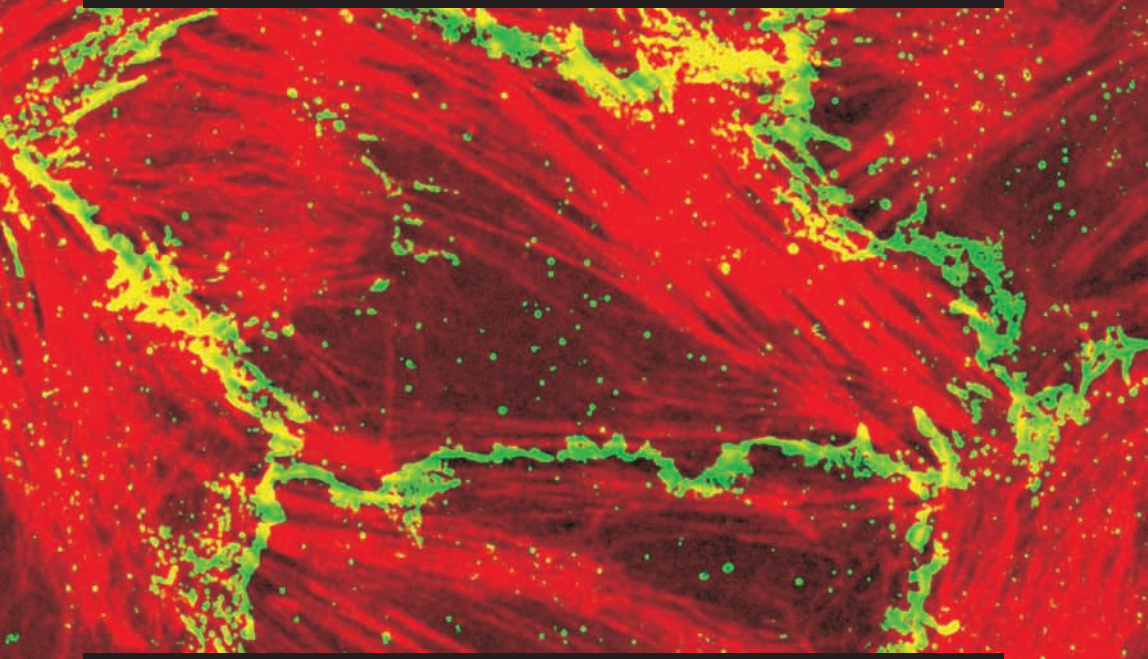
EFFECT OF CAMP/PKA SIGNALING MECHANISM ON BARRIER FUNCTION OF CULTURED ENDOTHELIAL CELLS:

ROLE OF MYOSIN LIGHT CHAIN PHOSPHATASE

MUHAMMAD ASLAM



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vorgelegt von

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Gießen 2007

Tag der Disputation:

Friday, September 28, 2007. 14:00 hrs.

Gutachter: Prof. Dr. W. Clauß (Fachbereich Biologie und Chemie)

Gutachter: Priv. Doz. Dr. T. Noll (Fachbereich Humanmedizin)

In the Name of Allah (God),

the Most Gracious, the Most Merciful

Proclaim! (or read!) in the name of thy Lord and Cherisher, Who created- Created man, out of a (mere) clot of congealed blood: Proclaim! And thy Lord is Most Bountiful,- He Who taught (the use of) the pen,- Taught man that which he knew not.

Man We did create from a quintessence (of clay); Then We placed him as (a drop of) sperm in a place of rest, firmly fixed; Then We made the sperm into a clot of congealed blood; then of that clot We made a (foetus) lump; then we made out of that lump bones and clothed the bones with flesh; then we developed out of it another creature. So blessed be Allah, the best to create! After that, at length ye will die. Again, on the Day of Judgment, will ye be raised up.

(Al-Quran)

To

My Mother

LIST OF ABBREVIATIONS

AC	Adenylyl cyclase
app.	Approximately
APS	Ammonium per sulfate
ATP	Adenosine-5-triphosphate
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	3'-5'-cyclic adenosine monophosphate
cGMP	3'-5'-cyclic guanosine monophosphate
CPI-17	PKC-potentiated inhibitor 17-kDa protein
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EC	Endothelial cells
ECGS	Endothelial cell growth supplement
ECL	Enhanced chemiluminescence
EC-MLCK	Endothelial cell myosin light chain kinase
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
F-actin	Filamentous actin
FCS	Fetal calf serum
FSK	Forskolin
GPCR	G-protein-coupled receptors
G-actin	Globular actin
HBSS	Hanks' balanced salt solution
hEGF	Human epidermal growth factor
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HUVEC	Human umbilical vein endothelial cells
IU	International unit
KCl	Potassium chloride

KH_2PO_4	Potassium dihydrogen phosphate
ILK	Integrin linked kinase
kDa	Kilo dalton
$MgCl_2$	Magnesium chloride
min	Minutes
MLC	Myosin light chain
MLC~P	Monophosphorylated myosin light chain
MLC~PP	Diphosphorylated myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
$MnCl_2$	Manganese chloride
MYPT1	Myosin phosphatase targeting subunit 1
NaCl	Sodium chloride
NaF	Sodium fluoride
Na_2HPO_4	Di-sodium hydrogen phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
Na-orthovanadate	Sodium orthovanadate
NP-40	Nonidet P-40
PAK	p21-activated kinase
PAR1	Protease-activated receptor-1
PBS	Phosphate-buffered saline
pH	Negative log of H^+ concentration
PKA	Protein kinase A
PKC	Protein kinase C
PKI	Protein kinase A inhibitor
PMA	Phorbol-12-myristate-13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PP1	Protein phosphatase 1
rec. I-2	Recombinant inhibitor 2
Rhotekin-RBD	Rho-binding domain of rhotekin
Rock	RhoA-dependent kinase
S19	Serine 19
SDS	Sodium dodecyl sulfate
SMC	Smooth muscle cells

SMC-MLCK	Smooth muscle cell myosin light chain kinase
Soln.	Solution
T18	Threonine 18
T38	Threonine 38
T696	Threonine 696
T850	Threonine 850
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
Thr	Thrombin
TEMED	N, N, N', N',-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
VE-cadherin	Vascular endothelial cadherin
% vol/vol	Volume by volume percentage
% wt/vol	Weight by volume percentage
ZIPK	Zipper-interacting protein kinase

1 INTRODUCTION

1.1 Barrier function of the vascular endothelium

The vascular endothelium acts as a semi-permeable barrier between the vascular lumen and the interstitial spaces and extends over a wide surface area. It controls the passage of ions, solutes, macromolecules and leukocytes across the vessel wall. It is well known that loss of this barrier function leads to extravasation of blood components and may finally result in edema formation (Mehta and Malik, 2006; Bazzoni, 2006).

The endothelial barrier function is maintained by an equilibrium of competing contractile and adhesive forces generated by the actomyosin cytoskeleton and adhesive molecules located at cell-cell and cell-matrix contacts. Endothelial cells are tightly connected with each other via interactions of adherens and tight junctional proteins of adjacent cells. These proteins are linked to the cortical actin cytoskeleton present directly under the cell membrane (Furuse et al., 1994; Ben-Ze'ev and Geiger, 1998; Vleminckx and Kemler, 1999). Inflammatory mediators like thrombin cause activation of the contractile apparatus, derangement of the actomyosin cytoskeleton, and loss of cell adhesions. This results in barrier failure, increased macromolecule extravasation, and edema formation in the inflamed tissue (Lum and Malik, 1996; van Hinsbergh 1997; Wojciak-Stothard et al., 1998).

The present study focuses on the role of the endothelial contractile machinery in regulating endothelial barrier function under pathophysiological inflammatory conditions, during which activation of endothelial cells leads to edema formation and organ failure. It is well documented that agents which counteract contraction in smooth muscle cells can also reduce endothelial permeability (van Hinsbergh and van Nieuw Amerongen, 2002). In accordance, maneuvers increasing the intracellular levels of cyclic adenosine monophosphate (cAMP) can counteract imminent barrier failure induced by inflammatory mediators like thrombin (Qiao et al., 2003). Presently, the molecular mechanisms of this barrier protection are not completely understood. Thrombin activates the contractile machinery of endothelial cells mainly via inhibition of the myosin light chain phosphatase (MLCP) and activation of myosin light chain kinase (MLCK)

leading to phosphorylation of the small regulatory myosin light chains (MLC) (Zhao and Davis, 1999; Gündüz et al., 2003), the key regulatory element of the contractile machinery. Activation of the cAMP-dependent protein kinase A (PKA) pathway leads to activation of MLCP in smooth muscle cells (Azam et al., 2007) as well as in endothelial cells (Bindewald et al., 2004) which leads to dephosphorylation of MLC. In endothelial cells, dephosphorylation of MLC goes along with inactivation of the contractile machinery and this causes stabilization of the endothelial barrier (Tinsley et al., 2004).

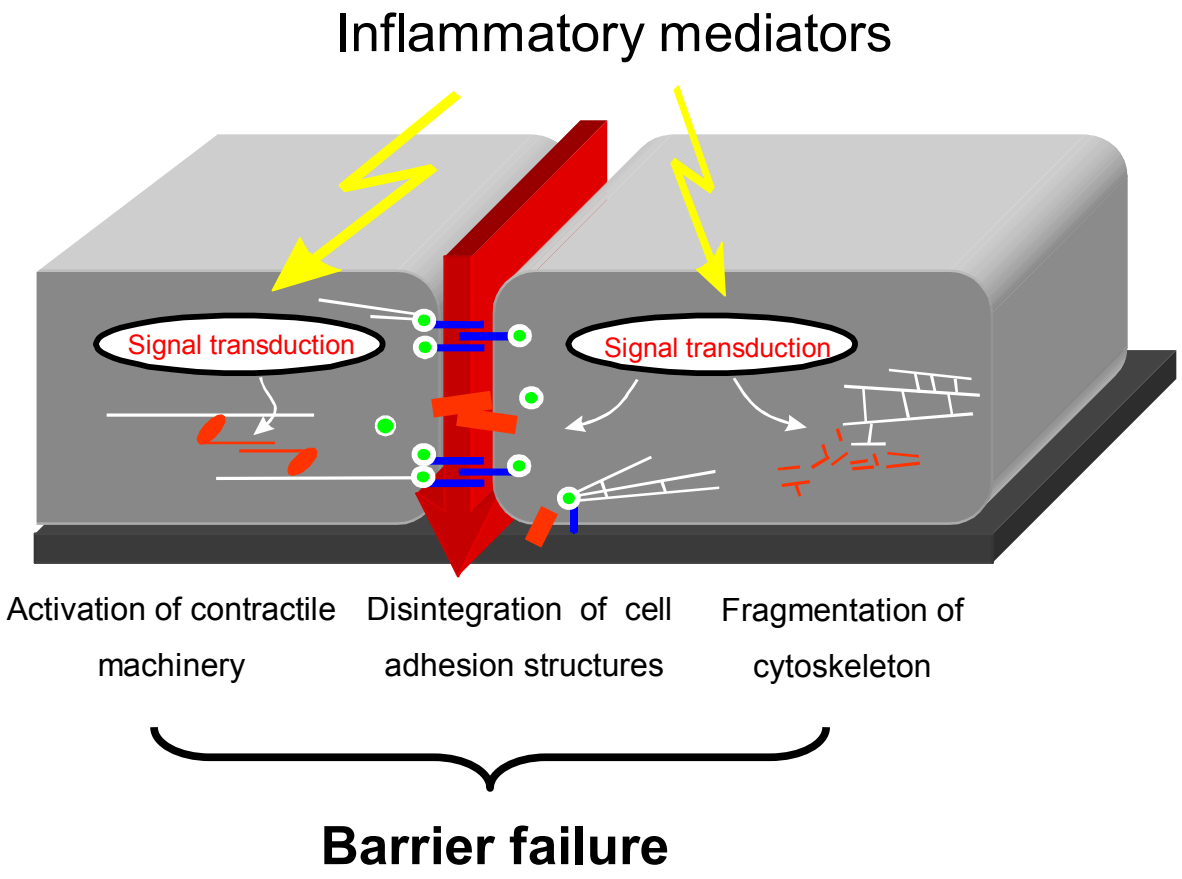

Fig. 1.1 Schematic presentation of the mechanism of endothelial barrier failure induced by inflammtory mediators. Mediators like thrombin cause failure of endothelial barrier function mediated by intracellular signal transduction mechanism. Effectors of these mechanisms are: the contractile machinery, cell-cell and cell-matrix adhesion structures and the actin cytoskeleton.

The main objective of the present study was to elucidate the mechanism of cAMP/PKA-mediated activation of MLCP as an important molecular target for endothelial barrier protection. The study was performed with a well-established *in vitro* cell culture model, using monolayers of human umbilical vein endothelial cells (HUVEC). Thrombin was used as an inflammatory mediator to simulate the *in vivo* state of hyperpermeability while forskolin (FSK), a direct activator of adenylyl cyclase, was used to activate the cAMP/PKA pathway.

1.2 Endothelial actomyosin cytoskeleton

Like other eukaryotic cells, endothelial cells possess a functional cytoskeleton consisting of actin and myosin filaments. The first direct evidence of endothelial cell contraction, in response to permeability-increasing agonists, was given by Majno and co-workers (1961a; 1961b). Using electron microscopy, they demonstrated endothelial cell contraction and gap formation in intact capillaries exposed to inflammatory mediators. A primary function of the contractile apparatus in endothelial cells is to regulate endothelial barrier integrity. Endothelial cell-cell adhesion and thus barrier integrity is mainly dependent on the actin cytoskeleton. Disruption of actin cytoskeleton by C2 toxin and cytochalasin D led to loss of endothelial cell-cell and cell-matrix contacts and detachment of cells from the substratum (Schnittler et al., 2001), whereas phallacidin, an actin stabilizer, prevented agonist-mediated barrier dysfunction (Phillips et al., 1989).

