cAMP/PKA antagonizes thrombin-induced inactivation of endothelial myosin light chain phosphatase: role of CPI-17

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Aims	Activation of cAMP signalling abrogates thrombin-induced hyperpermeability. One of the mechanisms underlying this protective effect is the inactivation of endothelial contractile machinery, one of the major determinants of endothelial barrier function, mainly via the activation of myosin light chain phosphatase (MLCP). To date, the mechanisms of cAMP-mediated MLCP activation are only partially understood. Here the contribution of two cAMP effectors, PKA and Epac, in the regulation of endothelial contractile machinery and barrier function was studied.
Methods and results	Endothelial contractile machinery and barrier function were analysed in cultured human umbilical vein endothelial cells (HUVEC). The cAMP analogues 8-CPT-cAMP and 6-Bnz-cAMP were used to activate Epac and PKA, respectively, and forskolin (FSK) was used to activate adenylyl cyclase. The cells were challenged by thrombin to inhibit MLCP via the RhoA/Rock pathway. Activation of either PKA or Epac partially blocked thrombin-induced hyperpermeability. Simultaneous activation of PKA and Epac had additive effects that were comparable to that of FSK. Activation of PKA but not Epac inhibited thrombin-induced phosphorylation of MLC and the MLCP regulatory subunit MYPT1, partly via inhibition of the RhoA/Rock pathway. FSK activated the MLCP catalytic subunit PP1 via dephosphorylation and dissociation of the PP1 inhibitory protein CPI-17. FSK blunted thrombin-induced CPI-17 phosphorylation, CPI-17/PP1 complex formation, and PP1 inactivation. Down-regulation of CPI-17 attenuated thrombin-induced hyperpermeability and abolished the antagonistic effect of the PKA activator, whereas the Epac activator retained its antagonistic effect.
Conclusion	cAMP/PKA regulates the endothelial barrier via inhibition of the contractile machinery, mainly by the activation of MLCP via inhibition of CPI-17 and RhoA/Rock. The permeability-lowering effect of the cAMP/Epac pathway is independent of CPI-17.
Keywords	Myosin light chain phosphatase • CPI-17 • Endothelial permeability • PKA • Epac

1. Introduction

Vascular endothelium forms a selective barrier and regulates the trafficking of macromolecules and blood cells across the vessel wall. The integrity of the endothelial barrier is highly dependent on the actin–myosin-based endothelial contractile machinery.[1,2] Activation of the contractile machinery is triggered by phosphorylation of the regulatory myosin light chains (MLC), which is balanced by endothelial Ca^{2+}–calmodulin-dependent MLC kinase and MLC phosphatase (MLCP).[3,4] In the context of the present study MLCP is of special interest. The endothelial MLCP is a heterotrimeric enzyme composed of a catalytic subunit, PP1, a regulatory myosin phosphatase targeting subunit (MYPT1), and a 20-kDa subunit of yet unknown function.[5,6] In smooth muscle cells, it is well established that the activity of MLCP is controlled by Rho kinase (Rock), which phosphorylates MYPT1 at its inhibitory sites,[7] and the endogenous inhibitor of PP1, CPI-17 (PKC-potentiated inhibitory protein for PP1 of 17 kDa).[8,9] However, in endothelial cells, the regulation of MLCP is not completely understood.

Previous reports from us[10] and others[11] have shown that RhoA/Rock regulates MLCP activity via phosphorylation of MYPT1 at its

inhibitory sites. However, the studies have also revealed that there exists another regulatory mechanism controlling the activity of MLCP: in smooth muscle cells, MLCP can be inhibited through the endogenous inhibitor of the catalytic subunit PP1, CPI-17.[8,9] Phosphorylation of CPI-17 at threonine 38 (Thr38) residue increases its inhibitory potential 1000-fold.[12] Several kinases including Rock have been reported to phosphorylate CPI-17 at Thr38.[13] Recently, CPI-17 has been identified in endothelial cells and has been shown to be involved in the regulation of endothelial cytoskeleton.[14]

Inflammatory mediators like thrombin stimulate a repertoire of signalling events that activate the contractile machinery of endothelial cells leading to the development of intercellular gaps and, hence, barrier failure.[4,11,15] We and others have shown that manoeuvres elevating intracellular cAMP levels inactivate the contractile machinery and antagonize hyperpermeability induced by a wide range of inflammatory mediators.[16–19] However, the molecular mechanism of contractile inhibition via cAMP signalling is not fully understood.

The major cAMP effector PKA acts via inhibition of RhoA/Rock-mediated reduction of MLC phosphorylation and thus inhibition of actomyosin-based contractile machinery.[6,16,18] Apart from PKA, cAMP may also promote cell–cell adhesion via activation of Epac, an exchange protein directly activated by cAMP for the GTPase Rap1.[20–22] The role of Epac in the regulation of endothelial contractility has not been analysed yet. Some recent reports describe that PKA and Epac act independently in the control of endothelial barrier and adhesion.[23,24] However, the relative contribution of both effectors in either control of contractile machinery or barrier function is still missing.

In the present study, we analysed the effects of specific activation of PKA and Epac signalling on barrier function and contractile machinery in a well-established model of human umbilical vein endothelial cells (HUVEC). The main goals of the study were to analyse: (i) the relative contribution of PKA and Epac in the control of endothelial barrier via inactivation of the contractile machinery in thrombin-induced barrier failure and (ii) the role of CPI-17 in cAMP-mediated activation of MLCP, contractile inactivation, and control of barrier function in thrombin-induced barrier failure. N^6-benzoyl-cAMP, 8-CPT-2Me-cAMP, and forskolin (FSK) were used to activate PKA, Epac, or adenylyl cyclase, respectively. Thrombin was applied for RhoA/Rock-mediated activation of contractile machinery.

2. Methods

2.1 Materials

For detailed Materials, see Supplementary material online.

2.2 Cell culture

The study was approved by the local institutional Ethics Committee and conforms to the principles outlined in the 'Declaration of Helsinki' (Cardiovascular Research 1997; 35: 2–3). HUVEC were isolated from umbilical cords and cultured as described previously.[15] All the experiments were performed with passage 1 (see Supplementary material online).

2.3 Experimental protocols

The basal medium used in incubations was modified Tyrode's solution as described previously.[15] Stock solutions of PKI, Y27632, 8-CPT-Me-cAMP, 6-Bnz-cAMP, and thrombin were prepared immediately before use with basal medium. Stock solutions of calyculin-A, H89, and FSK were prepared with dimethyl sulfoxide (DMSO). Appropriate volumes of these solutions were added to the cells yielding final solvent concentrations <0.1%

(vol/vol). The same final concentrations of DMSO or basal medium were included in all respective control experiments.

