Epac/Rap1 pathway regulates microvascular hyperpermeability induced by PAF in rat mesentery


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First published January 4, 2008; doi:10.1152/ajpheart.00937.2007.—Experiments in cultured endothelial cell monolayers demonstrate that increased intraacellular cAMP strongly inhibits the acute permeability responses by both protein kinase A (PKA)-dependent and -independent pathways. The contribution of the PKA-independent pathways to the anti-inflammatory mechanisms of cAMP in intact mammalian microvessels has not been systematically investigated. We evaluated the role of the cAMP-dependent activation of the exchange protein activated by cAMP (Epac), a guanine nucleotide exchange factor for the small GTPase Rap1, in rat venular microvessels exposed to the platelet-activating factor (PAF). The cAMP analog 8-pCPT-2′,3′-O-methyl-cAMP (O-Me-cAMP), which stimulates the Epac/Rap1 pathway but has no effect on PKA, significantly attenuated the PAF increase in microvessel permeability as measured by hydraulic conductivity (Lp). We also demonstrated that PAF induced a rearrangement of vascular endothelial (VE)-cadherin seen as numerous lateral spikes and frequent short breaks in the otherwise continuous peripheral immunofluorescent label. Pretreatment with O-Me-cAMP completely prevented the PAF-induced rearrangement of VE-cadherin. We conclude that the action of the Epac/Rap1 pathway to stabilize cell-cell adhesion is a significant component of the activity of cAMP to attenuate an acute increase in vascular permeability. Our results indicate that increased permeability in intact microvessels by acute inflammatory agents such as PAF is the result of the decreased effectiveness of the Epac/Rap1 pathway modulation of cell-cell adhesion.

VASCULAR ENDOTHELIUM (VE) is the principal barrier to, and regulator of, material exchange between circulating blood and the body tissues. In most organs with continuous endothelium (heart, lung, skin, and muscle), an increase in endothelial permeability to water and macromolecules leads to edema formation and possible loss of organ function. Function is restored only after the barrier is restored. Inflammatory mediators induce the transient formation of gaps between endothelial cells both in vivo and in cell culture (3, 23, 24). Studies based primarily on thrombin-stimulated cultured endothelial cells suggest that gaps form through the generation of an active contractile force within endothelium coupled with the loss of endothelial-endothelial adhesion. Contractile mechanisms include a calcium-dependent myosin light chain kinase (MLCK) phosphorylation of myosin and RhoA-dependent polymerization of actin stress fibers. RhoA also activates RhoA-dependent kinase (ROCK), which contributes to myosin activation both through direct myosin light chain phosphorylation and the inactivation of myosin phosphatase. Loss of endothelial (cell-cell) adhesion implies a disassembly of multiple junctional molecules (13). Adhesion regulation through the phosphorylation of adherens complex components has been demonstrated in various studies but questioned in others (21, 22, 33, 38). It is widely recognized that conditions leading to increased intraendothelial cAMP strengthen barrier function and attenuate increased permeability in both cultured endothelial cell monolayers and intact microvessels (40). Although the most generally accepted model of the cAMP mechanism to attenuate increased permeability suggests that increased cAMP, acting via a protein kinase A (PKA)-dependent pathway that includes MLCK and RhoA, leads to the dephosphorylation of myosin light chains and reduced actin/myosin contraction, the general applicability of this model in vivo is questioned. In rat mesentery microvessels, inhibition of MLCK or ROCK failed to block platelet-activating factor (PAF)-stimulated permeability (1, 3). Recent investigations from our laboratory in intact microvessels and cultured endothelial cells also suggest that a cAMP-dependent mechanism regulates cell-cell adhesion and cell-matrix adhesion to modulate endothelial barrier permeability (43, 44). Moreover, independent studies using cultured endothelial cells show that these adhesion mechanisms involve an alternate cAMP pathway that does not include the activation of PKA (11, 15, 19). The role of PKA-independent pathways in the regulation of intact venular microvessel permeability has not been tested. Thus the primary goals of the present experiments were to evaluate the activity of a cAMP analog that does not activate PKA but does activate an alternate cAMP target that modulates cell-cell adhesion mechanisms in individually perfused mammalian microvessels and to test the hypothesis that the activation of the latter cAMP-dependent pathway could block PAF-stimulated permeability.