In endothelial cells actin and myosin filaments represent ~16% of total cellular protein (Wong and Gotlieb, 1990). Actin comprises about 5% of the total protein and exists in two different forms: in a filamentous form, called F-actin, and in a monomeric form, called G-actin (Tobacman and Korn, 1983). Actin filaments are dynamic structures, and the shift between the monomeric and the polymeric form of this protein plays a central role in several cell functions, especially in cell contraction and migration. In endothelial cells, about half of the actin is present in F-actin form, and half in G-actin form. Stress fibers are composed of bundles of F-actin and myosin filaments and are the primary elements of the contractile machinery of endothelial cells (Dudek and Garcia, 2001). Inhibition of actin polymerization by cytochalasin D or latrunculin leads to actin depolymerisation and abrogates the contractile response to inflammatory mediators (Goeckeler and

Wysolmerski, 1995; Moy et al., 1996, Mehta et al., 2002). Stimulation of endothelial cells with thrombin increases polymerization of actin filaments, as determined by the conversion of G-actin to F-actin (Thurston and Turner, 1994; Ehringer et al., 1999) reduces cortical actin content and leads to reorganization of actin to form stress fibers (Goeckeler and Wysolmerski, 1995; Ehringer 1999, van Nieuw Amerongen et al., 2000a; 2000b). This increase in stress fiber formation leads to a change in endothelial cell shape (Vouret-Craviari et al., 1998), which is an important factor in increased endothelial gap formation.These results provide a concept that actin polymerization plays a key role in induction of endothelial contraction.

1.3 Endothelial contractile machinery

Endothelial contractile machinery consists of actin and myosin filaments, the activation of which is mainly regulated by the phosphorylation state of the regulatory MLC. The phosphorylation state of MLC is precisely regulated by balanced activities of MLCK and MLCP. Thus, the major components of endothelial contractile machinery are actin-myosin filaments, MLC, MLCK and MLCP.

1.3.1 Myosin light chains

MLC is a 20 kDa small protein and an important determinant of the state of contractile activation in endothelial cells. It was shown that phosphorylation of serine 19 (S19) (monophosphorylation) and/or threonine 18 (T18) (diphosphorylation) of the regulatory MLC not only increases actomyosin ATPase activity, but also shifts the equilibrium from the folded to unfolded myosin forms (Kamisoyama et al., 1994), thus providing the assembly and function of the contractile apparatus of the cells. After thrombin treatment, MLC phosphorylation is accompanied by an increase in the F-actin and decrease in G-actin contents in endothelial cells (Goeckler and Wysolmerski, 1995). Phosphorylation of MLC has been shown to be involved in the regulation of permeability of cultured endothelial cells as well as intact isolated postcapillary venules and pulmonary microvessels in response to histamine and thrombin (Yuan et al., 1997; Vogel et al., 2000). The

relationship between MLC phosphorylation and interendothelial gap formation is only partly understood. It is clear, however, that activation of the contractile machinery leads to an increase in paracellular permeability. The maximal increase in MLC phosphorylation in response to an agonist such as thrombin precedes tension development and the increase in endothelial barrier permeability (Goeckeler and Wysolmerski, 1995; Moy et al., 1996; Moy et al., 2002).

1.3.2 Myosin light chain kinase

MLCK is a Ca^{2+}/calmodulin-dependent enzyme that phosphorylates MLC at S19 and/or T18 (Goeckeler and Wysolmerski, 1995). In contrast to smooth muscle cell MLCK (SMC-MLCK), which is 110-130 kDa, endothelial cell MLCK (EC-MLCK) is a 210-kDa protein (Dudek and Garcia, 2001). Structurally, EC-MLCK contains all the domains present in the smooth muscle form, but in addition, has a unique 922 amino acid NH_2-terminal domain containing consensus sites that may be phosphorylated by diverse protein kinases, including PKA (de Lanerolle et al., 1984; Garcia et al., 1997), PKC (Bogatcheva et al., 2003), p21-activated kinase (PAK) (Goeckeler et al., 2000), Src (Birukov et al., 2001), and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Verin et al., 1998). It is well established that activation of EC-MLCK leads to endothelial cell contraction and barrier dysfunction in response to inflammatory mediators like thrombin and histamine (Dudek and Garcia, 2001), while inhibition of EC-MLCK, using pharmacological inhibitors like KT5926 or ML-7, abrogates the increase in vascular permeability (Yuan et al., 1997; Tinsley et al., 2000). Recently, it has been shown that expression of a constitutive active form of EC-MLCK resulted in an elevated basal permeability of venules as well as venular endothelial cells in culture (Tinsley et al., 2000). The selective knockout of EC-MLCK in a mouse model resulted in protection against barrier failure induced by lipopolysaccharides (Wainwright et al., 2003) and burn injury (Reynoso et al., 2007). These studies indicate that EC-MLCK plays a critical role in agonist-induced endothelial barrier failure via activation of the contractile machinery.

1.3.3 Myosin light chain phosphatase

MLCP is a holoenzyme consisting of a catalytic subunit (PP1), a regulatory subunit (MYPT1), which targets PP1 to myosin and a smaller subunit of 20 kDa (M20) of unknown function. In endothelial cells the primary function of MLCP is to dephosphorylate MLC at S19 and T18.

PP1 catalytic subunit. The PP1 catalytic subunit in mammalian cells is encoded by three genes, α, γ, and δ (also called β). Alternative splicing generates α_1, α_2, γ_1, and γ_2 variants (Sasaki et al., 1990; Durfee et al., 1993). The resulting five isoforms have high sequence homology and are expressed in many different cell types. They share over 95% identity in the core catalytic domain. With the exception of α_2, which has an N-terminal insert, the other isoforms differ mostly in their C-terminal sequence, sharing less than 50% identity. Substrate specificity is modulated by association with a large number of regulatory and/or inhibitory subunits. In endothelial cells it is well established that PP1δ is the predominant isoform of PP1 existing in the MLCP holoenzyme (Verin et al., 2000; Härtel et al., 2007).

Myosin phosphatase targeting subunit. The specific activity of PP1 of MLCP to dephosphorylate the MLC is dependent upon its binding to the regulatory subunit MYPT1. MYPT1 is a 130 kDa protein and plays a key role in determining the physical and functional integrity of the trimeric myosin phosphatase (Khatri et al., 2001). PP1δ binds to the N-terminus and M20 to the C-terminus of MYPT1. Myosin binds to both C- and N-terminal of MYPT1. The N-terminal of MYPT1 shows ~15 fold increased activity and ~10-fold higher affinity for phosphorylated MLC than the isolated catalytic subunit (Hartshorne, 1998; Hartshorne et al., 2004; Ito et al., 2004).

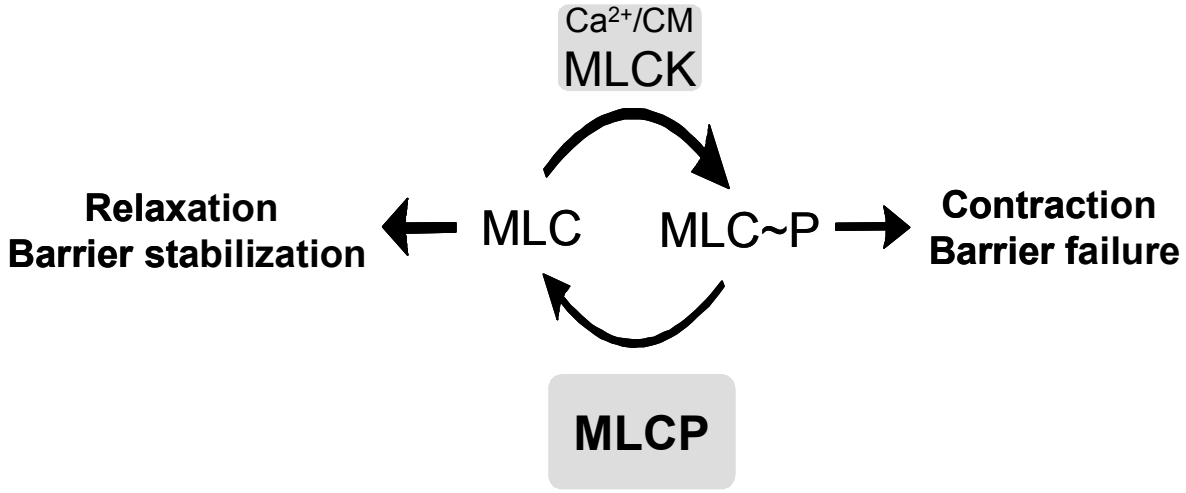

Fig. 1.2 Regulation of endothelial cell contractility: Phosphorylation of myosin light chain (MLC~P) is a key step in the regulation of the activation of the contractile machinery. Calcium/calmodulin (Ca^{2+}/CM)-dependent myosin light chain kinase (MLCK) phosphorylates, while myosin light chain phosphatase (MLCP) dephosphorylates MLC. Activation of the contractile machinery leads to endothelial cell contraction and barrier failure, while inactivation to relaxation and barrier stabilization.

1.3.4 Regulation of myosin light chain phosphatase activity via MYPT1 phosphorylation

The activity of MLCP can be regulated either through its regulatory subunit MYPT1 or through direct inhibition of the catalytic subunit PP1 by low molecular weight endogenous inhibitors. Stimulation of endothelial and nonendothelial cells with thrombin leads to activation of RhoA-dependent kinase (Rock), which induces phosphorylation of MYPT1 and inhibition of MLCP activity (Goeckeler and Wysolmerski, 2005; Pandey et al., 2006).

Most of our understanding about the regulation of the contractile machinery by MYPT1 is based on data obtained primarily from smooth muscle cells and little from endothelial cells. Several studies have well documented that MLCP activity is regulated by phosphorylation of its targeting subunit (MacDonald et al., 2001; Wooldridge et al., 2004). The two main inhibitory phosphorylation sites identified in smooth muscle cells as well as in endothelial cells are threonine 696 (T696) and threonine 850 (T850). However, the mechanism of MLCP inhibition remains unclear. Several kinases have been reported to phosphorylate MYPT1 at one or

both of these sites. The major well-known kinase is Rock, which phosphorylates MYPT1 at both sites and inhibits its catalytic activity (Hartshorne, 1998; Fukata et al., 2001). Other kinases phosphorylating MYPT1 in a Ca^{2+}-independent manner include zipper-interacting protein kinase (ZIPK) and the so called MYPT1-associated kinase, which is highly homologous to ZIPK (Hartshorne, 1998; Borman et al., 2002). Phosphorylation at T696 by Rock or ZIPK inhibits the activity of MLCP (Fukata et al., 2001; MacDonald et al., 2001; Borman et al., 2002). Accordingly, phosphorylation of T850 by Rock was shown to induce dissociation of MYPT1 from myosin and thus inactivation of MLCP (Velasco et al., 2002). These data confirm that the phosphorylation of MYPT1 plays an important role in the regulation of MLCP activity.

1.3.5 Regulation of myosin light chain phosphatase activity by CPI-17

Recent data, mainly from smooth muscle cells suggests an alternative mechanism for the regulation of the activity of MLCP holoenzyme. A small 17 kDa protein, the PKC-potentiated inhibitor 17-kDa protein (CPI-17), can directly interact with, and inactivate the catalytic subunit of MLCP (Murthy et al., 2003; Somlyo and Somlyo, 2003). CPI-17 is a soluble globular protein and was purified as a myosin phosphatase specific inhibitor from pig aorta (Eto et al., 1995). Phosphorylation of CPI-17 at threonine 38 (T38) was shown to enhance its inhibitory potency by more than 1000-fold (Eto et al., 2004). Several kinases such as PKCα/δ, PAK, ZIPK, integrin linked kinase (ILK), and Rock were shown to activate CPI-17 by phosphorylation at T38 (Koyama et al., 2000; MacDonald et al., 2001; Erdödi et al., 2003). Initially, CPI-17 was assumed to be present only in smooth muscle cells, but recently it was found in platelets (Watanabe et al., 2001), brain (Dubois et al., 2003) and also in endothelial cells of different origins (Kolosova et al., 2004). The phosphatase activity of purified MLCP towards phosphorylated MLC was completely inhibited in the presence of phosphorylated CPI-17 (Senba et al., 1999). Agonists like histamine and thrombin, cause phosphorylation of CPI-17 in smooth muscle cells (Kitazawa et al., 2003), as well as in platelets (Watanabe et al., 2001) and endothelial cells (Kolosova et al., 2004).

1.4 cAMP and endothelial permeability

cAMP is a well-known intracellular second messenger mediating stabilization of barrier function in isolated vessels as well as in cultured endothelial monolayers (Adamson et al., 1998; Stelzner et al., 1989; He et al., 2000). Consistently, activation of adenylyl cyclase via G-protein-coupled receptor (GPCR) agonists like, adenosine, prostacyclin, prostaglandin E2, and β-adrenergic agonists, direct activation of adenylyl cyclase (by FSK) as well as elevation of cytosolic cAMP concentration by blocking its degradation via phosphodiesterases, reduce endothelial hyperpermeability induced by inflammatory stimuli both *in vitro* and in *in vivo* (Carson et al., 1989; Langeler and van Hinsbergh, 1991; Suttorp et al., 1993; He and Curry, 1993; Fu et al., 2006). The effect of cAMP is fast and occurs in endothelial cells both under basal conditions as well as after exposure to inflammatory mediators. Its efficacy is independent on whether cAMP is elevated by activation of adenylyl cyclase or by inhibition of cAMP-degrading phosphodiesterases (Van Hinsbergh, 2001).

cAMP exerts its effect primarily through direct activation of PKA (Yuan, 2002). It has been shown in endothelial cells that inhibition of PKA by overexpression of a specific peptide inhibitor of PKA (PKI) as well as by pharmacological inhibitors (e.g. Rp-cAMP), resulted in increased endothelial permeability (Lum et al., 1999; Liu et al., 2001). The barrier protective effects of cAMP towards inflammatory mediators were abolished in these cells. In an *in situ* study in intact frog and rat microvessels it was shown that inhibition of PKA leads to increased basal permeability (He et al., 2000). Liu and co-workers (2005) showed in cultured endothelial cells that inhibition of PKA leads to an increase in stress fiber formation and basal endothelial permeability. These studies demonstrate clearly that cAMP exerts its effects on endothelial barrier function mainly by activating PKA.

Recently, it has been shown that cAMP can also strengthen barrier function by PKA-independent mechanism. cAMP can activate the small GTPase Rap1 via activation of the exchange factor Epac1/2 (Cullere et al., 2005; Fukuhare et al., 2005) leading to stabilization of adherens junctions. However, there is no evidence that activation of Epac causes dephosphorylation of MLC and inactivation of the contractile machinery.