In a set of pilot experiments, concentration–response relationships were determined to find the optimal effective concentration. The following agents were applied in their optimal effective concentrations: FSK (5 μM), PKI (20 μM) thrombin (0.2 U/mL), Y-27632 (10 μM), 8-CPT-Me-cAMP (200 μM), and 6-Bnz-cAMP (200 μM).

In sets of experiments where PKI was used, the cells were preincubated with PKI for 60 min followed by the addition of relevant stimulators as stated in the figure legends.

2.4 Determination of MLC phosphorylation

The phosphorylation of MLC was determined by glycerol–urea polyacrylamide gel electrophoresis and western blot analysis as described previously[16] (see Supplementary material online).

2.5 Immunoprecipitation and western blot analysis

PP1 was immunoprecipitated using pre-immobilized PP1 antibody on protein G-coated magnetic beads as recently described.[10] The precipitated proteins were analysed by western blot analysis using antibodies against PP1 and CPI-17.

2.6 Protein phosphatase assay

The PP1 holoenzyme was immunoprecipitated from cell homogenates using anti-PP1 antibody and the phosphatase activity of the precipitate was determined as described recently.[10]

2.7 RhoA translocation

The activation of RhoA was assessed by analysing its translocation from the cytosolic to the particulate fraction by ultracentrifuge cell fractionation (see Supplementary material online).

2.8 Depletion of endogenous CPI-17

To reduce the content of CPI-17, endothelial cells were transfected with CPI-17-specific siRNA using FuGene® as described by Kolosova et al. (2004)[14] (see Supplementary material online).

2.9 Macromolecule permeability measurement

The macromolecule permeability of HUVEC monolayers cultured on polycarbonate filters was measured as described previously.[25]

2.10 Statistical analysis

Data are given as means ± SD of five experiments using independent cell preparations. The comparison of means between groups was performed by one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls post hoc test. Changes in parameters within the same group were assessed by multiple ANOVA analysis. Probability (P) values of less than 0.05 were considered significant.

3. Results

3.1 Activation of either PKA or Epac protects endothelial barrier function

To establish the relative contribution of PKA and Epac signalling pathways in the cAMP-mediated endothelial barrier protection, two pharmacological approaches were applied. First, two selective cAMP analogues, N^6-benzoyl-cAMP (6-Bnz) and 8-CPT-2Me-cAMP (8-CPT) were applied to specifically activate PKA or Epac,[26] respectively. For simultaneous stimulation of both cAMP signalling pathways FSK, a direct activator of adenylyl cyclase, was applied. Thrombin
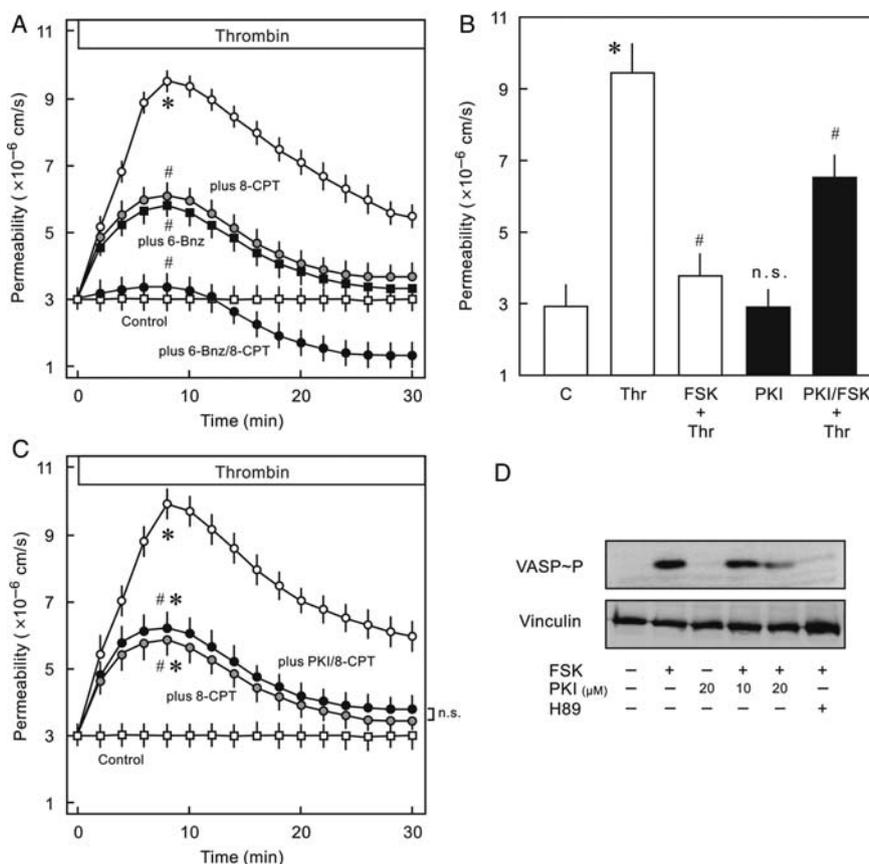
Figure 1 Effect of cAMP signalling on thrombin-induced hyperpermeability. (A) Endothelial monolayers were treated with thrombin (Thr; 0.2 U/mL), Epac activator 8-CPT-cAMP (8-CPT; 200 μM) plus Thr, PKA activator 6-Bnz-cAMP (6-Bnz; 200 μM) plus Thr, or vehicle (control) as indicated. Data are means ± SD of three separate experiments with independent cell preparations. *$P < 0.05$ vs. control; #$P < 0.05$ vs. Thr alone. (B) Endothelial monolayers were treated with Thr (0.2 U/mL), adenylyl cyclase activator forskolin (FSK; 5 μM) plus Thr, PKA inhibitor (PKI; 20 μM), FSK plus Thr plus PKI or vehicle (C; control) for 10 min, as indicated. The cells were incubated with PKI for 60 min before FSK plus Thr were added. Data are means ± SD of three separate experiments with independent cell preparations. *$P < 0.05$ vs. C; #$P < 0.05$ vs. Thr alone; n.s.: not significantly different from control. (C) Endothelial monolayers were treated with Thr, 8-CPT (200 μM) plus Thr (0.2 U/mL), 8-CPT plus Thr plus PKI (20 μM) or vehicle (control), as indicated. Data are means ± SD of three separate experiments with independent cell preparations. *$P < 0.05$ vs. C; #$P < 0.05$ vs. Thr alone. n.s.: not significantly different. (D) Effect of FSK, PKI, and H89, a pharmacological PKA inhibitor, on VASP phosphorylation at Ser157. Representative western blots of VASP phosphorylation. Endothelial cells were exposed to FSK (5 μM), PKI (20 μM), PKI (10 or 20 μM) plus FSK, H89 (10 μM) plus FSK or vehicle (C; control) for 10 min. The cells were incubated with PKI for 60 min or H89 for 30 min before FSK was added. The western blots are representative of three separate experiments with independent cell preparations.