The cAMP analog 8-pCPT-2′,3′-O-methyl-cAMP (O-Me-cAMP) activates the guanine nucleotide (GDP/GTP) exchange factor (GEF) known as exchange protein activated by cAMP (Epac). O-Me-cAMP is a highly potent activator of Epac but a very poor activator of PKA and is thereby used to discriminate between signaling through Rap1 and signaling through PKA (6, 8). The primary target of Epac in endothelial cells is the small GTPase Rap1 (6). Rap1 may play an important role to maintain normal permeability because it has been shown to promote polymerization of the cortical band actin, strengthen-
Confocal microscopy of immunolabeled mesenteries. Venular microvessels (2 to 4 vessels for each treatment group) were perfused first with control solution and then with PAF or pretreated with cAMP analogs before PAF. After 5 min of PAF (near the peak of $L_p$ response), mesenteries were flooded with ice-cold fixative (1% freshly depolymerized paraformaldehyde in phosphate-buffered saline, pH 7.2, 5 min). The tissues were labeled with primary antibody against VE-cadherin (sc6458; Santa Cruz) and a fluorescent secondary antibody and then mounted for confocal microscopy. Tissues were mounted whole to retain the three-dimensional structure of the vessels and enable a separate collection of either the front (near lens) or rear half of each vessel. Images stacks (about 10 from each vessel), typically composed of 15 to 30 images taken at 0.5-μm steps, were collected (Zeiss LSM510 laser scanning microscope, 63 × 1.4 numerical aperture lens) with pinhole settings to achieve 0.8-μm optical section thickness. Stacks were projected onto a single plane for analysis. An intensity profile of the VE-cadherin label was measured perpendicularly to the cell border at randomly selected locations in each treatment group, enabling the measurement of mean intensity profile and the calculation of mean kurtosis (peakedness of the profile). For each gap in the VE-cadherin fluorescence, a value of one-half was assigned to each cell sharing the gap. The mean number of gaps per cell was calculated within each group.

**Solutions and reagents.** Mammalian Ringer solution was composed (in mM) 132 NaCl, 4.6 KCl, 2.1 CaCl$_2$, 1.2 MgSO$_4$, 5.5 glucose, 5.0 NaHCO$_3$, and 20 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and Na-HEPES. The ratio of acidi-HEPES to Na-HEPES was adjusted to achieve a pH of 7.40–7.45. All perfusates were mammalian Ringer solution additionally containing BSA at 10 mg/ml. The following stock solutions were prepared in advance and diluted into the final perfusate immediately before use. PAF (1-O-hexadecyl-2-acetyl-sn-glycer-3-phosphocholine; 511075; Calbiochem) was prepared at 1 mM in ethanol. Rolipram (PD-175; Biomol) was prepared at 50 mM in ethanol. Forskolin (CN-100; Biomol) was prepared at 25 mM in ethanol. Stock solutions of rolipram and forskolin were stored for up to 2 mo and diluted to working concentrations on the day of use. O-Me-cAMP (C041-05; Biolog) was prepared as a 100-mM stock in Ringer and stored frozen. Isoproterenol (I-6504; Sigma) was dissolved in water to 10 mM on the day of use.

**Analysis and statistics.** $L_p$ measurements during the control period were averaged to establish a single value for control $L_p$ for each vessel. Peak $L_p$ values attained ($L_{p_{peak}}$) were the single highest measurements recorded after treatment with PAF. $L_p$ values were normalized to the control $L_p$ values for each vessel before averaging. To examine the modulation of the response to PAF, we tested the response in each vessel twice, first in the absence of other agents and second in the presence of a test reagent. Therefore, each vessel acted as its own control. Throughout, averaged $L_p$ values were reported as means ± SE. The indicated statistical tests were performed assuming significance for probability levels of <0.05.