The present study focuses on the molecular mechanism of the cAMP/PKA pathway which has been related to barrier stabilisation and protection via inhibition of the contractile machinery. A number of studies have shown that activation of PKA causes dephosphorylation of MLC (Moy et al., 1993), dissociation of F-actin from myosin (Langeler et al., 1991) and stabilization of cytoskeleton filaments (Hastie et al., 1997). Several studies have tried to elucidate the molecular mechanisms by which cAMP/PKA stabilizes the endothelial cytoskeleton. It has been proposed that cAMP/PKA inhibits the small GTPase RhoA and this in turn results in inhibition of phosphorylation of the regulatory MLC and thus activation state of the contractile machinery (Essler et al., 2000; Qiao et al., 2003). In an early study, Garcia and co-workers (1995) showed that cAMP/PKA activation causes MLCK phosphorylation and proposed that this might lead to MLCK inhibition. In a recent study Goeckeler and Wysolmerski (2005) clearly excluded that activation of cAMP/PKA pathway can lead to MLCK phosphorylation and inactivation.

A number of studies in smooth muscle cells and few in endothelial cells (Birukov, 2003; Goeckeler and Wysolmerski, 2005) have shown that activation of cAMP/PKA pathway leads to activation of MLCP but a detailed molecular analysis has not yet been performed.

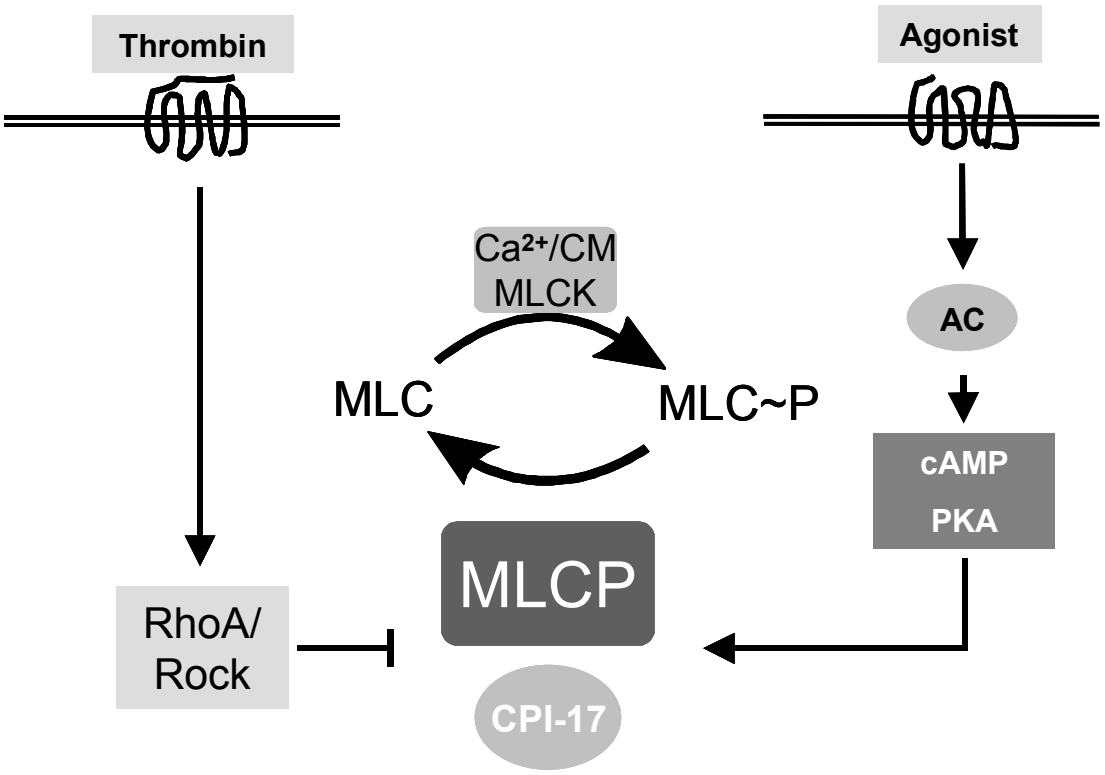

Fig 1.3 Regulation of myosin light chain phosphatase (MLCP). Thrombin, via protease-activated receptor-1 (PAR1), activates RhoA/Rock pathway. Rock inactivates myosin light chain phosphatase (MLCP) leading to increased myosin light chain (MLC) phosphorylation and contractile activation. Agonists like adenosine, via activation of adenosine A_2 receptors activate adenylyl cyclase (AC) leading to increased production of cAMP and activation of protein kinase A (PKA). Activation of cAMP/PKA pathway can activate MLCP, which dephosphorylates MLC causing inactivation of the contractile machinery. CPI-17 is an endogenous inhibitor of MLCP, activated by several kinases including PKC and Rock.

1.5 Aims and objectives of the study

The present study was conducted to elucidate the molecular mechanisms by which activation of the cAMP/PKA pathway leads to inactivation of the endothelial contractile machinery and protection of endothelial barrier function. Since previous studies in endothelial as well as smooth muscle cells demonstrated that cAMP/PKA exerts its main effect on contractile machinery via activation of MLCP, the present study was focused to analyze the molecular mechanism of MLCP activation via the cAMP/PKA signaling pathway.

The study was performed using an established model of cultured monolayers of human umbilical vein endothelial cells (HUVEC). The following questions were addressed:

- Does cAMP/PKA induce complex formation of the MLCP holoenzyme? Special emphasis was laid on analysis of recruitment of PP1 to MYPT1 and and the translocation of both to myosin.
- Is this cAMP/PKA-induced MLCP holenzyme complex formation dependent on inhibition of RhoA/Rock pathway?
- Does cAMP/PKA pathway cause inactivation of CPI-17, a specific inhibitor of PP1?
- Does the RhoA/Rock pathway play a role in the cAMP/PKA-mediated effect on CPI-17?
- Does inhibition of CPI-17 have any functional role in endothelial barrier failure caused by inflammatory mediators (e.g. thrombin)?

The following experimental strategies were used to answer these questions.

- Recruitment of MYPT1 and PP1 to myosin was analyzed by co-immunoprecipitation analysis.
- Activation of MLCP was analyzed by direct determination of phosphatase activity in co-immunoprecipitated complexes.
- Activation of RhoA/Rock pathway was analyzed by pulldown assay and RhoA translocation to membranes by cell fractionation.
- Interaction of CPI-17 with PP1 was analyzed by co-immunoprecipitation analysis and CPI-17 phosphorylation (i.e. activation) was analyzed by western blot analysis.
- Macromolecule permeability across endothelial monolayers was used as a functional assay to evaluate the impact of CPI-17. in these experiments CPI-17 was downregulated by siRNA technique.

2 Methods

2.1 Cell culture

Isolation of human umbilical vein endothelial cells (HUVEC)

Collagenase solution

HBSS	x ml
Collagenase II, 293 IU/mg (wt/vol)	0.025 %
$MgCl_2$	0.5 mM
$CaCl_2$	1.5 mM

Endothelial cell culture medium

Endothelial cell basal medium (PromoCell®) supplemented with

FCS (vol/vol)	10 %
Endothelial cell growth supplement/Heparin (wt/vol)	0.4 %
Hydrocortisone (wt/vol)	0.1 %
bFGF (wt/vol)	1 ng/ml
hEGF (wt/vol)	0.1 ng/ml
Penicillin/streptomycin (vol/vol)	2 %

Procedure. The study conforms with the principles outlined in the ‘’Declaration of Helsinki’’ (*Cardiovascular Research* 1997;35:2–3). Human umbilical vein endothelial cells (HUVEC) were isolated from freshly collected umbilical cords (from Gynecology Department, University Hospital Giessen) according to Jaffe et al., (1973) with some modifications. After cleaning, the umbilical vein was canulated and perfused with HBSS to remove traces of blood. Afterwards, the lumen of the vein was filled with collagenase solution and incubated for 20-30 minutes at 37 °C. Afterwards, the collagenase solution, containing endothelial cells, was gently flushed from the vein by perfusion with 30 ml of HBSS containing 3% (vol/vol) FCS, to inactivate the collagenase. The effluent was collected in a 50 ml Falcon tube and centrifuged at 250 x g for 5 minutes at room temperature. The supernatant was removed and the cell pellet was resuspended in endothelial cell culture medium containing 0.1% gentamycin. The cell suspension was seeded on

3-4 primary cell-culture dishes and incubated at 37 °C with 5% CO_2 for 2 hours. Thereafter, cells were washed with HBSS to remove erythrocytes, non-adherent cells, and cell debris and were incubated with cell culture medium containing 0.1% (vol/vol) gentamycin at 37 °C with 5% CO_2. After 24 hours the medium was replaced with normal endothelial cell culture medium.

Sub-culturing of HUVEC. Confluent monolayers of primary endothelial cells were trypsinized (5-7 days after isolation) in phosphate-buffered saline [PBS; composition in mM: 137 NaCl, 2.7 KCl, 1.7 KH_2PO_4, and 10 Na_2HPO_4; pH 7.4, supplemented with 0.05% (wt/vol) trypsin, and 0.02% (wt/vol) EDTA] and seeded at a density of 7 x 10^4 cells/cm^2 on Transwell® filters (for permeability) or on cell culture dishes (for western blot analysis, immunoprecipitation and pulldown assay). Experiments were performed with confluent endothelial monolayers of either primary or passage 1, 3 days after seeding.

2.2 General incubation conditions

The basal medium used in incubations was HBSS supplemented with 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 2 % (vol/vol) FCS. After an initial equilibration period of 20 minutes, agents were added as indicated. Stock solutions of thrombin, Y27632 and PKI were prepared immediately before use with basal medium. Stock solutions of forskolin were prepared with dimethyl sulfoxide (DMSO). Appropriate volumes of these solutions were added to the cells yielding final solvent concentrations $\leq$ 0.1% (vol/vol). The same final concentration of DMSO was included in all respective control experiments. Stock solutions of all other substances were prepared in basal medium (composition as described above). Appropriate volumes of these solutions were added to the cells. Identical additions of basal medium were included in all respective control experiments.

In a set of pilot experiments, concentration-response relationships were determined to find the optimum effective concentration of the agents to be used in this study. The agents were applied in their optimum effective concentrations as follows: forskolin (5 µM), thrombin (0.2 IU/ml), PKI (100 µM), Y-27632 (10 µM).

2.3 Protein analysis

Sample preparation

Endothelial cells were washed with HBSS and subsequently lysed in 150 µl 2x SDS sample buffer (250 mM Tris/HCl; pH 6.8, 20% (vol/vol) glycerol, 4% (wt/vol) SDS, 1% (vol/vol) β-mercaptoethanol, 10 µM cantharidin, 0.001% (wt/vol) bromphenol blue, and 10 mM DTT added freshly before use). Subsequently, 50 IU/ml Benzonase® and 2 mM $MgCl_2$ was added and lysate was collected in a 1.5 ml Eppendorf tube. Samples were denatured for 3 minutes at 95 °C and used immediately or stored at –20 °C.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Resolving gel buffer: Tris/HCl; pH 8.8 120 mM
Stacking gel buffer: Tris/HCl; pH 6.8 120 mM

10x Gel running buffer

Tris	250 mM
Glycine	2.0 M
SDS (wt/vol)	10 %

SDS gels

The composition of gels of different percentages is given below:

Gels	**Resolving gels**				**Stacking gel**
Solutions	7.5 %	10 %	12.5 %	15 %	6 %
Acrylamide 40% (wt/vol)	7.7 ml	10.2 ml	12.7 ml	15.3 ml	3.8 ml
Bisacrylamide 2% (wt/vol)	4.2 ml	5.6 ml	7.0 ml	8.4 ml	2.0 ml
Millipore water	17.7 ml	13.8 ml	9.8 ml	5.8 ml	17.5 ml
Resolving gel buffer	9.5 ml	9.5 ml	9.5 ml	9.5 ml	------
Stacking gel buffer	------	------	------	------	6.0 ml
SDS 10% (wt/vol)	0.4 ml	0.4 ml	0.4 ml	0.4 ml	0.25 ml
TEMED	30 µl	30 µl	30 µl	30 µl	20 µl
APS 10% (wt/vol)	0.4 ml	0.4 ml	0.4 ml	0.4 ml	0.25 ml

Procedure. After cleaning the glass plates and spacers with water and ethanol, the gel apparatus was assembled and the resolving gel solution was poured (app. 10 cm height), and layered with water. The gel was let to polymerize for 3-4 hours or overnight at room temperature.

The layer of water was removed, the stacking gel solution was poured on top of the resolving gel, the comb was inserted and the stacking gel was let to polymerize for 1 hour at room temperature. After removing the comb 1x running gel buffer was added to the chamber and the wells were washed with a syringe. Protein samples were loaded into the wells and the gel was run overnight at 45 volts. The run was stopped when bromophenol blue had passed through the gel.

Electroblotting and immunodetection of proteins (Western Blot)

Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane by semi-dry blotting. Afterwards specific proteins were immunodetected using specific antibodies.

Materials and solutions

- Nitrocellulose transfer membrane, cut to the same dimensions as the gel
- Six pieces of Whatman® 3 MM filter paper, cut to the same dimensions as the gel
- Blotting chamber
- Anode buffer 1: 0.3 M Tris/HCl; pH 10.4, 20% (vol/vol) methanol
- Anode buffer 2: 30 mM Tris/HCl; pH 10.4, 20% (vol/vol) methanol
- Cathode buffer: 25 mM Tris/HCl; pH 9.4, 40 mM 6-amino-n-hexanoic acid, 20% (vol/vol) methanol
- Millipore water

Procedure. The blotting chamber was assembled as follows: Two sheets of filter paper (Whatman® 3MM) soaked in anode buffer 1, were placed in the centre of the graphite anode of the blotting chamber. On top of these sheets, two sheets of filter paper, soaked in anode buffer 2, were placed followed by nitrocellulose membrane equilibrated in anode buffer 2 for 10-15 minutes. After briefly equilibrating with

cathode buffer, the SDS-gel (devoid of stacking gel) was layered on top of the nitrocellulose membrane, avoiding air bubbles. Two sheets of filter paper, pre-soaked in cathode buffer, were placed on top of the gel followed by the graphite cathode of the blotting chamber. Transfer was achieved by application of 0.8-0.9 mA/cm^2 current for approximately 2-2.5 hours.

Ponceau staining of proteins

To estimate the efficiency of protein transfer after blotting, the membrane was stained with ponceau S. This stain is reversible and produces pink bands on a light background. The nitrocellulose membrane was washed with Millipore water for 1 minute, incubated in Ponceau-S solution for 2-3 minutes with constant shaking at room temperature. Subsequently the membrane was destained by washing in Millipore water to the desired contrast and photographed. To remove the stain completely, the membrane was washed with TBST (1x TBS plus 0.1% tween 20) under constant shaking.