was used to challenge endothelial barrier function. Thrombin alone induced a phasic increase in albumin permeability which peaked at 8–10 min and decreased towards basal level within approximately 60 min (*Figure 1A*). Addition of either 6-Bnz or 8-CPT reduced the thrombin-induced permeability increase by 60 or 55%, respectively. Simultaneous application of both agents had an additive effect, which was comparable with FSK. Secondly, the barrier protective effect of FSK was reduced by 55% when endothelial cells were preincubated with PKI, a specific cell-permeable PKA inhibitory peptide[27] (*Figure 1B*), further strengthening the concept that half of the FSK-mediated barrier protective effect is mediated by cAMP/PKA pathway. PKI alone had no significant effect on 8-CPT-mediated barrier protection (*Figure 1C*).

The inhibition of PKA by PKI was assessed by the phosphorylation state of vasodilator-stimulated phosphoprotein (VASP). VASP is phosphorylated by PKA at the specific phosphorylation site Ser157. PKI abolished the FSK-mediated VASP phosphorylation in a

concentration-dependent manner (*Figure 1D*). The effect of PKI was compared with that of H89, a pharmacological inhibitor of PKA. Approximately 80% of FSK-induced VASP phosphorylation was blocked by 20 μM PKI, a concentration found to be maximally effective on permeability (see above).

3.2 Activation of PKA but not Epac inactivates the endothelial contractile machinery

As endothelial contractile machinery is one of the major regulators of endothelial barrier function,[4] we analysed the effect of PKA and Epac activation on MLC phosphorylation. The effect of FSK was tested on MLC phosphorylation under basal conditions as well as under thrombin stimulation. As shown in *Figure 2A*, FSK caused a dephosphorylation of MLC, thrombin an increase in phosphorylation, and the combined addition of FSK plus thrombin abolished the thrombin effect.
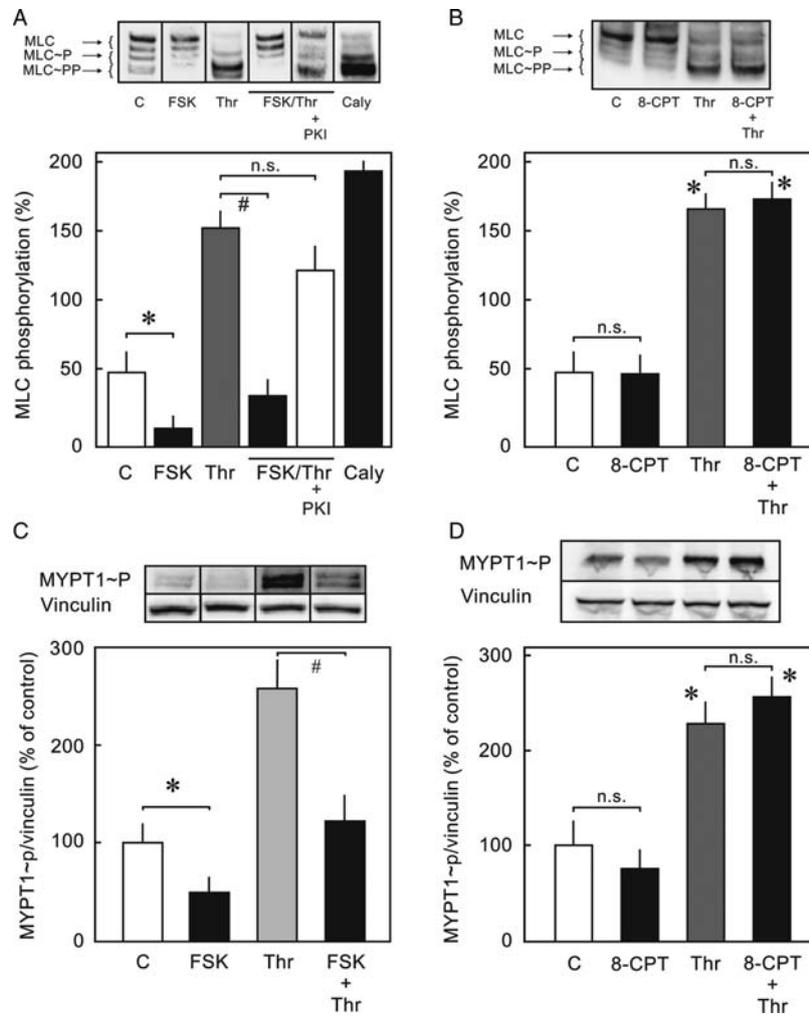
Figure 2 Effect of cAMP signalling on thrombin-induced MLC and MYPT1 phosphorylation. (*A*) Upper panel: representative western blots of MLC phosphorylation. Endothelial cells were exposed to FSK (5 μM), Thr (0.2 IU/mL), FSK plus Thr or vehicle (C; control) for 10 min. In a set of experiments, cells were incubated with PKI (20 μM) for 60 min before FSK plus Thr were added, as indicated. As a positive control 1 nM calyculin-A (Caly), a protein phosphatase inhibitor was added for 20 min. The bands represent, from top to bottom, non-(MLC), mono-(MLC $\sim$ P), and di-phosphorylated MLC (MLC $\sim$ PP), respectively. Lower panel: densitometric analysis of the western blots of MLC phosphorylation. As all MLC can become diphosphorylated, MLC phosphorylation varies between 0 and 200%. (*B*) Upper panel: representative western blot of MLC phosphorylation. Endothelial cells were exposed to 8-CPT (200 μM), Thr (0.2 IU/mL), 8-CPT plus Thr or vehicle (C; control) for 10 min. Lower panel: densitometric analysis of western blots of MLC phosphorylation. (*C*) Upper panel: representative western blots of MYPT1 phosphorylation at Thr850. Endothelial cells were treated with FSK (5 μM), Thr (0.2 IU/mL), FSK plus Thr or vehicle (C; control) for 10 min. Lower panel: densitometric analysis of western blots. MYPT1 phosphorylation relative to vinculin (loading control) is given as % increase compared with control. The ratio of control was set to 100%. (*D*) Upper panel: representative western blot of MYPT1 phosphorylation at Thr850. Endothelial cells were exposed to 8-CPT (200 μM), Thr (0.2 IU/mL), 8-CPT plus Thr or vehicle (C; control) for 10 min. Lower panel: densitometric analysis of western blots. Data are means $\pm$ SD of five separate experiments of independent cell preparations. *$P < 0.05$ vs. C; #$P < 0.05$; n.s.: not significantly different.