**RESULTS**

Effect of cAMP and O-Me-cAMP to block acute inflammatory response. Figure 1 shows that the cAMP agonist O-Me-cAMP, which activates Epac but not PKA, significantly attenuates the PAF-induced (10 nM) increase in $L_p$. A typical response to PAF alone and the near complete inhibition of that response by increased cAMP (stimulated by rolipram and forskolin) are shown in Fig. 1A. A representative experiment to test the effectiveness of stimulating the Epac pathway shows a typical PAF response and a much-reduced PAF response following pretreatment with O-Me-cAMP (100 μM; Fig. 1B). This concentration is known to maximally stimulate Rap1 in an ovarian carcinoma cell line (31) and to strongly reduce baseline
permeability of cultured endothelial cell monolayers (11). The reduction in the $L_p$ response using O-Me-cAMP was not as complete as that measured when cAMP was increased by using rolipram with forskolin. A representative control experiment shows that the action of O-Me-cAMP or cAMP was not due to tachyphylaxis; within 30 min, vessels recovered the potential for full responsiveness to PAF (Fig. 1C). Figure 1D summarizes these experiments where the mean peak PAF response is presented as the ratio of the second PAF-induced $L_p^{\text{peak}}$ value to the first PAF $L_p^{\text{peak}}$. Increasing the O-Me-cAMP concentration to 500 µM does not induce any further attenuation of increased permeability. The data in Fig. 1D summarize the dose response to O-Me-cAMP in these experiments. Both 100 and 500 µM O-Me-cAMP significantly attenuate PAF-induced increases in $L_p$. These results are the clearest evidence to date that a PKA-independent pathway stimulated by cAMP, in this case the Epac/Rap1 pathway, contributes strongly to the attenuation of permeability in intact microvessels.

Measured $L_p$ values throughout this study can be typified by the mean $L_p$ measured at the end of the initial control perfusion and the initial peak PAF $L_p$ response of various protocols. For the 22 vessels represented in Fig. 1D, mean ($\pm$ SE) baseline $L_p$ was $0.62 \pm 0.05 \times 10^{-7}$ cm/(s cmH$_2$O), ranging from 0.35 to 1.01 $\times 10^{-7}$ cm/(s cmH$_2$O). The mean ($\pm$ SE) initial PAF peak $L_p$ was $16.9 \pm 2.1 \times 10^{-7}$ cm/(s cmH$_2$O), ranging from 4.6 to 44.8 $\times 10^{-7}$ cm/(s cmH$_2$O).

Baseline $L_p$: the action of O-Me-cAMP compared with that of cAMP increased by rolipram plus forskolin or by isoproterenol. Treatment of microvessels with O-Me-cAMP did not affect baseline $L_p$ (Fig. 2A). The mean $L_p$ measured over 40 min in the presence of O-Me-cAMP was not different from that measured in a control group perfused with vehicle solution (Fig. 2A). This was true for both 100 and 500 µM O-Me-cAMP. This result is an important control because it demonstrates that there were no changes in baseline permeability that might have complicated the interpretation of the results in Fig. 1.

However, the conditions that increased intracellular cAMP did reduce baseline $L_p$. Figure 2B summarizes the results from
two such protocols shown compared with the same control vessels as in Fig. 2A. The group perfused with rolipram and forskolin fell to 65% of baseline $L_p$ over 40 min of treatment. As a further test of the effects of increased cAMP, we treated vessels with isoproterenol (10 μM), an agonist of the β-adrenergic receptor linked to adenylyl cyclase. The $L_p$ measured in these vessels also significantly decreased. Together, these results demonstrate that elevated cAMP but not O-Me-cAMP, which is specific for Epac, can reduce baseline $L_p$.

Use of 6-Phe-cAMP to investigate further cAMP-dependent effects. We attempted further experiments to test whether a cAMP-dependent mechanism acting via PKA would account for the difference seen in the PAF hyperpermeability attenuation. To do this we used 6-Phe-cAMP, the analog that activates both PKA and Epac but preferentially stimulates PKA. Figure 3 shows that 6-Phe-cAMP (either 200 or 500 μM) combined with O-Me-cAMP (100 μM) attenuated the PAF response no more than O-Me-cAMP alone. This was not due to a failure of 6-Phe-cAMP to have any effect because a third group of experiments using 6-Phe-cAMP (500 μM) alone showed that this analog could also attenuate the PAF-induced increase in permeability (Fig. 3).