Immunodetection of proteins

Solutions

10x Tris-buffered saline (TBS)

Tris/HCl (pH 7.4)	100 mM
NaCl	1.6 M

TBS Tween (TBST)

1x TBS

0.1% (vol/vol) Tween 20

Blocking-buffer and antibody-dilution buffer

3% (wt/vol) BSA in 1x TBST (BSA) or

5% (wt/vol) non-fat dried milk powder in 1x TBST (Milk)

Primary Antibodies

Antibody	Dilution	Dilution buffer
CPI-17 (Rabbit IgG, polyclonal)	1:1000	Milk
Phospho CPI-17 (Rabbit IgG, polyclonal)	1:1000	BSA
MLC (Clone MY-21, mouse IgM, monoclonal)	1:2000	Milk
MYPT1 (Sheep IgG, polyclonal)	1:1000	Milk
Phospho MYPT696 (Rabbit IgG, polyclonal)	1:1000	BSA
Phospho MYPT850 (Rabbit IgG, polyclonal)	1:1000	BSA
PP1δ (Rabbit IgG, polyclonal)	1:1000	BSA
RhoA (Mouse IgG, monoclonal)	1:1000	BSA
Vinculin (Clone hVIN-1, mouse IgG, monoclonal)	1:1000	BSA

Secondary antibodies, horseradish peroxidase (HRP)-labeled

Antibody	Dilution	Dilution buffer
Anti-rabbit IgG	1:1000	BSA or Milk
Anti-mouse IgG	1:1000	BSA or Milk
Anti-mouse IgM	1:2000	BSA or Milk
Anti-sheep IgG	1:1000	BSA or Milk

Procedure. After a brief washing with Millipore water and TBST, the membrane was blocked with either 5% (wt/vol) non-fat milk powder or 3% (wt/vol) BSA in TBST for 2 hours at room temperature. After blocking, the membrane was incubated with primary antibody overnight at 4°C. The membrane was then washed with TBST 3-4 times for 5-10 minutes each at room temperature and incubated with secondary antibody for 1 hour at room temperature. The membrane was then washed with TBST 3-4 times for 10-15 minutes (each) and was then incubated with enhanced chemiluminescence (ECL) solution (30 seconds to 1 minute) and the luminescence was detected and recorded with *Bio-Rad Quantity One* gel documentation system.

2.4 Determination of MLC phosphorylation

The phosphorylation of MLC was determined by glycerol-urea polyacrylamide gel electrophoresis and Western blot analysis (Persechini et al., 1986). This procedure allows separation of non-phosphorylated from phosphorylated MLC protein, the latter of which migrates more rapidly.

Urea-glycerol gel

Following solutions were used to make 6 small gels:

Glycerol (87%)	20.70 ml
Acrylamide solution 40% (wt/vol)	11.25 ml
Bisacylamide solution 2% (wt/vol)	9.50 ml
Urea-gel buffer	3.80 ml

To remove air bubbles, the solution was degassed for 10 minutes with a water vacuum pump.

TEMED	7.10 µl
APS 10% (wt/vol)	200 µl

Urea-gel buffer

Tris/HCl	240 mM
Glycine	276 mM

Adjust pH to 8.8

Anode buffer

Urea-gel buffer	83 ml
Millipore water	917 ml

Cathode buffer

Anode buffer	450 ml
DTT	2.3 mM
Sodium thioglycolate	2.4 mM

Procedure. Experimental incubations of cultures were terminated by a rapid removal of the medium and addition of 10% (wt/vol) ice-cold trichloroacetic acid and incubation on ice for 30-60 minutes. Precipitated proteins were transferred

into 1.5 ml Eppendorf tubes and centrifugated at 10,000 x g for 10 minutes at 4 °C. Sediments were washed 2-3 times with diethylether. After evaporation of diethylether, sediments were suspended in 30 µl lysis buffer (8.8 M urea, 60 mM imidazole, 23 mM glycine, 20 mM Tris/HCl; pH 8.8, 10 mM DTT, 5 mM sodium thioglycolate, 10 µM cantheridin, 0.001 % (wt/vol) bromophenol blue). Before loading the lysates, the gels were pre-run at 400 V for 1 h. Approximately 20-40 of µg protein per lane was loaded on 10% urea-glycerol polyacrylamide gels and allowed to run at 400 V and 18 °C for 80 minutes. Separated proteins were blotted on nitrocellulose membranes (0.2 µm) and incubated as described under section 2.3, with an anti-MLC antibody (1:2000) over night, followed by incubation of HRP-labeled anti-mouse IgM antibody (1:2000) for 1 hour at room temperature. Luminescence was detected and recorded with Bio-Rad Quantity One gel documentation system. The percentage of MLC phosphorylation (expressed as % of total MLC) was calculated from densitometrical values of non- (MLC), mono- (MLC~P), and diphosphorylated MLC (MLC~PP) as follows:

$$\text{MLC phosphorylation (\%)} = \frac{(2 \times \text{MLC} \sim \text{PP}) + \text{MLC} \sim \text{P}}{\text{Total MLC}} \times 100$$

As all MLC can become diphosphorylated, MLC phosphorylation (%) varies between 0 and 200 %.

2.5 Co-immunoprecipitation of proteins

Preparation of beads. Protein G-coated magnetic beads (6 µl beads suspension for approximately 1 mg of total cell lysate) were washed 3-4 times with 0.1 M sodium phosphate buffer (composition: 80 mM Na_2HPO_4, 20 mM NaH_2PO_4; pH 7.4) and incubated with the respective antibody (4-5 µg for 1 mg total cell lysate) overnight at 4 °C with end-over-end rotation. Afterwards the beads were washed 3-4 times with 0.1 M sodium phosphate buffer containing 0.1 % (vol/vol) Tween 20 and stored in 50 µl of PBS.

Immunoprecipitation. Confluent endothelial monolayers cultured on a 10-cm cell culture dish were stimulated as indicated in the text. Cells were incubated with 600

µl lysis buffer containing 50 mM Tris/HCl; pH 7.4, 150 mM NaCl, 1% (vol/vol) Triton X-100, 0.5% (vol/vol) NP-40, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1.5 mM Na-orthovanadate, 10 mM DTT, 0.5 mM PMSF, and Complete® protease inhibitor cocktail, for 10 minutes on ice and subsequently harvested by scraping with a rubber policeman and lysed by passing through a 26G needle (4-5 times). Lysates were cleared by centrifugation at 1,000 x g for 5 minutes at 4 °C. The supernatant was transferred to another tube and incubated with the respective antibodies pre-immobilized on protein G-coated magnetic beads for 1.5 hours at 4 °C with end-over-end rotation. After incubation, beads were washed three times with PBS containing 0.1 % (vol/vol) Tween 20. The beads were collected and the bound proteins were eluted in 2x SDS sample buffer and analyzed by western blot analysis.

2.6 Protein phosphatase assay

Preparation of [^{32}P]-labeled substrate. [^{32}P]-labeled phosphorylase-a was prepared according to Essler et al., (1998) with some modifications. Briefly, phosphorylase-b (5 mg/ml) and phosphorylase-kinase (200 IU/ml) were incubated in a 2 ml incubation mixture (composition: 20 mM $MgCl_2$, 31 mM β-mercaptoethanol, 0.5 mg/ml BSA, 1 mM $CaCl_2$, 1 mM ATP, 1 mCi γ-[^{32}P]-ATP, and 50 mM Tris/HCl; pH 7.4) for 2.5 hours at 30 °C. The radioactive-labeled phosphorylase-a was precipitated by addition of 2 volumes of ice-cold saturated ammonium sulfate solution. The tube was incubated for 20 minutes on ice and centrifuged for 30 minutes at 12,000 x g at 4 °C. The precipitate was solubilized in 2 ml dialysis buffer (10 mM Tris/HCl; pH 7.4, 1 mM EDTA) dialyzed two times at room temperature against 2 liter dialysis buffer and finally stored at 4 °C. Radioactive labeling was verified by measuring the product in a liquid scintillation counter (Tri-Carb 1600 TR liquid scintillation counter).

Determination of protein phosphatase activity. Protein phosphatase activity was determined according to Neumann et al. (1991). For determination of protein phosphatase activity of MLCP, the holoenzyme was immunoprecipitated using an anti-MYPT1 specific antibody pre-immobilized on protein G-coated magnetic beads (see section 2.5). Aliquots were preincubated in a total volume of 30 µl for

10 minutes at 30 °C in the presence or absence of 5 nM okadaic acid, a concentration inhibiting protein phosphatase 2A, or 0.5 µM human recombinant inhibitor 2 (rec. I-2), a specific inhibitor of PP1. The reaction was started by addition of 20 µl [^{32}P]-labeled phosphorylase-a in an incubation mixture containing 50 mM Tris/HCl; pH 7.4, 12.5 mM caffeine, 0.25 mM EDTA, 1.25 mM $MnCl_2$, 0.25% (vol/vol) β-mercaptoethanol. After incubation for 20 minutes at 30 °C, the reaction was terminated on ice by addition of 20 µl of 50% (wt/vol) ice-cold trichloroacetic acid (TCA) and 30 µl of 2% (wt/vol) bovine serum albumin (BSA). After 15 minutes on ice, the suspension was centrifuged at 12,000 x g for 5 minutes at 4 °C. 70 µl of the supernatant was measured in a liquid scintillation counter. Reactions were carried out in duplicate or triplicate. To ensure linear rates of dephosphorylation, the extent of dephosphorylation of [^{32}P]-labeled phosphorylase-a was restricted to <25%.

2.7 Detection of activated RhoA

The assay was performed according to the manufacturer's instructions using the Rho binding domain of rhotekin (Rhotekin-RBD) to specifically bind and isolate activated GTP-bound RhoA.

Procedure. Endothelial cells were stimulated with FSK, thrombin, or combination of both. Afterwards the cells were washed with ice-cold PBS and incubated with 600 µl of lysis buffer (25 mM Hepes; pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM NaF, 2 mM Na-orthovanadate, 5 mM DTT, 0.5 mM PMSF, 2% (vol/vol) glycerol, 0.5% (vol/vol) Triton X-100, and Complete® protease inhibitor cocktail. The cells harvested by scraping with a rubber policeman and lysed by passing through a 26G needle (4-5 times). Lysates were cleared by centrifugation at 14,000 x g for 5 minutes at 4 °C. The supernatant was transferred into another tube and incubated with 10 µg of Rhotekin-RBD beads at 4 °C for 40 minutes. The beads were washed four times with wash buffer (25 mM Tris/HCl; pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1% (vol/vol) Triton X-100, 0.5 mM PMSF and Complete® protease inhibitor cocktail, heated to 95 °C for 5 minutes with 40µl of 2x SDS sample buffer (see section 2.3) and loaded on a 12.5% SDS-PAGE. RhoA protein was detected by western blot analysis using anti-RhoA mouse monoclonal

antibody. RhoA activation was estimated by correlation of isolated GTP-bound RhoA to total amount of RhoA in cell lysates.

2.8 Determination of RhoA translocation

Activation of RhoA leads to its translocation to the cell membrane (Takaishi et al., 1996), so RhoA translocation to membrane was also determined by cell fractionation. After stimulation the cells were washed briefly with ice-cold PBS and then incubated with lysis buffer (5 mM Tris/HCl; pH 7.4, 250 mM sucrose, 5 mM NaCl, 1 mM $MgCl_2$, 5 mM EDTA, 10 mM DTT, 0.5 mM PMSF, and Complete® protease inhibitor cocktail, for 10 minutes on ice. Subsequently, cells were collected with a rubber policeman and lysed by passing through a 26G needle (4-5 times). Cell debris and nuclei were removed by centrifugation at 1000 x g for 5 minutes at 4 °C to clear the lysate. Afterwards, the supernatant was centrifuged at 100,000 x g for 30 minutes at 4 °C. The pellet was washed three times with lysis buffer, dissolved in 2xSDS-sample buffer and analyzed by SDS-PAGE and western blot analysis.

2.9 Measurement of endothelial monolayer permeability

The endothelial permeability was measured according to Noll et al. (1999) using a two compartment system. The luminal and abluminal compartments were separated by a porous membrane (pore size 0.4 µm). The cells were cultured on the membrane (Transwell®) filters until confluent. HBSS [supplemented with 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 2% (vol/vol) fetal calf serum (FCS)] was added in both compartments as basal medium. The luminal compartment contained 2.5 ml the while the abluminal compartment contained 9.5 ml of this medium. There was no difference in hydrostatic pressure between the luminal and abluminal compartment. In the luminal compartment, trypan blue-labeled albumin was added in a final concentration of 60 µM. The diffusion of trypan blue-labeled albumin from the luminal to the abluminal compartment was measured with a spectrophotometer (Specord 10, Zeiss Jena, Germany) continuously every minute. To avoid measurement artefacts, two-wavelength measurement mode was used (trypan blue 600 nm versus control 720 nm).

The albumin flux (F, measured in mol/(sec x cm^2) through endothelial monolayer area (S) was calculated as the increase in albumin concentration ($d[A]_2$) during the time interval (dt) in the abluminal compartment with the volume (V) as follows:

$$F = \frac{d[A]_2 / dt \times V}{S} \qquad (1)$$

The combined permeability coefficient (P [cm/sec]) of both endothelial cell monolayer and filter membrane was calculated as:

$$P = \frac{F}{([A]_1 - [A]_2)} \qquad (2)$$

Where $[A]_1$ and $[A]_2$ are the albumin concentrations in luminal and abluminal compartments, respectively.

2.10 Downregulation of endogenous CPI-17

To reduce the content of CPI-17, endothelial cells were treated with CPI-17-specific siRNA duplex. siRNA was ordered from QIAGEN in purified, desalted, 2'-deprotected duplex form. Duplex of sense 5'-ACCUGUCGAGGACUUCAUCdTdT-3' and antisense 5'-GAUGAAGUCCUCGACAGGUdTdT-3' siRNA was used as described by Kolosova et al. (2004). Nonspecific RNA duplex was used as a control treatment. Endothelial cells were seeded on 35-mm cell culture dishes (for western blotting), and on Transwell® filters (for permeability experiments). When 70% confluence was reached siRNA (100 nM) was transfected with FuGENE® 6 transfection reagent. Experiments were performed 48 hours after the incubations. Downregulation of CPI-17 was determined by western blot analysis.

Transfection

Transfection was carried out with 100 nM of siRNA. Calculations for 7 wells (6 well culture dish) are given below:

Two mixtures (siRNA and FuGENE® 6) were prepared in two different tubes.