To test whether the effect of FSK on thrombin-induced MLC phosphorylation is mediated via PKA, endothelial cells were preincubated for 60 min with PKI (20 μM). PKI abolished the effect of FSK on thrombin-induced MLC phosphorylation, whereas PKI applied alone did not change the basal MLC phosphorylation (data not shown). The role of Epac on MLC phosphorylation was analysed by using the Epac activator 8-CPT (200 μM). 8-CPT did neither affect basal nor thrombin-induced MLC phosphorylation (*Figure 2B*). These results indicate that the cAMP/PKA pathway but not cAMP/Epac pathway is involved in the regulation of endothelial contractile machinery.

3.3 FSK but not Epac antagonizes thrombin-induced MYPT1 phosphorylation and RhoA activation

The dephosphorylation of MLC is triggered by MLCP activation. The activity of MLCP is regulated by the phosphorylation state of its regulatory subunit MYPT1 at threonine 850 (Thr850), therefore, the phosphorylation of MYPT1 was analysed. FSK caused dephosphorylation of MYPT1 at Thr850 under basal as well as under thrombin stimulated condition (*Figure 2C*). In contrast, Epac activator 8-CPT did neither

affect basal nor thrombin-induced MYPT1 phosphorylation (*Figure 2D*), indicating that the cAMP/Epac pathway is not involved.

MYPT1 is directly phosphorylated by Rock, a downstream effector of RhoA. Therefore, it was analysed whether FSK affects translocation of RhoA. To accomplish that particulate fractions were prepared from cell lysates and probed for RhoA by western blot analysis. FSK reduced the amount of RhoA in the particulate fraction to half of the control value (*Figure 3A*). Thrombin increased the translocation of RhoA to the particulate fraction by 1.8-fold compared with control. This thrombin effect was abolished by FSK. When, instead of FSK, the Epac activator 8-CPT was applied, the thrombin effect was not antagonized (data not shown).

3.4 FSK activates MLCP catalytic subunit PP1

Since stimulation of endothelial cells with FSK but not Epac activator resulted in dephosphorylation of endothelial MLC and MYPT1, we analysed the activation of MLCP by FSK. Endothelial cells were exposed to FSK, thrombin, or FSK plus thrombin and MLCP holoenzyme was immunoprecipitated using an antibody against PP1, the catalytic subunit. The MLCP activity was measured by using an *in vitro* enzyme assay. FSK increased MLCP activity to 165%, thrombin decreased the MLCP activity to 60% compared with control, while FSK completely abolished the thrombin effect on MLCP activity (*Figure 3B*). The MLCP activity of both control- and FSK-treated immunoprecipitates was completely blocked by the addition of 0.5 μM of the recombinant inhibitor 2, a PP1-specific inhibitor, indicating that the phosphatase activity is solely due to PP1.

3.5 Effect of FSK and thrombin on CPI-17 phosphorylation and CPI-17/PP1 complex formation

CPI-17 is one of the well-known endogenous regulators of PP1 activity in smooth muscle cells[8,13] and has also been identified in endothelial cells.[14] Here it was analysed whether CPI-17 is expressed in HUVEC. As confirmed by western blot analysis, CPI-17 content is unchanged in cultured HUVEC up to passage 2, when compared with freshly isolated HUVEC (*Figure 4A*). In the experiments, only passage 1 cultures were used.

The activation of CPI-17 is dependent on its phosphorylation at Thr38. When phosphorylated, it can directly interact with PP1 and inactivate it. Therefore, in the first step, it was analysed whether FSK can decrease CPI-17 phosphorylation. FSK caused a dephosphorylation of CPI-17 at Thr38 by 50% compared with control within 10 min (*Figure 4B*), whereas thrombin increased CPI-17 phosphorylation to 150%. FSK abrogated this thrombin effect on CPI-17 phosphorylation. Next, the influence of FSK and thrombin on CPI-17/PP1 interaction was analysed. Under basal conditions, CPI-17 is co-immunoprecipitated with PP1 (*Figure 4C*). Exposure of endothelial cells to FSK reduced this co-immunoprecipitation of CPI-17 with PP1 by 0.5-fold within 10 min. This reduction was comparable to the increase in PP1 activity (*Figure 3B*). Thrombin caused a 1.7-fold increase in co-immunoprecipitation of CPI-17 with PP1, while simultaneous addition of FSK plus thrombin completely abolished thrombin-induced CPI-17/PP1 complex.
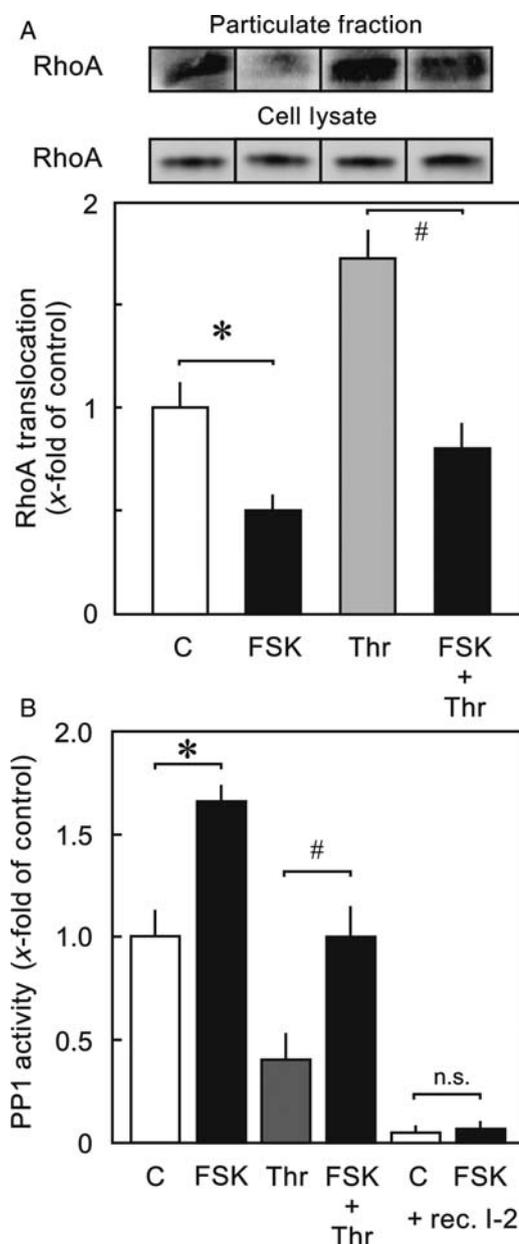