We also tested the effects of 6-Phe-cAMP on baseline $L_p$. In a series of experiments similar to those of Fig. 2, we found that 6-Phe-cAMP had no consistent effect on baseline $L_p$ over the range of 100 to 500 μM (Fig. 4).

Reorganization of VE-cadherin. To test for structural correlates of the O-Me-cAMP inhibitory effect on the acute high permeability state induced by PAF, we observed the distribution of VE-cadherin under control and PAF-treated conditions with and without O-Me-cAMP pretreatment. PAF (10 nM, 5 min) alone induced a rearrangement of the VE-cadherin (Fig. 5B) compared with vessels perfused with control solution only (Fig. 5A). In PAF-treated vessels, there were regions where the VE-cadherin was nonuniformly distributed. In some cleft segments, the label appeared in spikes that were oriented transversely to the endothelial perimeter. In some regions, the label became discontinuous, leaving gaps in the perimeter label. In control vessels, the label appeared as a nearly uniform, continuous peripheral band ~0.5 μm in width and without lateral spikes or discontinuities. In vessels pretreated with O-Me-cAMP, the subsequent PAF treatment did not induce any changes in the VE-cadherin pattern (Fig. 5C) compared with either vehicle control or perfusion with O-Me-cAMP alone (Fig. 5D). In summary, PAF induced a rearrangement of VE-cadherin, and that rearrangement was completely blocked by O-Me-cAMP. Increased cAMP with forskolin and rolipram (Fig. 5E) did not alter the VE-cadherin pattern relative to the vehicle control or the O-Me-cAMP.

To quantify the changes in VE-cadherin organization, we examined the fluorescence intensity profile across the cell-cell junctions at random locations around the periphery of endothelial cells in the several treatment groups. The fluorescence intensity profiles demonstrated that in the presence of PAF, VE-cadherin was distributed more widely across the clefts than under control conditions. This was not associated with an increased amount of VE-cadherin. Rather, it reflected the disruption of continuity and the formation of lateral spikes in the VE-cadherin label and, therefore, a broadening of the profile coupled with a lowering of the mean peak (Fig. 6A). One measure of this change in distribution was the mean width of the VE-cadherin fluorescent label determined at the value of half of the maximum intensity. PAF treatment resulted in a significantly wider average VE-cadherin distribution (0.9 ± 0.1 μm) than for the control group (0.5 ± 0.1 μm). The difference was also revealed by a much lower mean kurtosis for the PAF group than the control group (Fig. 6B). The pretreatment with O-Me-cAMP prevented the rearrangement and disruption of VE-cadherin, and thus the mean width of the VE-cadherin in the O-Me-cAMP/PAF group was different from the control. Interestingly, the profiles of the O-Me-cAMP group and the rolipram/forskolin group were not different from those of the control group. The latter result was in contrast to the results using cultured endothelium for which O-Me-cAMP treatment induced an intensified distribution of the VE-cadherin near cell-cell junctions. This observation highlights differences in baseline conditions between cultured endothelium and endothelium in intact vessels, which is further addressed in DISCUSSION.

Numerous studies indicate that not only is VE-cadherin a principal adhesion protein in VE but it also may play a
regulatory role in maintaining the integrity of the endothelial barrier (13). The continuity of peripheral VE-cadherin in endothelial monolayers is often used as an indicator of an intact barrier in situ and in cultured cells (35, 37, 46). Therefore, a second approach to quantify the effects of O-Me-cAMP on PAF-induced hyperpermeability was to count the number of discontinuities (gaps) in the peripheral label. The frequency of gaps in the peripheral VE-cadherin label was about 10-fold higher in the PAF group than in the control group (Fig. 6C). The group pretreated with O-Me-cAMP and then perfused with PAF was not different from the control group, further indicating an inhibition of VE-cadherin rearrangement.