Tube 1. 35 µl of 40 µM siRNA was mixed with 315 µl of serum-free medium.

Tube 2. 6 µl of FuGENE® 6 was mixed with 344 µl of serum-free medium.

The content of Tube 1 were added to Tube 2 and mixed by vortexing for 10 seconds and were incubated for 25 minutes at room temperature. Afterwards, the mixture was added to 6.3 ml of serum free medium and mixed properly. Subsequently, 1 ml of this mixture was added to each well already containing 1 ml of serum free medium. Cells were then incubated at 37 ºC in 5% CO_2 for 12 hours. Afterwards the transfection medium was replaced with the normal medium. After 48 hours, respective experiments were performed.

2.11 Statistical analysis

Data are given as means $\pm$ S.D. of 5 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 ($P< 0.05$).

3 Results

3.1 Effect of FSK on endothelial permeability

In the first instance, experiments were performed to confirm that, in the endothelial cell culture model used, activation of adenylyl cyclase can reduce basal endothelial permeability as well as thrombin-induced hyperpermeability, and that this effect is associated with reduction in the contractile activity of endothelial cells. Under control conditions endothelial monolayers exhibited a stable permeability for albumin (Fig. 3.1). When 5 µM FSK was added, to directly activate the adenylyl cyclase, permeability rapidly declined. Conversely, permeability rapidly rose when thrombin (0.2 IU/ml) was applied. Simultaneous addition of both agents reduced the thrombin effect nearly to basal level. To test whether the effect of FSK on thrombin-induced hyperpermeability is mediated by PKA, endothelial cells were incubated for 30 minutes in the presence of 100 µM PKI, a specific cell permeable peptide inhibitor of PKA. As shown in Fig. 3.1, PKI abolished the effect of FSK on thrombin-induced hyperpermeability.

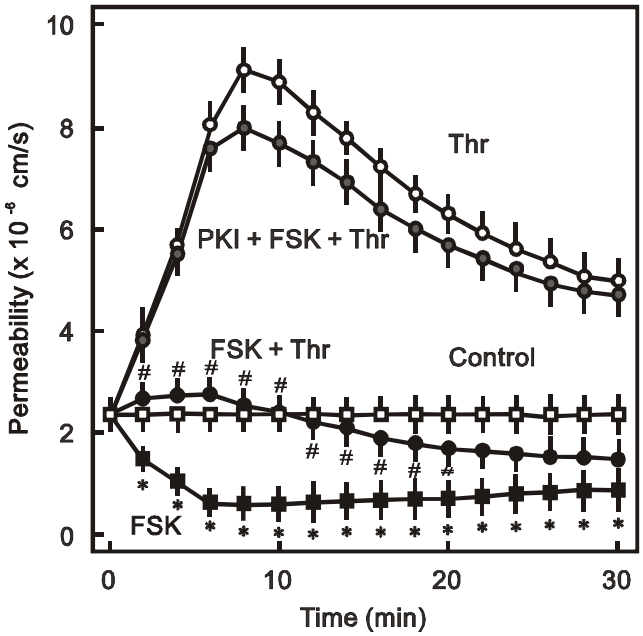

Fig. 3.1 Effects of forskolin (FSK), thrombin (Thr), and FSK plus Thr on albumin permeability of human umbilical vein endothelial monolayers. Endothelial cells were exposed to 5 µM FSK, 0.2 IU/ml Thr, FSK plus Thr or vehicle (Control). In a set of experiments cells were incubated with 100 µM PKI (a specific peptide inhibitor of PKA) for 30 minutes before FSK plus Thr was added (PKI+FSK+Thr). Data are means ± SD of 5 separate experiments with independent cell preparations. As indicated at time points between 2.5 and 30 minutes permeability is significantly different. $P < 0.05$: *FSK vs. Control; $^{\#}$FSK plus Thr vs. Thr alone. PKI plus FSK plus Thr is not significantly different from Thr alone.

3.2 Effect of FSK on MLC phosphorylation

Since MLC phosphorylation controls the activation of the endothelial contractile machinery, this parameter of contractile activation was analyzed (Fig. 3.2). FSK caused a decrease and thrombin an increase in MLC phosphorylation. The combined addition of FSK plus thrombin abolished the thrombin effect and reduced MLC phosphorylation below basal level. To test whether the effect of FSK on thrombin-induced MLC phosphorylation is mediated by PKA, endothelial cells were incubated for 30 minutes in the presence of 100 µM of PKA inhibitor (PKI). As shown in Fig. 3.2, PKI abolished the effect of FSK on thrombin-induced MLC phosphorylation.

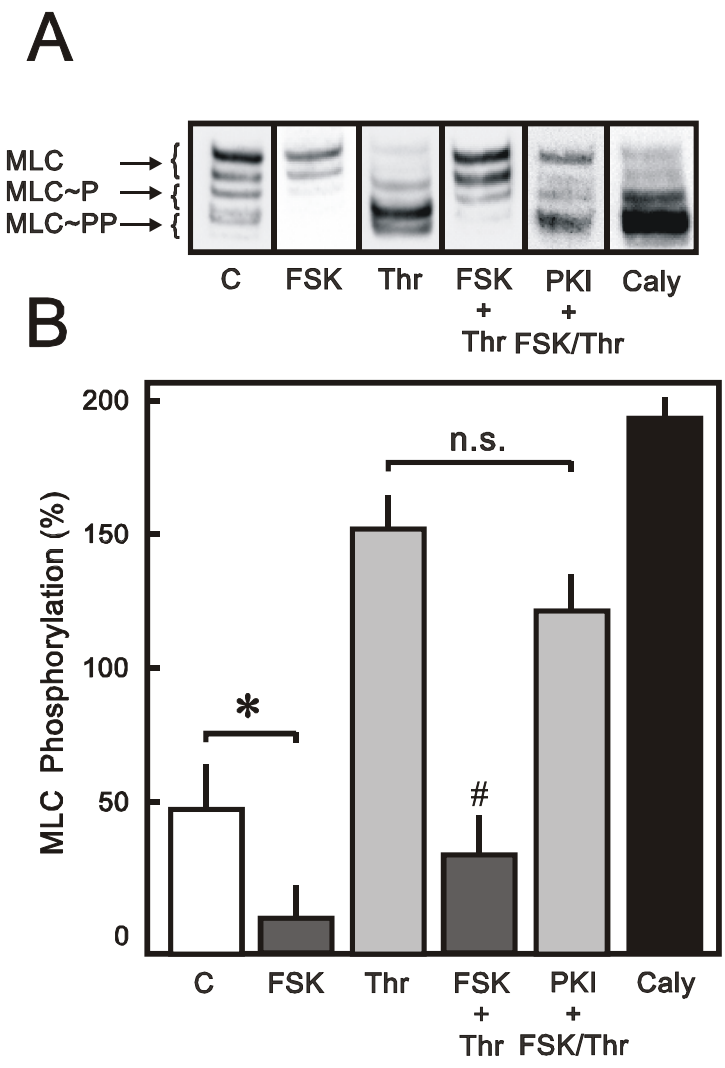

Fig. 3.2 Effects of forskolin (FSK), thrombin (Thr), FSK plus Thr and calyculin A on endothelial MLC phosphorylation. **(A)** Representative western blots of MLC phosphorylation. Endothelial cells were exposed to 5 µM FSK, 0.2 IU/ml Thr, FSK plus Thr or vehicle (C; control) for 10 minutes. In a set of experiments cells were incubated with 100 µM PKI (a specific peptide inhibitor of PKA) for 30 minutes before FSK plus Thr was added (PKI+FSK+Thr), as indicated. As a positive control 1 nM Calyculin A (Caly), a protein phosphatase inhibitor, was added for 20 minutes. The bands represent, from top to bottom, non- (MLC), mono- (MLC~P), and diphosphorylated MLC (MLC~PP), respectively. **(B)** Densitometric analysis of the western blots shown in A. As all MLC can become diphosphorylated, MLC phosphorylation varies between 0 and 200 %. Data are means ± SD of 5 separate experiments with independent cell preparations. $P < 0.05$: *FSK vs. Control; $^{\#}$FSK plus Thr vs. Thr alone. n.s: not significantly different.

MLC phosphorylation is mediated via RhoA/Rock pathway in endothelial cells. Here it was analyzed whether cAMP/PKA causes dephosphorylation of MLC via inhibition of the RhoA/Rock pathway. For that reason RhoA/Rock pathway was blocked by Y27632, a specific inhibitor of Rock. At optimum concentration (10 μM), Y27632 reduced MLC phosphorylation to 20 ± 6 % in 10 minutes (Fig. 3.3). Addition of 20 μM Y27632 could not further reduce MLC phosphorylation. Simultaneous addition of FSK plus Y27632 (10 μM) reduced MLC phosphorylation to 3 ± 5% . These data indicate that even under basal conditions the level of MLC phosphorylation is influenced by Rock pathway and is reduced by cAMP/PKA at least in part by a RhoA/Rock-independent pathway.

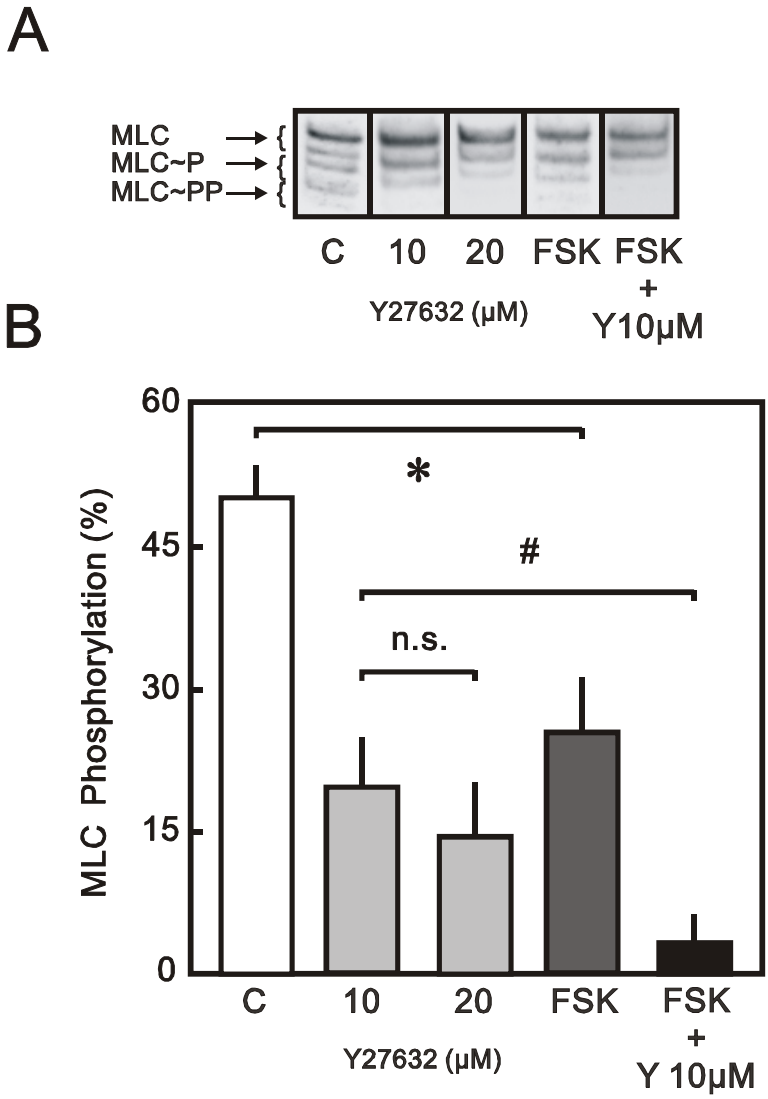

Fig. 3.3 Effects of forskolin (FSK), Y27632 (Y), or FSK plus Y on endothelial MLC phosphorylation. **(A)** Representative western blots of MLC. Endothelial cells were exposed to FSK (5 μM), Y (10 or 20 μM), FSK plus Y (10 μM) or vehicle (C; control) for 10 minutes. The bands represent, from top to bottom, non- (MLC), mono- (MLC~P), and diphosphorylated MLC (MLC~PP), respectively. **(B)** Densitometric analysis of the western blots shown in A. Data are means ± SD of 5 separate experiments with independent cell preparations. $^{*}P < 0.05$, $^{\#}P < 0.05$. n.s.: not significantly different.

3.3 Effect of FSK on MLC phosphatase complex formation and activity

Dephosphorylation of MLC may result either from inactivation of MLCK or activation of MLCP. Previously it has been shown (Bindewald et al., 2004) that stimulation of cAMP/PKA pathway can attenuate MLCK activity in endothelial cells. However, this attenuation can not explain the reduction of MLC phosphorylation observed. This concept is supported by recent data from Goeckeler and Wysolmerski (2005) who reported that cAMP/PKA has no significant effect on MLCK activity. Therefore, in the present study the effect of cAMP/PKA on MLCP activation was analyzed. The activation of MLCP requires that the PP1 catalytic subunit interacts with MYPT1, the myosin phosphatase targeting subunit, leading to formation of the MLCP holoenzyme complex. This MLCP holoenzyme complex has higher affinity to phosphorylated MLC. To analyze whether FSK induces MLCP complex formation, recruitment of PP1 and MYPT1 to myosin, was determined by immunoprecipitation using either a MYPT1 or a PP1-specific antibody. In the first step, PP1 and MLC were co-immunoprecipitated with MYPT1 in non-stimulated cells, indicating that an MLCP complex is already formed under basal conditions in endothelial cells (Fig. 3.4A, C). Exposure of endothelial cells to FSK increased the recruitment of PP1 and MLC to MYPT1 by 2.4 ± 0.5 and 4.3 ± 0.6-fold, respectively. This recruitment of PP1 lead to a 2-fold increase in phosphatase activity of the immunoprecipitated MLCP complex (Fig. 3.4B). Phosphatase activity of the immunoprecipitates, both of control and FSK-treated, was completely blocked by addition of 0.5 µM recombinant inhibitor 2 (rec. I-2), a PP1 specific inhibitor, indicating that the phosphatase activity is solely due to PP1.

In the second step, the assembly of MLCP complex was confirmed by immunoprecipitation using a PP1 specific antibody. As shown in Fig. 3.4D, under basal conditions MLCP complex is already formed and FSK induced the recruitment of MYPT1 and MLC to the catalytic subunit PP1.