Figure 3 Effect of FSK and thrombin on RhoA translocation and PP1 activity. (*A*) Upper panel: representative western blots of RhoA of particulate fraction and whole cell lysate. Endothelial cells were treated with FSK (5 μM), Thr (0.2 IU/mL), FSK plus Thr or vehicle (C; control) for 10 min. Particulate fractions from equal amounts of cell lysates were prepared and analysed by western blot analysis using an anti-RhoA antibody. Lower panel: densitometric analysis of western blots. RhoA in the particulate fraction relative to total RhoA is given as *x*-fold increase compared to control. The ratio of control was set to 1. (*B*) Endothelial cells were exposed to FSK (5 μM), Thr (0.2 IU/mL), FSK plus Thr or vehicle (C; control) for 10 min. PP1 was immunoprecipitated using an anti-PP1 antibody and the activity of immunoprecipitated phosphatase was measured in the absence or presence of 0.5 μM recombinant inhibitor 2 (rec. I-2). PP1 activity is given as *x*-fold increase compared with control. The mean of PP1 activity of control was set to 1. Data are means ± SD of three separate experiments with independent cell preparations. *$P < 0.05$; #$P < 0.05$; n.s.: not significantly different.
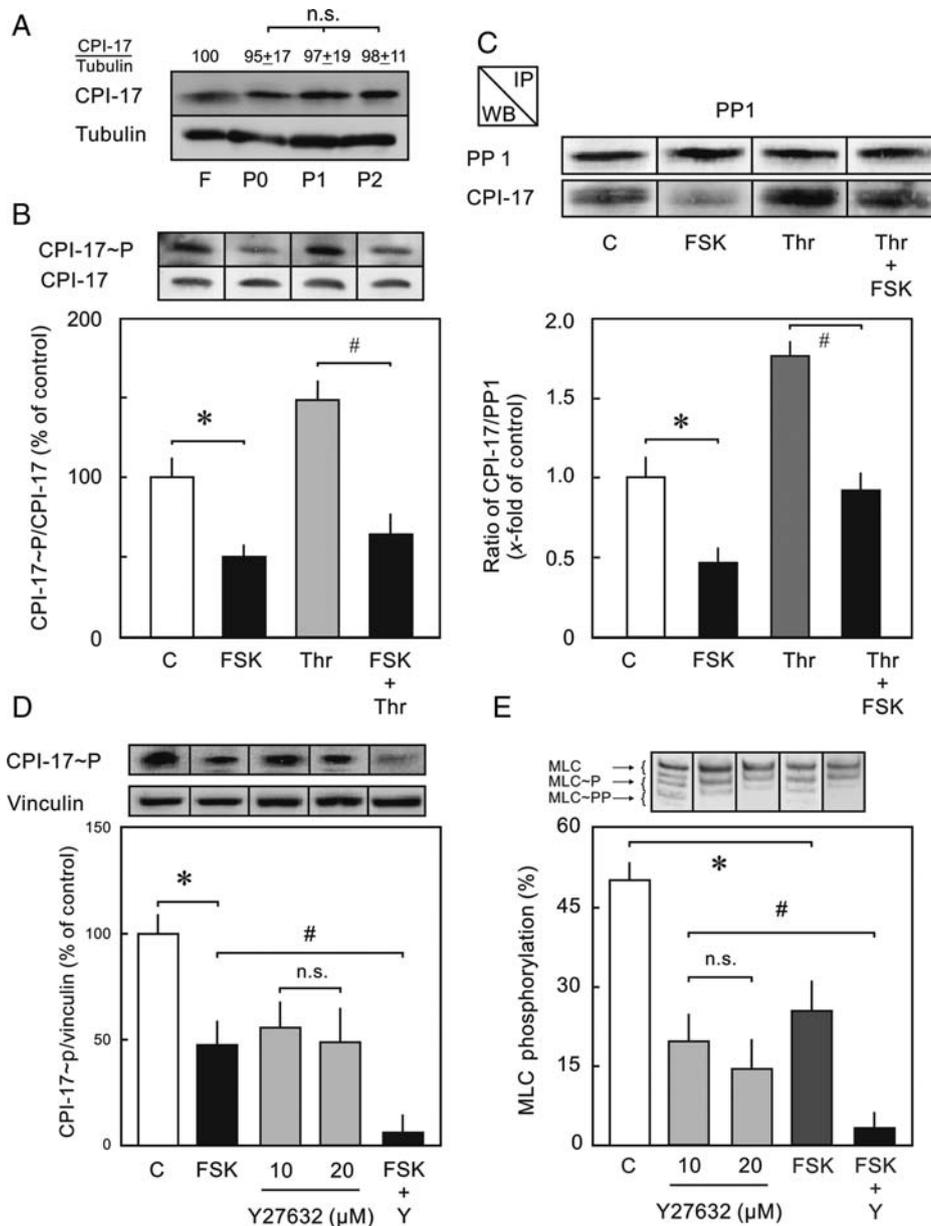
Figure 4 Effect of FSK and thrombin on CPI-17 phosphorylation and CPI-17/PP1 complex formation. (*A*) CPI-17 content in freshly isolated and passaged HUVEC. Cell lysates of HUVEC, from freshly isolated (F), primary, passage 1 and 2 (P0, P1, P2) were analysed by western blot. Representative western blots of CPI-17 and tubulin as loading control and densitometric analysis of western blots. CPI-17 relative to tubulin is given as % increase compared with F. The ratio of F was set to 100%. (*B*) Upper panel: representative western blots of Thr38-phosphorylated of CPI-17. Endothelial cells were exposed to FSK (5 μM), Thr (0.2 U/mL), FSK plus Thr or vehicle (C; control) for 10 min. The same blot was striped and reblotted against pan anti-CPI-17 antibody for loading control. Lower panel: densitometric analysis of western blots. CPI-17 phosphorylation relative to total CPI-17 is given as % of control. The ratio of control was set to 100%. (*C*) Upper panel: representative western blots of PP1 and CPI-17 co-immunoprecipitated with PP1. Endothelial cells were exposed to FSK (5 μM), Thr (0.2 U/mL), FSK plus Thr or vehicle (C; control) for 10 min. PP1 was immunoprecipitated using an anti-PP1 antibody and analysed by western blot. Lower panel: densitometric analysis of western blots. CPI-17 relative to PP1 is given as *x*-fold increase compared with control. The ratio of control was set to 1. Data are means ± SD of five separate experiments with independent cell preparations. *$P < 0.05$; #$P < 0.05$; n.s.: not significantly different. (*D* and *E*) Effects of FSK or Rho kinase inhibitor Y27632 on CPI-17 and MLC phosphorylation. Endothelial cells were exposed to FSK (5 μM), Y27632 (Y; 10 or 20 μM), FSK plus Y (10 μM), or vehicle (C; control) for 10 min. (*D*) Upper panel: representative western blots of Thr38 phosphorylated of CPI-17. Lower panel: densitometric analysis of western blots. CPI-17 phosphorylation relative to vinculin is given as % of control. The ratio of control was set to 100%. (*E*) Upper panel: representative western blots of phosphorylated MLC. The bands represent, from top to bottom, non- (MLC), mono- (MLC~P), and di-phosphorylated MLC (MLC~PP), respectively. Lower panel: densitometric analysis of the western blots. Data are means ± SD of five separate experiments with independent cell preparations. *$P < 0.05$; #$P < 0.05$; n.s: not significantly different.

