. MyEnd cells were treated with either LT (200 ng/ml) or F/R (5 and 10 μM, respectively) alone or in combination for 60 or 120 min. Degree of in vivo glucosylation was determined by incorporation of 3HClabeled UDP-glucose into cell lysates incubated with 200 ng/ml LT. Cell lysates were subjected to SDS-PAGE and phosphorimaging. Increased cAMP significantly reduced Rac-1 glucosylation (n = 3 for each condition).](image)
vitro. However, it must be emphasized that the mechanisms involved in formation of large gaps like those observed in cultured endothelium do not necessarily represent the mechanisms of increased permeability in vivo (41). So it is possible that other mechanisms might disguise the contribution of PKA-dependent mechanisms to intercellular gap formation in vitro. One of these mechanisms might be myosin-dependent contraction, because in our study, cAMP resulted in reduced formation of stress fibers. Previously we showed (41) that myosin-dependent contraction contributes to formation of large gaps in cultured endothelium. Thus an important question for further study is whether there are several cAMP-dependent mechanisms regulating junction stability. These possibly include both VE-cadherin binding to the actin cytoskeleton and the insertion of new junction strand proteins into the tight junction. Furthermore, the mechanisms may be regulated via cAMP in different ways, as suggested by some of the experiments in cultured cells discussed below. For example, studies investigating the role of Rac-1 in integrin function showed that Rac-1, in contrast to Rho A and Cdc42, is activated by PKA (12, 32). This does not appear to be due to direct phosphorylation of the GTPase itself, because Rac-1 lacks a PKA phosphorylation site, but by phosphorylation of its GTP exchange factors like Tiam-1 and Trio (14, 32). Phosphorylation of these or other factors may in turn protect Rac-1 from glycosylation by LT. However, it must be emphasized that further experiments have to investigate the role of cAMP-mediated Rac-1 regulation in the signaling of physiological inflammatory mediators rather than LT.

**Actions of cAMP in intact microvessels.** The effects of inflammatory mediators such as thrombin in cultured endothelial cells (30, 33) as well as bradykinin and platelet-activating factor (PAF) in vivo (4, 10, 19) have been demonstrated to be abolished or largely reduced by increased cAMP. One common mechanism suggested for the action of cAMP is modulation of actin-myosin contraction. However, we have shown (4) that actin-myosin contractile mechanisms do not contribute significantly to increased permeability in rat microvessels exposed to bradykinin and PAF. Specifically, inhibition of neither myosin light chain kinase nor myosin ATPase attenuated the increase in permeability in rat microvessels after exposure to PAF or bradykinin. This was not due to a lack of action of the inhibitors. After inhibition of actin-myosin contractile mechanisms, baseline permeability was reduced and recovery toward control permeability levels was accelerated. In summary, the lack of a direct contribution of an actin-myosin contractile mechanism to increased permeability argues against an action of cAMP to regulate actin-myosin contraction. Thus in intact microvessels it is clear that the primary target of cAMP to regulate permeability is via the modulation of adhesion rather than modulation of contraction.

**Rho proteins and endothelial barrier regulation in cultured endothelial cells.** On the basis of experiments in cultured endothelial cells there is detailed evidence that the GTPases of the Rho family are substantially involved in endothelial permeability regulation of cultured endothelial cell monolayers (42). In studies using cultured endothelial cells, both Rho A and Rac-1 have been demonstrated to be involved in endothelial barrier regulation; both GTPases were thought to act primarily by regulation of cell contraction. Indeed, our studies indicate that Rac-1, independently from Rho A, seems to reduce myosin-dependent contraction, because inhibition of Rac-1 led to increased formation of stress fibers. Moreover, formation of large intercellular gaps could be prevented by inhibition of myosin light chain kinase or myosin ATPase (41). However, given our investigations showing the regulation of VE-cadherin binding by Rac-1 discussed above, the fact that we have found no direct action of contractile mechanisms to increase permeability in intact microvessels, and the fact that endothelial cells in culture have a strong tendency to form large gaps after exposure to inflammatory conditions, the results of experiments on cultured endothelial cells require careful analysis to determine the contribution of Rho protein-dependent mechanisms to regulate permeability in intact microvessels. In particular, the results from cultured cells point to further experiments to test the role of Rho proteins in regulation of permeability and the role of cAMP in modulation of the mechanisms of contraction and adhesion.

First, we note that most experiments suggesting that Rho proteins contribute to the regulation of permeability have been carried out after the permeability of the endothelial monolayers was increased with thrombin. However, intact microvessels in the rat do not respond to thrombin unless they have been exposed previously to inflammatory stimuli (11). Thus the Rho-dependent endothelial cell phenotypes studied in the experiments summarized below may be different from the endothelial phenotype studied in intact rat microvessels. In studies using various cultured cell lines and cell-free systems it has been shown that Rho protein function is modulated by PKA. For Rho and Cdc42, inhibition of GTPase-dependent effects has been demonstrated after phosphorylation of serine residues of the GTPase itself, resulting in increased association with GDP dissociation inhibitors (13, 14, 25, 31, 33, 34, 38). Recently, protection of endothelial barrier functions against thrombin by PKA-dependent phosphorylation of Rho A has provided the first indication that these mechanisms may be relevant in endothelial cells as well (33).

Finally, we note that our previous studies did not favor an important stabilizing role of Rho A and Cdc 42 for barrier properties both in culture and in vivo because the effects of toxin B, which inhibits Rho A, Rac-1, and Cdc42, was equal in effect to LT, which only inhibits Rac-1, when both toxins were used under conditions in which they induce comparable glycosylation (1, 40). However, recent studies also indicate a supportive role of Cdc42 in lung microvessels in vivo, mainly as a feedback mechanism to restore barrier properties after permeability increases by inflammatory mediators like thrombin (24).

**ACKNOWLEDGMENTS**

We are grateful to Gabriele König, Agnes Weth, and Joyce Lenz for skillful technical assistance.

**GRANTS**

These studies were supported in part by grants from the National Heart, Lung, and Blood Institute (HL-44485 and HL-28607) and by a grant from the Deutsche Forschungsgemeinschaft (SFB 487, TP B5).

**REFERENCES**