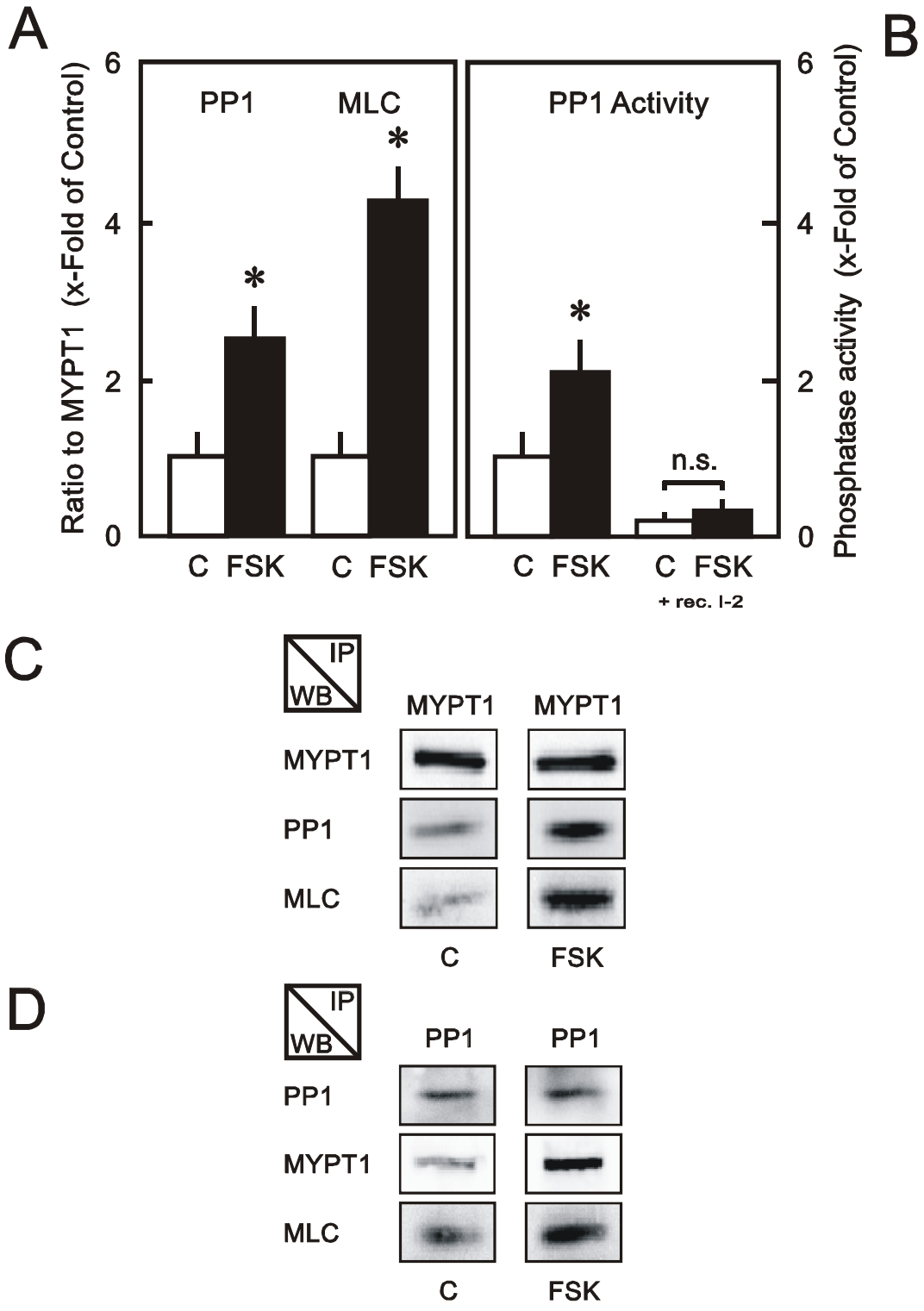

Fig. 3.4 Effect of forskolin (FSK) on MLCP complex assembly and activity of the immunoprecipitated phosphatase complexes. Endothelial cells were exposed to 5µM FSK or vehicle (C; control) for 10 minutes. MLCP was immunoprecipitated with an anti-MYPT1 antibody coupled to protein G-coated magnetic beads and analyzed by western blot analysis. Phosphatase activity of the immunoprecipitates was determined by phosphatase assay. **(A)** Densitometric analysis of the western blots shown in C. PP1 and MLC relative to MYPT1 are given as x-fold increase

compared to control. The ratio of control was set to 1. **(B)** Phosphatase activity of the immunoprecipitated MCLP complex, measured in the absence or presence of 0.5 µM recombinant inhibitor 2 (rec. I-2). The mean phosphatase activity of the control cells was set to 1. **(C)** Representative western blots of MYPT1, PP1 and MLC co-immunoprecipitated with MYPT1. **(D)** Representative western blots of PP1, MYPT1, and MLC co-immunoprecipitated with PP1. PP1 was immunoprecipitated with an anti-PP1 antibody coupled to protein G-coated magnetic beads. Data are means ± SD of 5 separate experiments of independent cell preparations. $^{*}P < 0.05$, FSK vs. control; n.s.: not significantly different.

To analyze whether RhoA/Rock is involved in cAMP/PKA-induced MLCP complex formation, endothelial cells were exposed to 10 µM Y27632, a concentration with maximum effect on MLC dephosphorylation (see Fig. 3.3). The assembly of MLCP complex was determined by co-immunoprecipitation using the same MYPT1 specific antibody as in the previous set of experiments. If cAMP/PKA mediates the formation of the MLCP complex via inhibition of RhoA/Rock, then Rock inhibition should influence MLCP complex formation. However, Y27632 did not lead to an increase in PP1 recruitment to MYPT1. It was found that Y27632 does not affect FSK-induced recruitment of PP1 to MYPT1. These data indicate that inhibition of RhoA/Rock is not involved in cAMP/PKA-induced MLCP complex formation.

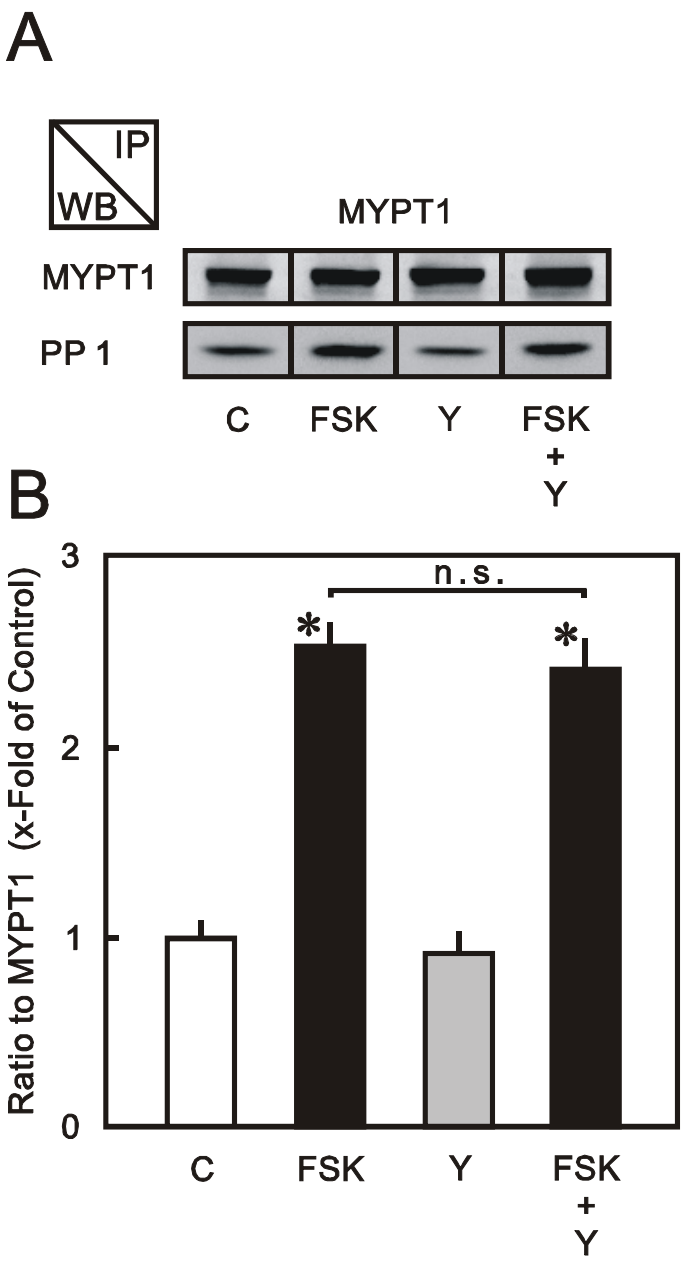

Fig. 3.5 Effect of forskolin (FSK), Y27632 (Y) or FSK plus Y on MLCP complex formation. Endothelial cells were exposed to FSK (5 µM), Y (10 µM), FSK plus Y for 10 minutes or vehicle treated (C; control). MYPT1 was immunoprecipitated using an anti-MYPT1 antibody coupled to protein G-coated magnetic beads. Co-immunoprecipitation of MYPT1 and PP1 was analyzed by western blot analysis. **(A)** Representative western blots of MYPT1 and PP1 co-immunoprecipitated with anti-MYPT1 antibody. **(B)** Densitometric analysis of western blots shown in A. PP1 relative to MYPT1 is given as x-fold increase compared to control. The ratio of control was set to 1. Data are means ± SD of 5 separate experiments of independent cell preparations. $^{*}P < 0.05$, FSK plus Y or FSK alone vs Control. n.s.: not significantly different.

3.4 Effect of FSK on MYPT1 phosphorylation

There is evidence that the affinity of the MLCP complex to phosphorylated MLC is controlled by phosphorylation of MYPT1 at T850. Here it was studied whether activation of cAMP/PKA signaling can affect MYPT1 phosphorylation and whether this signaling mechanism can counteract thrombin-induced MYPT1 phosphorylation. Under control conditions exposure of endothelial cells to FSK reduced MYPT1 phosphorylation at T850 to half of the basal level (Fig. 3.6). Addition of thrombin for 10 minutes caused a 2.5-fold increase in MYPT1 phosphorylation. Simultaneous addition of FSK plus thrombin abolished the thrombin-induced MYPT1 phosphorylation.

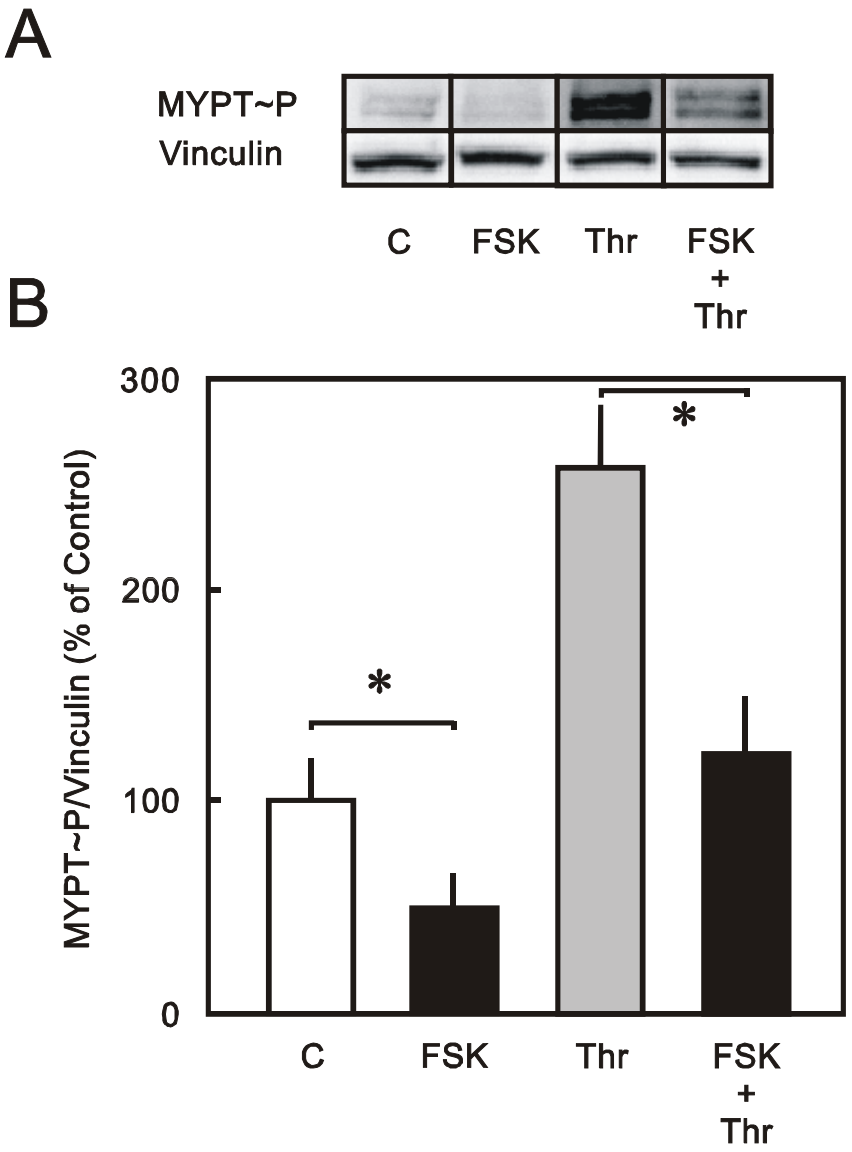

Fig. 3.6 Effect of forskolin (FSK), thrombin (Thr) or FSK plus Thr on MYPT1 phosphorylation at threonine 850 (T850). Endothelial cells were exposed to FSK (5µM), thrombin (0.2 IU/ml), FSK plus Thr or vehicle (C; control) for 10 minutes. **(A)** Representative western blots with an anti-phospho-T850 MYPT1 and anti-vinculin antibody. **(B)** Densitometric analysis of western blots shown in A. MYPT1 phosphorylation relative to vinculin is given as % increase compared to control. The ratio of control was set to 100%. Data are means ± SD of 5 separate experiments of independent cell preparations. $^{*}P < 0.05$.

3.5 Effect of FSK on RhoA translocation

Activation of RhoA/Rock pathway requires that RhoA is translocated to the plasma membrane. To analyze whether FSK affects translocation of RhoA, membrane fractions were prepared and analyzed by western blot analysis. Stimulation of endothelial cells with FSK reduced the amount of RhoA in the membrane fraction to half of the control value (Fig. 3.7). Thrombin increased the translocation of RhoA to membranes by 1.8-fold, compared to control. This thrombin effect was abolished when cells were incubated in the presence of FSK plus thrombin.