3.6 Effect of FSK on CPI-17 phosphorylation is both Rock dependent and Rock independent

As cAMP signalling inactivates the RhoA/Rock pathway, it was analysed whether FSK-induced dephosphorylation of CPI-17 is due to an inhibition of RhoA/Rock pathway. Y27632 was used to inhibit Rock. At 10 μM, a concentration that causes maximum effect on MLC dephosphorylation, Y27632 reduced CPI-17 phosphorylation to approximately 50% after 10 min (*Figure 4D*). Addition of 20 μM, Y27632 did not further reduce CPI-17 phosphorylation significantly. However, simultaneous addition of FSK (10 μM) plus 10 μM Y27632 decreased CPI-17 phosphorylation to almost zero. This indicates that CPI-17 is regulated by both Rock-dependent and Rock-independent pathways which are, at least partially, inactivated by cAMP signalling.

3.7 Effect of FSK on MLC phosphorylation is both Rock dependent and Rock independent

The question was addressed whether the dephosphorylating effect of cAMP signalling on MLC can be attributed entirely to interference with the MLCP inhibitory RhoA/Rock pathway. At the maximum effective concentration of 10 μM, the Rock inhibitor Y27632 reduced the basal state of MLC phosphorylation to 20% (*Figure 4E*). Addition of 20 μM Y27632 did not further reduce MLC phosphorylation. However, simultaneous addition of FSK plus Y27632 entirely suppressed MLC phosphorylation. These data indicate that the effect of cAMP signalling on MLC phosphorylation is, in part, independent of the RhoA/Rock pathway. As a corollary, it follows that MLCP activation under cAMP signalling must be due to at least in part, mechanisms independent of RhoA/Rock signalling.

3.8 Effect of CPI-17 downregulation on endothelial permeability

To analyse directly the role of CPI-17 in endothelial barrier function, the content of endogenous CPI-17 was reduced by siRNA-mediated gene silencing. Treatment of endothelial cells with CPI-17-specific siRNA caused a 70% reduction in the amount of CPI-17 protein (*Figure 5A*). Silencing of CPI-17 did not affect the cellular PP1 content. The maximum effect of thrombin on permeability in CPI-17-silenced cells after 10 min was reduced by 40% compared with the corresponding effect on endothelial cells treated with control siRNA (*Figure 5B*). CPI-17 depletion did not significantly affect the basal permeability. To further analyse which cAMP signalling wing is involved in endothelial barrier protection via CPI-17, permeability experiments were performed in CPI-17 downregulated cells in the presence of either PKA or Epac activation. If PKA activation modulates the endothelial contractile machinery via CPI-17, its downregulation should abolish the protective effect of PKA on thrombin-induced hyperpermeability. Accordingly, the PKA activator, 6-Bnz (200 μM), failed to reduce the thrombin effect on permeability (*Figure 5C*). In contrast, the Epac activator, 8-CPT lowered the thrombin-induced hyperpermeability under the same conditions. This indicates that the permeability lowering effect elicited by cAMP/PKA pathway is CPI-17 dependent, whereas that of cAMP/Epac pathway is CPI-17 independent.
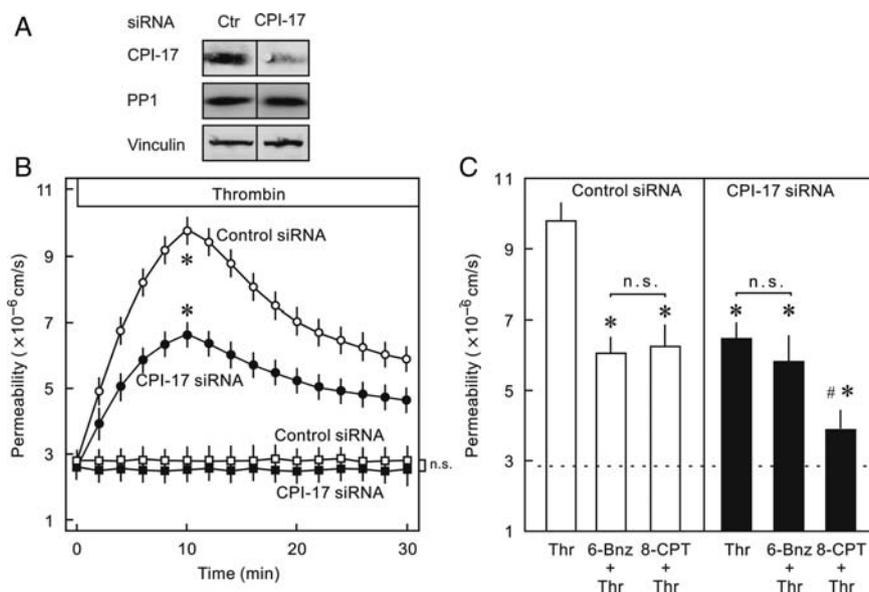