DISCUSSION

The principal observation is that, in intact venular microvessels, the cAMP analog O-Me-cAMP that does not activate PKA but does activate the GEF Epac (6, 11) strongly attenuated the PAF-induced permeability increase. Stimulation of the Epac/Rap1 pathway also completely blocked the PAF-induced rearrangement of junction-associated VE-cadherin. Thus, under the conditions of our experiments, we conclude that cAMP activation of the Epac/Rap1 pathway provides the major attenuation of the acute permeability response in intact microvessels and is associated with the stabilization of the endothelial-endothelial junction complex. These results do not conform to the hypothesis that rapid modulation of microvessel permeability by cAMP can be described in terms of a single dominant mechanism such as the attenuation of contractile actin/myosin interaction by a cAMP-stimulated, PKA-dependent phosphorylation of MLCK. At the same time, our results do not rule out a contribution of a cAMP/PKA-dependent mechanism. This is because the conditions that increase intracellular cAMP (activation of adenylate cyclase and inhibition of phosphodiesterase by a combination of rolipram and forskolin) completely attenuate the PAF-induced increase in permeability and reduce baseline permeability (3).

These results are the first in intact microvessels to use the newly available cAMP analogs to discriminate between the contributions of PKA-dependent and -independent pathways to the regulation of baseline and increased permeability. Previous investigations were carried out in cultured endothelial cell monolayers (11, 15, 19). Exposure of cultured human umbilical vein endothelial cells (HUVECs) to O-Me-cAMP over the same range of concentrations used in the present investigations caused a dose-dependent activation of the small GTPase Rap1.
but no activation of PKA under the same conditions. After the exposure of HUVECs to the inflammatory agent thrombin, the Epac pathway stimulated by O-Me-cAMP attenuated the thrombin-induced increase in solute permeability but did not restore permeability to the same extent as cAMP (11). In that study using HUVECs, O-Me-cAMP accounted for only about one-third of the attenuation of the maximum response achieved with cAMP. The latter observations are consistent with the results of our present experiments showing that the Epac-stimulated pathway regulates some but not all of the cAMP-dependent attenuation of increased permeability. The results also suggest that the contribution of the PKA-independent pathway to attenuate increased permeability may be different for different inflammatory stimuli and for different endothelial cells. In particular, the GEF Epac has been shown to directly regulate Rap1, and the activation of Rap1 leads to the stabilization of adhesion between endothelial cells via multiple mechanisms including 1) Rap1-dependent assembly of junction complexes (e.g., binding with AF-6, an intracellular binding partner of several tight and adherens junction proteins); 2) stabilization of adherens junctions by modulating VE-cadherin adhesion to the actin cytoskeleton; and 3) increased peripheral band actin polymerization, possibly due to cross talk between activated Rap1 and Rho family GTPases (6, 11, 19, 26, 31, 34, 36, 45).

Models of cAMP-dependent inhibition of acute inflammatory response. The widely accepted model of acute permeability increase states that receptor binding by inflammatory mediators leads to the activation of MLCK and the contraction of actin/myosin structures within the endothelium. This pathway also activates RhoA, enhancing the polymerization of actin bundles and activating RhoA-dependent kinase (ROCK), which both activates myosin light chain and inactivates myosin phosphatase (40). These pathways have been extensively investigated using thrombin-stimulated cultured endothelial cells and are generally accepted for cultured endothelium. Widely accepted is also the model for cAMP-dependent inhibition of this pathway (Fig. 7, left), which has been shown to rely on the PKA phosphorylation of MLCK and RhoA, thus inhibiting both myosin phosphorylation and actin polymerization (16, 30). This general model has been less well investigated in vivo, and although some reports lend support to the model in vivo (7, 41), other studies do not support this model for intact microvessels. In particular, we have previously investigated this pathway by use of both PAF and bradykinin stimulation of endothelial permeability in intact rat microvessels. Our experiments demonstrated that the inhibition of myosin ATPase, MLCK, and ROCK all failed to block acute permeability.