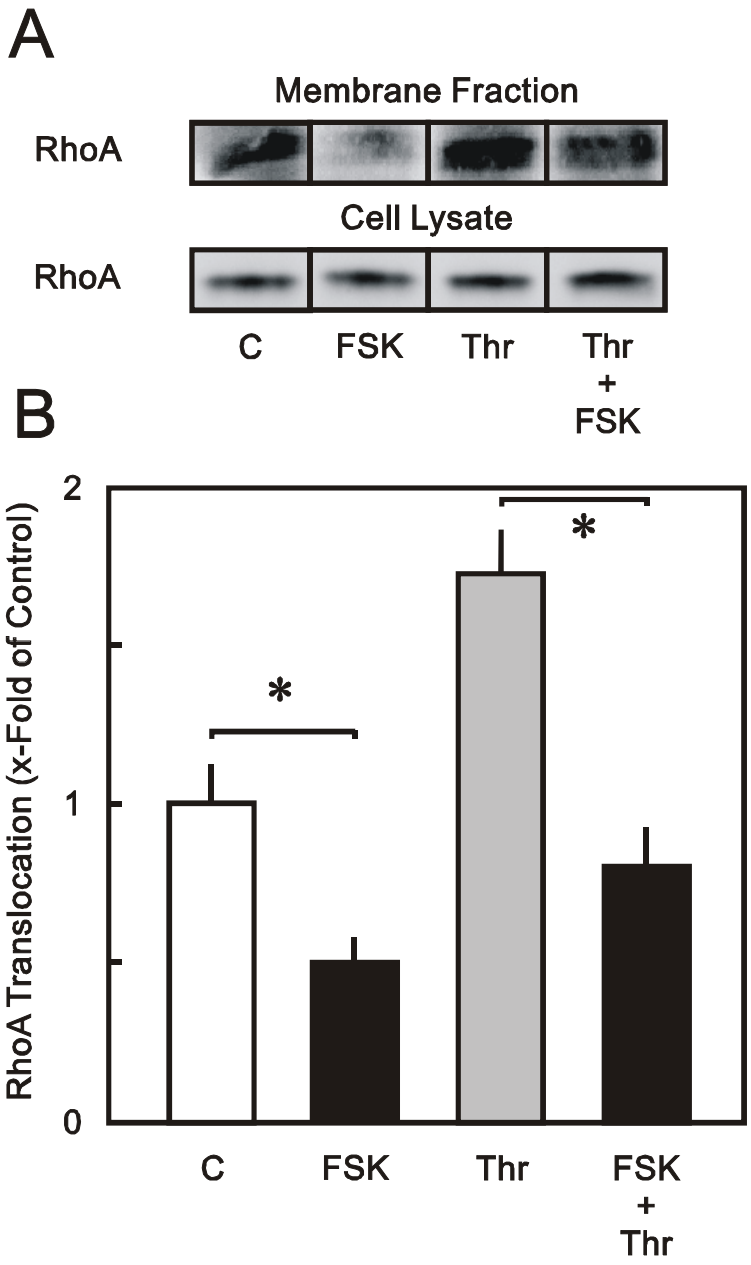

Fig. 3.7 Effect of forskolin (FSK), thrombin (Thr) or FSK plus Thr on RhoA translocation. Cells were treated with FSK (5 µM), thrombin (0.2 IU/ml), FSK plus thrombin or vehicle (C; control) for 10 minutes. Membrane fractions from equal amounts of cell lysates were isolated and analyzed by western blot analysis using an anti-RhoA antibody. **(A)** Representative western blots of membrane fraction and whole cell lysate with an anti-RhoA antibody **(B)** Densitometric analysis of western blots shown in A. RhoA in the membrane fraction relative to total RhoA is given as x-fold increase compared to control. The ratio of control was set to1. Data are means ± SD of 5 separate experiments of independent cell preparation. $^{*}P < 0.05$.

3.6 Effect of forskolin on CPI-17 phosphorylation and co-immunoprecipitation with PP1

The activity of the catalytic subunit PP1 is regulated by an endogenous inhibitor CPI-17, which interacts and inactivates PP1 when phosphorylated at threonine 38 (T38). Here the influence of FSK and thrombin on CPI-17/PP1 interaction and PP1 activity were analyzed. Therefore, PP1 was immunoprecipitated by using an anti-PP1 antibody coupled to magnetic beads. The immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by western blot analysis. Under basal conditions CPI-17 is co-immunoprecipitated with PP1 (Fig. 3.8). Exposure of endothelial cells to FSK reduced co-immunoprecipitation of CPI-17 with PP1 by 0.5-fold within 10 minutes. This reduction was accompanied by 60% increase in PP1 activity corresponding to a 2-fold increase in PP1 activity in MYPT1 co-immunoprecipitates. Thrombin caused a 1.7-fold increase of CPI-17 with PP1 immunoprecipitates and a 40% reduction in PP1 activity. Simultaneous addition of FSK plus thrombin completely abolished thrombin-induced complex formation and PP1 inhibition. Phosphatase activity of the immunoprecipitates, both of control and FSK-treated, was completely blocked by addition of 0.5 µM rec. I-2, a PP1 specific inhibitor, indicating that the phosphatase activity is solely due to PP1.

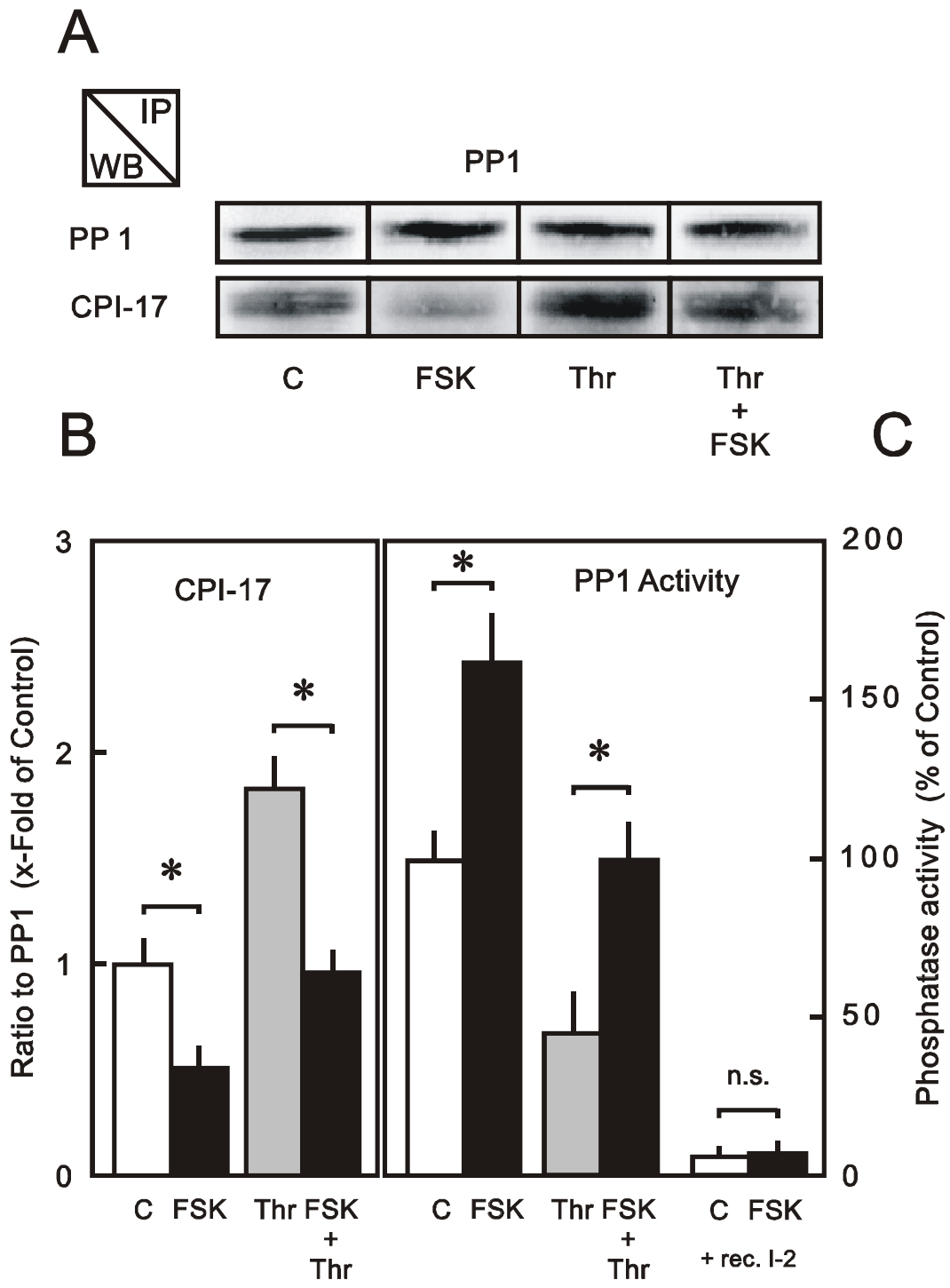

Fig. 3.8 Effect of forskolin (FSK), thrombin (Thr) or FSK plus Thr on CPI-17/PP1 complex and activity of the co-immunoprecipitaed complexes. Endothelial cells were treated with FSK (5 µM), Thr (0.2 IU/ml), FSK plus Thr or vehicle (C; control) for 10 minutes and PP1 was co-immunoprecipitated using anti-PP1 antibody coupled to protein G-coated magnetic beads and analyzed by western blot analysis. Phosphatase activity of the immunoprecipitated complex was measured by phosphatase assay. **(A)** Representative western blots of PP1 and CPI-17 co-immunoprecipitated with PP1. **(B)**. Densometric analysis of western blots shown in A. CPI-17 relative to PP1 are given as x-fold increase compared to control. The

ratio of control was set to 1. **(C).** Phosphatase activity of immunoprecipitated phosphatase, measured in the absence or presence of 0.5 µM recombinant inhibitor 2 (rec. I-2). PP1 activity is given as % increase compared to control. The mean of PP1 activity of control was set to 100%. Data are means ± SD of 5 separate experiments with independent cell preparations. $^{*}P < 0.05$. n.s.: not significantly different.

It is reported that the interaction of CPI-17 with PP1 is controlled by its phosphorylation at T38. Therefore it was analyzed whether the reduction of co-immunoprecipitation of CPI-17 with PP1 was accompanied by a decrease of CPI-17 phosphorylation. In accordance with the reduction in co-immunoprecipitation of CPI-17 with PP1, FSK caused a dephosphorylation of CPI-17 at T38 by 50% compared to control within 10 minutes (Fig. 3.9), whereas thrombin increased CPI-17 phosphorylation to 150%. FSK abrogates this thrombin effect on CPI-17 phosphorylation.

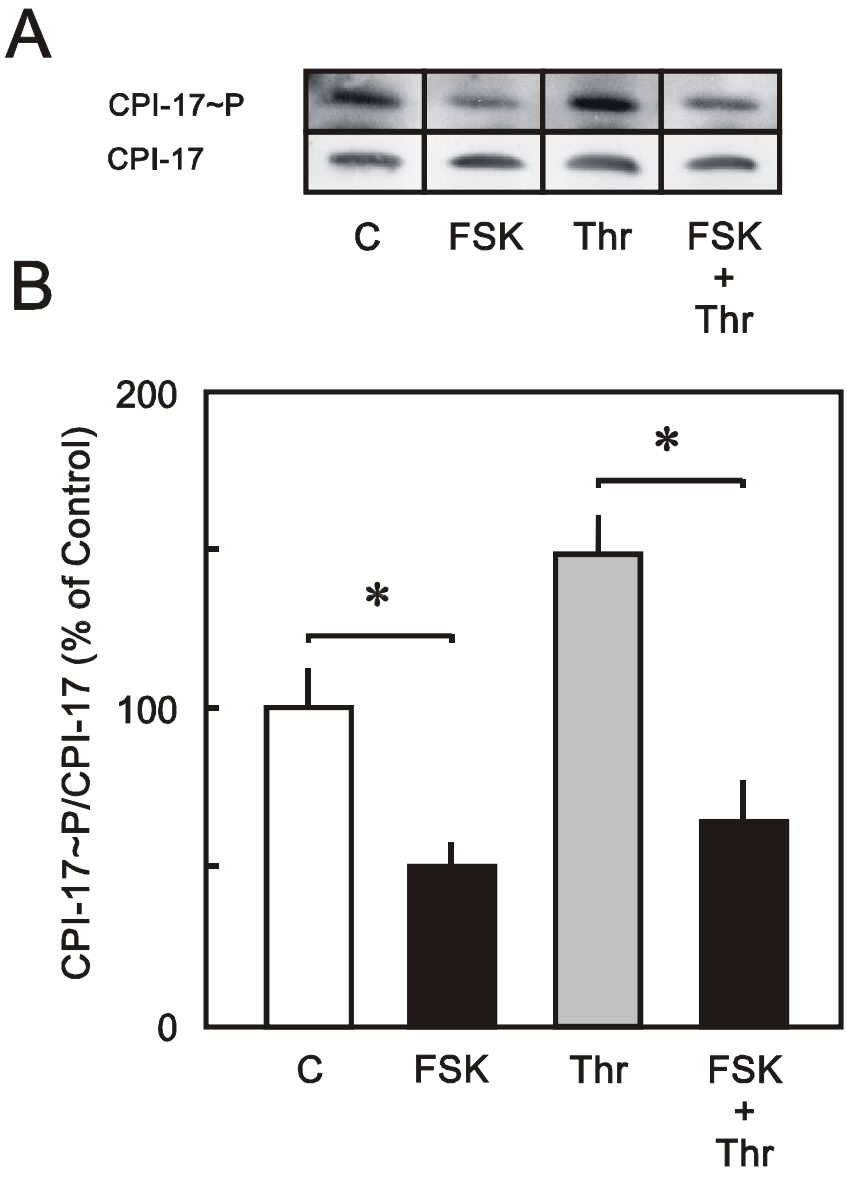

Fig. 3.9 Effect of forskolin (FSK), thrombin (Thr) or FSK plus Thr on CPI-17 phosphorylation at threonine 38 (T38). Endothelial cells were treated with FSK (5μM), Thr (0.2 U/ml), FSK plus Thr or vehicle (C; control) for 10 minutes. **(A)** Representative western blots with an anti-phospho-T38 CPI-17 and pan-specific anti-CPI-17 antibody. **(B)** Densitometric analysis of western blots shown in A. CPI-17 phosphorylation relative to total CPI-17 is given as % of control. The ratio of control was set to 100%. Data are means ± SD of 5 separate experiments with independent cell preparations. $^{*}P < 0.05$.