Figure 5 Effect of 6-Bnz and 8-CPT on thrombin-induced increase in macromolecule permeability in CPI-17 downregulated endothelial monolayers. (*A*) Representative western blots of CPI-17 in CPI-17 siRNA (CPI-17) and control siRNA (Ctr)-treated endothelial cells. Vinculin was used as loading control. (*B*) Endothelial monolayers, treated with CPI-17 siRNA or control siRNA, were exposed to Thr (0.2 U/mL). Data are means ± SD of five separate experiments with independent cell preparations. *$P < 0.05$, control siRNA vs. CPI-17 siRNA; n.s: not significantly different. (*C*) Endothelial cell treated with CPI-17 siRNA or control siRNA as in (*B*) were exposed to Thr (0.2 IU/mL), 8-CPT (200 μM) plus Thr, or 6-Bnz (200 μM) plus Thr, for 10 min as indicated. The dotted line shows the basal levels of endothelial permeability treated with control siRNA. *$P < 0.05$ vs. Thr (control siRNA); [#]$P < 0.05$ 8-CPT plus Thr (control siRNA) vs. 8-CPT plus Thr (CPI-17 siRNA); n.s.: not significantly different.

4. Discussion

The endothelial contractile machinery is one of the major determinants of endothelial barrier function. This study investigated the relative role of cAMP effectors, PKA, and Epac in the regulation of this mechanism. The novel findings are: (i) cAMP signalling via PKA but not via Epac inactivates the endothelial contractile machinery via activation of MLCP, (ii) this MLCP activation occurs via inhibition of CPI-17, endogenous inhibitor of the catalytic subunit PP1, by dephosphorylation and induction of its dissociation from PP1, thus increasing the free pool of active PP1. This inhibition of CPI-17 is partly Rock dependent and partly Rock independent, and (iii) down-regulation of CPI-17 by siRNA attenuates the thrombin-induced hyperpermeability.

Accumulating evidence[18,19,28,29] suggests that elevation of the intracellular cAMP level results in increased endothelial barrier integrity and efficiently counter regulates endothelial hyperpermeability induced by a variety of agents. Among various physiological agonists are the β-adrenergic agents,[16] prostaglandins,[23] and adrenomedullins,[30] which via G protein-coupled receptors, activate adenylyl cyclase leading to increased intracellular cAMP levels.

cAMP may exert its endothelial barrier stabilizing effect both via activation of PKA and Epac.[19,22,23,29] In accordance to Cullere *et al.*,[21] we here show that the activation of Epac antagonizes the thrombin effect by 50%. Likewise, activation of PKA antagonized thrombin effect by 60%. Accordingly, simultaneous activation of both pathways has an additive effect on barrier stabilization, which is comparable to the effects of FSK, a direct activator of adenylyl cyclase. In accordance, inhibition of PKA could only partially block the FSK effect and did not interfere with the Epac-mediated stabilization of endothelial barrier function, indicating Epac mediates its effect independent of PKA. This explains that PKA and Epac partly stabilize endothelial barrier independent of each other.

Inflammatory mediators like thrombin, histamine, and platelet-activating factor (PAF) activate a repertoire of signalling mechanisms in endothelial cells leading to vascular leakage. In cultured cells, thrombin activates RhoA/Rock pathway via its receptor PAR1.[11] This leads to the inactivation of myosin phosphatase, phosphorylation of MLC, and actin–myosin-driven cell contraction, which contributes to thrombin-induced barrier dysfunction. A similar mechanism has been reported for VEGF- and neutrophil-induced vascular leakage.[31,32] However, there exist also other mechanisms in which Rho/Rock-induced contractile inactivation seems not to play an important role in mediating the hyperpermeability response. Recent reports show that bradykinin and PAF-induced vascular leakage in rat and mouse mesentery venules results from disruption of endothelial adherens junctions without interfering with the RhoA/Rock pathway.[33−35] Similarly, Göggel and Uhlig[36] show that PAF-mediated lung oedema results independent of RhoA/Rock activation. However, PAF-induced lung oedema was in part attenuated by MLCK-inhibitor, ML-7.[36] Similarly, ML-7 induced a fast recovery of the barrier in PAF-challenged venules.[34] In these cases, other mechanisms involving loss of adherens junctions may be more important than RhoA/Rock-mediated contractile activation.

Activation of cAMP signalling leads to the inhibition of endothelial contractile machinery and protects against agonist-induced loss of barrier function.[18,29,35] It is evident from recent studies that cAMP-mediated cell adhesion and spreading is regulated via both PKA and Epac,[23,24] but not in all cell types to the same extent. In macrovascular

endothelial cells, PKA has been found dominant in regulating cell adhesion and spreading compared with microvascular cells, where both PKA and Epac have been found responsible.[24] A role of Epac in regulating the endothelial contractile machinery is not established. Stimulation of endothelial cells with FSK induced a rapid dephosphorylation of MLC which was abolished by a specific PKA inhibitor, indicating a PKA-dependent effect of cAMP. Conversely, specific pharmacological activator of Epac had no effect on basal as well as thrombin-induced MLC phosphorylation, supporting the concept that it is PKA but not Epac via which cAMP mediates contractile inactivation.

It has previously been shown that Rock can phosphorylate MYPT1 leading to the inhibition of MLCP.[7] Using a site-specific antibody against phospho-Thr850-MYPT1, we demonstrate that cAMP caused MYPT1 dephosphorylation under basal conditions as well as thrombin stimulation. Likewise, cAMP inactivated the upstream RhoA under both conditions. These data are in line with previous reports, both from us and others[16,29] showing that cAMP signalling inactivates RhoA and activates MLCP. Contrariwise, Epac activation neither dephosphorylated MYPT1 nor inactivated RhoA, suggesting a role of PKA and not Epac in the regulation of endothelial contractile machinery. These data clearly demonstrate that Epac is not involved in the regulation of endothelial contractile machinery.