Fig. 6. O-Me-cAMP blocks redistribution of VE-cadherin. A: mean intensity profiles of VE-cadherin label distribution indicated that VE-cadherin was distributed slightly more widely across the clefts during stimulation with PAF, reflecting the disruption and lateral spikes of VE-cadherin label (n = 49 measured profiles from about 15 cell pairs in each group; SE suppressed for clarity). B: the kurtosis in the PAF group was significantly lower than in other groups. *P < 0.05, 1-way ANOVA with Bonferroni posttests. C: the number of gaps in the peripheral VE-cadherin label was significantly higher in the PAF group than in Ctrl vessels. *P < 0.05, 1-way ANOVA with Bonferroni posttests. The O-Me-cAMP/PAF group was not different from the Ctrl, further indicating that O-Me-cAMP inhibits rearrangement of VE-cadherin during stimulation with PAF.

Fig. 7. cAMP mechanisms that stabilize the endothelial barrier. Right: pathways where activation of Rap1 strengthens the endothelial barrier by stabilizing adhesion of adjacent endothelial cells. Strengthening of the actin peripheral band and cell-cell adhesion is a potential mechanism to attenuate increases in permeability. Other possible mechanisms include reducing the activity of the small GTPase RhoA, which regulates stress fiber formation and contraction. Thus the Rap1 pathway activated by exchange protein activated by cAMP (Epac) lies in parallel with well-known cAMP-protein kinase A (PKA)- dependent pathways (left), which modulate contractile mechanisms linked to stress fiber formation and myosin light chain kinase (MLCK) phosphorylation. The latter pathways are prominent in cell culture but appear to play a reduced role in intact, noninflamed microvessels. ROCK, RhoA-dependent kinase; GEF, guanine nucleotide exchange factor.
response (1, 3). Results of those studies suggested that the primary endothelial response to these acute inflammatory mediators does not include an active contraction of the actin/myosin apparatus for intact endothelium.

An alternate cAMP-dependent regulatory pathway has been described (Fig. 7, right). Epac1 binding of cAMP results in Rap1 activation that has been associated with the stabilization of cultured endothelial cells in several studies (6, 11, 15). Rap1 activation increases peripheral band actin while diminishing stress fibers and promotes the redistribution of both tight and adherens junction components including VE-cadherin to the cell-cell border (11, 15). A possible mechanism for these changes is that Rap has been shown to bind to and activate Tiam1 and VAV2, both GEFs for Rac1, in a cell-spreading assay (4). Rac1 is widely known as a regulator of actin dynamics and has been implicated in the permeability regulation of intact microvessels (42). In another link to actin regulation, ARAP3, when bound to Rap1, acts as a downstream effector of Rap1 through the activation of its GAP activity toward RhoA (20). The Rap1-dependent inhibition of thrombin-stimulated permeability was shown to act through the inhibition of RhoA, suggesting an important role for Rap/GAP function of ARAP3 in HUVECs (11, 15). Another effector that binds to several proteins including components of tight and adherens junctions is AF-6, also known as afadin (17, 36). Activation of the Epac/Rap1 pathway using O-Me-cAMP induced the enhanced distribution of AF-6 to cell-cell junctions in association with improved barrier function of HUVEC monolayers (11). Thus numerous effectors and structural components of both tight and adherens junctions are potentially regulated by the Epac/Rap1 pathway. Although further investigation of these pathways is required, our present data are the first to demonstrate inflammatory inhibition linked to Epac activation using an in vivo model.

Intact microvessels compared with endothelial cell monolayers. Cultured endothelial cell monolayers such as HUVECs are strongly stimulated to increase permeability after exposure to thrombin, resulting in the activation of RhoA signaling pathways, the development of tension, and the formation of large gaps between adjacent cells (5, 23, 27, 39, 47, 48). In contrast, we have shown that thrombin does not increase the permeability of normal rat mesenteric microvessels with no prior exposure to inflammatory conditions (12).