In addition, while dissecting the molecular mechanism of PKA-mediated MLCP activation, we show that the activity of the MLCP complex is also controlled by CPI-17, a specific endogenous inhibitor of PP1.[8] Initially, it was assumed that this inhibitor peptide plays a role only in smooth muscle cells. But Kolosova *et al.*[14] recently showed that overexpression of CPI-17 in lung microvascular endothelial cells lead to enhanced effects of agonist-induced cytoskeleton rearrangement. We here show, for the first time, that CPI-17 plays a functional role as a modulator of contractile machinery-mediated barrier property. Co-immunoprecipitation experiments demonstrate that CPI-17/PP1 complexes are formed in endothelial cells. Activation of the cAMP pathway led to dissociation of the CPI-17/PP1 complex, with a corresponding increase in PP1 activity. Thrombin induced CPI-17/PP1 complex formation and a corresponding decrease in PP1 activity. This complex formation and MLCP inactivation was effectively blocked by cAMP signalling. Phosphorylation of CPI-17 at Thr38 has been reported to induce its inhibitory activity towards PP1.[12] In the present study, FSK reduced the basal CPI-17 phosphorylation by 50%. This dephosphorylation corresponded with a decrease in CPI-17/PP1 complex formation to the same extent and a 1.6-fold increase in PP1 activity. Contrariwise, thrombin induced a 50% increase in CPI-17 phosphorylation corresponding to 1.6-fold increase in CPI-17/PP1 complex formation and a 0.5-fold reduction in PP1 activity. The nature of the protein phosphatase involved being PP1 was identified by use of a recombinant-specific inhibitor protein-2, which blocked the activity of the MLCP completely. Identification of PP1 is in line with previous investigations on MLCP in endothelial cells.[10]

To this point, the results left the question open whether the effect of the cAMP pathway on CPI-17 phosphorylation is due to inhibition of the RhoA/Rock pathway. At maximum effective concentrations, the Rho kinase inhibitor Y27632 induced a partial dephosphorylation of CPI-17 to 60% of the basal level. Presence of Y27632 plus FSK led to virtually complete dephosphorylation of CPI-17. This indicates the existence of at least two mechanisms regulating the phosphorylation state of CPI-17. One is Rock dependent and the other is
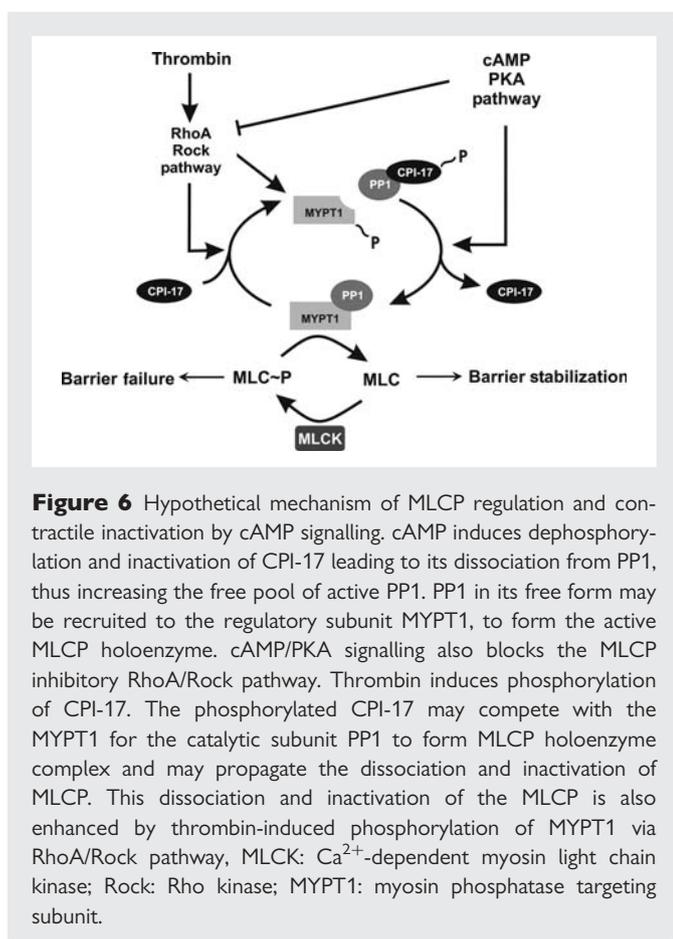
Figure 6 Hypothetical mechanism of MLCP regulation and contractile inactivation by cAMP signalling. cAMP induces dephosphorylation and inactivation of CPI-17 leading to its dissociation from PP1, thus increasing the free pool of active PP1. PP1 in its free form may be recruited to the regulatory subunit MYPT1, to form the active MLCP holoenzyme. cAMP/PKA signalling also blocks the MLCP inhibitory RhoA/Rock pathway. Thrombin induces phosphorylation of CPI-17. The phosphorylated CPI-17 may compete with the MYPT1 for the catalytic subunit PP1 to form MLCP holoenzyme complex and may propagate the dissociation and inactivation of MLCP. This dissociation and inactivation of the MLCP is also enhanced by thrombin-induced phosphorylation of MYPT1 via RhoA/Rock pathway, MLCK: Ca^{2+}-dependent myosin light chain kinase; Rock: Rho kinase; MYPT1: myosin phosphatase targeting subunit.

independent of it. Similarly, MLC phosphorylation, is also in part controlled in a Rock-dependent and in part Rock-independent manner.

Finally, experiments using siRNA directed against CPI-17 showed a functional role of CPI-17 in agonist-induced barrier failure. Downregulation of CPI-17 reduced the thrombin effect on permeability by one-third. This supports the notion that CPI-17 is an important but not the only mediator of agonist-induced activation of endothelial contractile machinery and barrier dysfunction. Activation of PKA in CPI-17 downregulated cells failed to reduce permeability, suggesting that cAMP-mediated CPI-17 inactivation is PKA dependent. However, activation of Epac alone in CPI-17 downregulated cells had an additive effect on Epac-mediated reduction in permeability. This indicates that the PKA wing of the cAMP signalling regulates the endothelial barrier function via contractile inactivation, although the involvement of other mechanisms is also evident. However, the Epac wing regulates the barrier function without affecting the contractile machinery and without interfering with PKA signalling, as indicated by the fact that PKI could not blunt the Epac effect on permeability.

Thus the robust action of cAMP signalling pathways on endothelial permeability is based on two factors: (i) it inactivates the contractile machinery and relaxes the endothelial cells releasing the centripetal force provided by the tense endothelial contractile machinery, mainly via PKA activation[6,16] and (ii) it strengthens the cell–cell adhesions by the stabilization of adherens junctions via Epac pathway.[22,23]

Figure 6 summarizes the results of this study and previous reports and depicts a potential mechanism of cAMP/PKA-mediated activation of MLCP and inactivation of CPI-17. Thrombin activates RhoA/Rock which inactivates MLCP by activating the inhibitory CPI-17 and phosphorylating MYPT1 at its inhibitory sites. cAMP can effectively antagonise this thrombin effect via inhibiting both RhoA/Rock pathway and CPI-17.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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