These observations may explain why the Epac/Rap1 pathway accounts for more of the cAMP-dependent attenuation of the PAF-stimulated permeability increase in intact microvessels than the thrombin-stimulated permeability increase in HUVECs. Specifically, both thrombin and PAF are assumed to activate mechanisms that weaken adhesion, but thrombin also stimulates active tension development via a RhoA-dependent pathway in cultured cells. Thus, although an O-Me-cAMP-stimulated Epac/Rap1 pathway may attenuate the tendency to weaken adhesion between adjacent endothelial cells in both intact venular microvessels and HUVEC monolayers, this action is not sufficient to withstand the active RhoA-dependent tension developed in HUVECs after thrombin stimulation. This would especially be the case if the resting level of adhesion between adjacent endothelial cells was less in the HUVEC monolayers than in the intact microvessels. When making this comparison, it is important to emphasize that the baseline permeability of the HUVEC monolayers to macromolecules [such as a 70K molecular weight dextran as used by Cullere and colleagues (11)] is close to two orders of magnitude larger than those in intact microvessels. Therefore, HUVEC monolayers appear to have weakened adhesion between the cells even in the resting state. Thus, if a primary action of the targets of the Epac/Rap1 pathway is to strengthen adhesion, appropriate activation of Rap1 could account for a significant part of the effect of cAMP to reduce baseline permeability in such monolayers.

In intact microvessels, we found that increased intracellular cAMP induced by exposure to rolipram and forskolin decreased baseline $L_p$, but O-Me-cAMP did not. This observation would be consistent with the idea that the Epac/Rap1 pathway does not activate all the mechanisms to reduce permeability in endothelial cells. However, we cannot exclude the possibility that under purely basal conditions, Epac is not active and that it is upregulated by inflammatory stimulation. Similarly, we found that 6-Phe-cAMP, which preferentially stimulates the PKA pathway, had no consistent effect to reduce baseline $L_p$. Thus attempts to separately activate Epac and PKA both failed to reproduce the reduction in baseline $L_p$ that was induced by the stimulation of cAMP using rolipram and forskolin. It is possible that an unknown cAMP target is responsible for the reduction in baseline $L_p$. Another possibility includes a requirement for a very high, highly localized (compartmentalized) concentration of cAMP (e.g., near receptor-linked forskolin stimulated adenylyl cyclase) that may be necessary to assemble and regulate the large multiprotein molecular complex consisting of binding proteins, phosphatases, phosphodiesterases, etc.) that is required for the effective function of a cAMP-dependent signaling system (10). Loading with the 6-Phe-cAMP or O-Me-cAMP analogs may not establish a sufficiently high local concentration to modify the baseline $L_p$. Similarly, the full inhibition of the PAF response that is seen with forskolin and rolipram is not achieved using O-Me-cAMP alone or in combination with 6-Phe-cAMP. The full inhibition of the inflammatory response may require specific localization or high concentrations of cAMP. These possibilities require much further investigation.

Potential PKA targets. There are many PKA targets that are associated with either tight junction strands or with the peripheral actin-cadherin adhesion complex, including claudin-5 (18), occludin (32), the actin-binding protein vasodilator-stimulated phosphoprotein (VASP) (9), and GEFs for Rac1 (28). Much remains to be investigated about the importance of the strength of the actin-adhesion protein complex, the assembly of occludin and claudins, and the role of the actin-binding proteins in the regulation of endothelial permeability, but an earlier study from our laboratory using frog mesentery microvessels indicated that cAMP, stimulated by forskolin plus rolipram or by the β-adrenergic agonist isoproterenol, could reduce $L_p$ to near 30% of control within 30 min (2). The fall in $L_p$ in the frog microvessels was associated with an increase in the number of tight junction strands, a structural end point reflecting the regulation of the adhesion structures.

In summary, new cAMP analogs such as O-Me-cAMP provide a novel strategy to investigate in vivo the endothelial barrier-promoting properties of cAMP. In particular, we find that O-Me-cAMP contributes significantly to the attenuation of acute inflammatory permeability response without affecting baseline permeability. These results conform to the hypothesis
that the Epac/Rap1 pathway is a principal signaling pathway in the regulation of normal endothelial barrier permeability in intact microvessels and also plays a key role in the recovery of normal permeability after acute inflammatory injury. An understanding of these mechanisms is also important because better-targeted agents with anti-inflammatory action would harness the powerful anti-inflammatory actions of cAMP that are already widely recognized but generally avoided because of the adverse effects of other actions of cAMP that currently limit clinical applications (14, 40).

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