Furthermore it was analyzed whether FSK-induced dephosphorylation of CPI-17 is due to an inhibition of RhoA/Rock pathway. For that reason endothelial cells were incubated in presence of Y27632 to inhibit Rock. At 10 µM, an optimum concentration to obtain maximum effects on MLC dephosphorylation, it reduced CPI-17 phosphorylation to approximately 50% after 10 minutes. (Fig. 3.10). Addition of 20 µM Y27632 did not further reduce CPI-17 phosphorylation significantly. However, simultaneous addition of FSK plus 10 µM Y27632 reduced CPI-17 phosphorylation to almost zero.

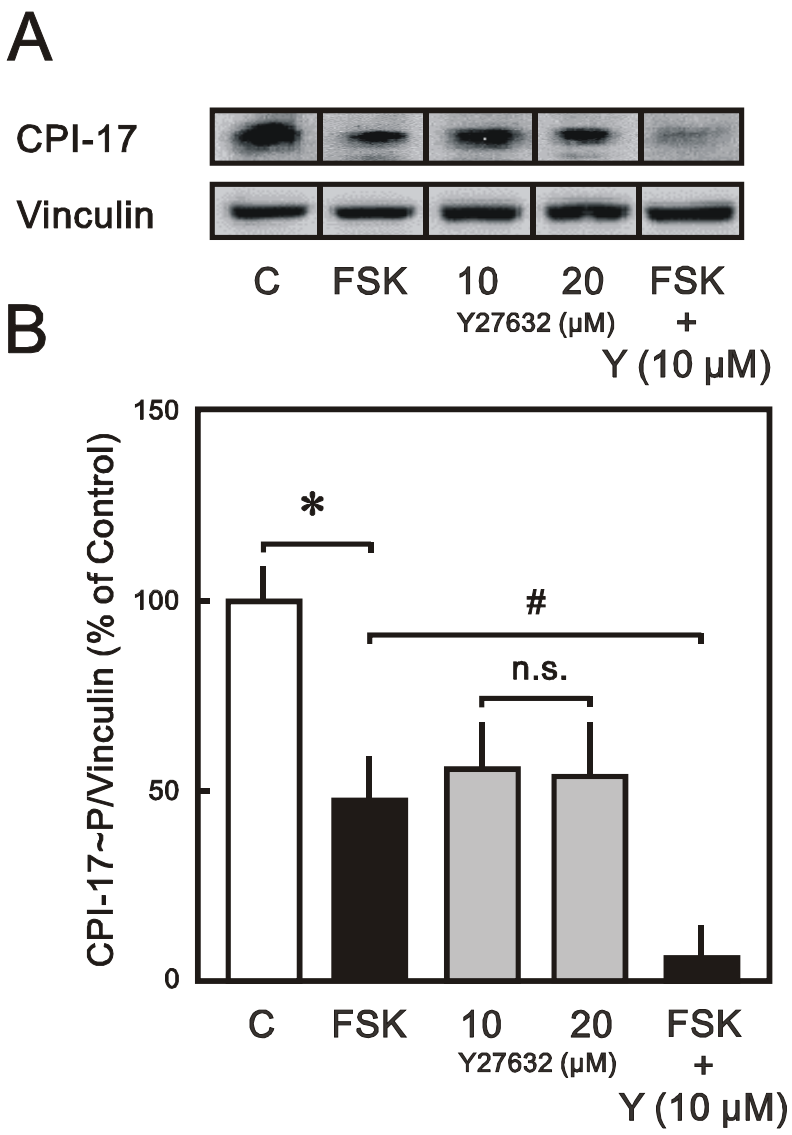

Fig. 3.10 Effects of forskolin (FSK), Y27632 (Y), or FSK plus Y on endothelial CPI-17 phosphorylation. Endothelial cells were exposed to FSK (5 μM), Y (10 or 20 μM), FSK plus Y (10 μM) or vehicle (C; control). **(A)** Representative western blots with an anti-phospho-T38 CPI-17 and anti-vinculin antibody. **(B)** Densitometric analysis of western blots shown in A. CPI-17 phosphorylation relative to vinculin is given as % of control. The ratio of control was set to 100%. Data are means ± SD of 5 separate experiments with independent cell preparations. $^{*}P < 0.05$; $^{\#}P < 0.05$. n.s: not significantly different.

3.7 Effect of CPI-17 downregulation on endothelial permeabiity

To analyze the role of CPI-17 in endothelial barrier function, the content of endogenous CPI-17 was reduced by gene silencing. Treatment of endothelial cells with CPI-17-specific siRNA caused a significant reduction in the amount of CPI-17 protein compared to the control siRNA treatment (Fig. 3.11A). Albumin permeability of endothelial monolayers of CPI-17-depleted cells in absence and presence of thrombin was determined compared to cells treated with non-specific control siRNA. CPI-17 depletion did not affect basal permeability. However, in CPI-17 depleted cells the maximum effect of thrombin on permeability after 10 minutes was reduced by 35% compared to the corresponding effect on endothelial cells treated with non-specific control siRNA (Fig. 3.11B).

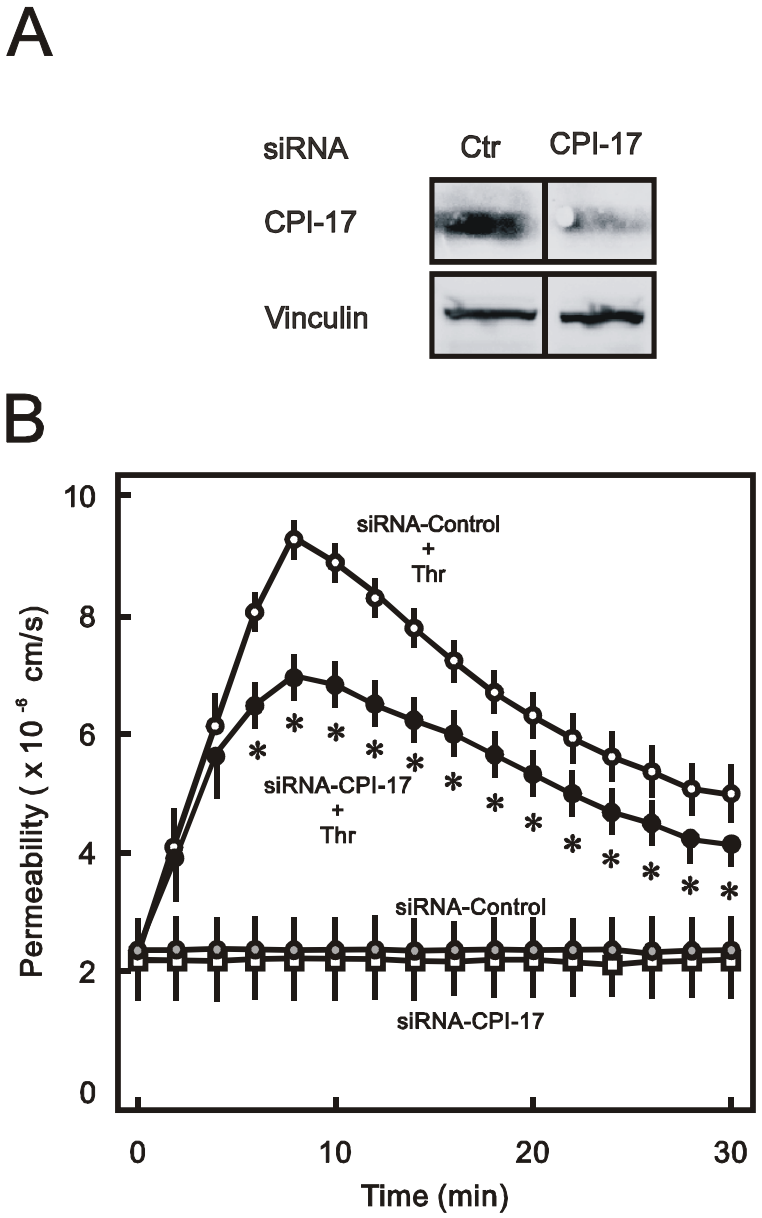

Fig. 3.11 Effect of thrombin (Thr) on albumin permeability of endothelial monolayers treated with CPI-17 or control siRNA. **(A)** Representative western blots with an anti-CPI-17 antibody or an anti-vinculin antibody. **(B)** Effect of Thr (0.2 IU/ml) on albumin permeability. Data are means ± SD of 5 separate experiments with independent cell preparations. At time points between 6 and 30 minutes, the albumin permeability in the presence of Thr in CPI-17 siRNA treated monolayers is significantly different from the control siRNA treated monolayers; $^{*}P < 0.05$.

4 Discussion

4.1 Main Findings

In the present study the molecular targets of the cAMP/PKA pathway controling the activation state of the endothelial contractile machinery, an important determinant of endothelial barrier function, were investigated. FSK, a direct activator of adenylyl cyclase, reduced basal permeability as well as antagonized thrombin-induced hyperpermeability. These effects on permeability were accompanied by related changes in MLC phosphorylation, the key regulator of the contractile machinery in endothelial cells. The effects of forskolin on both parameters were abolished by a specific inhibitor of PKA, PKI. PKI is a small (22 amino acid) peptide, and constitutes the inhibitory domain of PKI-protein (a 75 amino acid small protein), isolated originally from rabbit muscle (Scott et al., 1985). It can specifically bind to the catalytic subunit of PKA (Walsh et al., 1971) and provides greater selectivity to PKA inhibition than the pharmacological agents. These data show that in the cell model used in this study, the effects of activation of adenylyl cyclase on endothelial barrier function and the contractile machinery are mediated by PKA.

The present study focuses on the regulation of myosin light chain phosphatase. The major findings are (1) that activation of adenylyl cyclase by FSK reduces basal permeability and MLC phosphorylation. It also antagonizes the effect of thrombin on both parameters. (2) FSK induces the recruitment of the catalytic subunit PP1 and the myosin targeting subunit MYPT1 to myosin. This recruitment leads to an increase in phosphatase activity of the formed complex. (3) FSK reduces the inhibitory phosphorylation of MYPT1 at threonine 850 and also antagonizes the thrombin-induced phosphorylation at that site. (4) FSK reduces the interaction of PP1 with the endogenous inhibitor CPI-17. This reduction is accompanied by dephosphorylation of CPI-17 at its activation site threonine 38. FSK also antagonizes the thrombin effect on this endogenous inhibitor of PP1. The data of the present study shows that FSK can cause activation of the MLCP by two principal mechanisms: it induces the assembly of the MLCP holoenzyme complex and its recruitment to myosin, and activates the phosphatase by dephosphorylation and release of the regulatory inhibitor CPI-17.

It is well known that agents increasing intracellular levels of cAMP relax both endothelial and smooth muscle cells (Morel et al 1989; McDaniel et al., 1994; Stull et al., 1991). However, the target sites of cAMP/PKA on the contractile apparatus is still not clear. The phosphorylation level of MLC, the key regulator of contractile activity, is controlled by the balanced activities of at least two enzymes: the Ca^{2+}-calmodulin dependent myosin light chain kinase (MLCK) and myosin light chain phosphatase. Studies analyzing the mechanism of cAMP/PKA on MLCK inhibition have mainly focused on the Ca^{2+} lowering effect of cAMP in non-vascular smooth muscle cells. Recently Azam and co-workers (2007) have shown that cAMP relaxes vascular smooth muscle cells without affecting Ca^{2+} homeostasis. However, MLCK may also be modulated by direct PKA-dependent phosphorylation. In line with this concept, it has been reported that in smooth muscle cells PKA can phosphorylate MLCK (de Lanerolle et al., 1984). However, there is no report that this phosphorylation also affects the activity of MLCK.

Studies investigating the effects of cAMP on endothelial permeability found no alterations on Ca^{2+} homeostais, suggesting that cAMP protects endothelial barrier in an Ca^{2+}-independent manner (Carson et al., 1989). Garcia and co-workers (1997) reported that augmentation of cAMP levels increased phosphorylation and reduced the activity of MLCK endothelial cells. In contrast, Goeckeler and Wysolmerski (2005) have demonstrated that activation of cAMP/PKA pathway does not lead to phosphorylation of MLCK and does not affect the activity of MLCK. Previous data from our laboratory (Bindewald et al., 2004) has shown that maneuver increasing the cellular cAMP levels can lead to slight inhibition of MLCK activity in porcine aortic endothelial cells. However, this reduction in MLCK activity can not explain the increase in the velocity of MLC dephosphorylation in response to the elevation of cAMP level. Thus, inhibition of MLCK is of minor, if any, importance for the cAMP/PKA-induced inactivation the contractile machinery.

4.2 MLCP holoenzyme complex formation and activation

If the MLCK inhibition can be ruled out, activation of the MLCP is the most obvious target of the cAMP/PKA pathway to reduce the activation state of the contractile machinery. The activation of MLCP requires the assembly of the MLCP holoenzyme complex and its recruitment to phosphorylated MLC (Terrak et al.,

2004). In the present study, major components of the holoenzyme complex, i.e. PP1 and MYPT1, were found to be associated with MLC already under the basal conditions in non-stimulated cells. This recruitment was demonstrated by co-immunoprecipitation with specific antibodies, targeting both components of MLCP, MYPT1 and PP1. FSK increased the recruitment of PP1 to MYPT1. The binding of MLC to the MYPT1 containing complex was also increased. Western blot analysis showed a 4.5-fold increase in MLC/MYPT1 association as compared to a 2.5-fold increase in PP1/MYPT1 interaction. Irrespective of the possible differences in non-linearities of co-immunoprecipitations for MLC and PP1, the increase in MLC association with the MLCP complex correlates with the dephosphorylation of MLC, suggesting an increase in affinity of MLCP holoenzyme for MLC. Measurement of phosphatase activity of the immunoprecipitated MLCP complexes showed that the PP1 activity is increased corresponding to its binding to MYPT1. This indicates that recruitment of PP1 to MYPT1 is of central importance for the activation of the MLCP complex by the cAMP/PKA pathway.

The activity of the phosphatase in the immunoprecipitated complex was completely blocked by addition of recombinant inhibitor-2 (rec. I-2), which has been shown to specifically abrogate PP1 activity in phosphatase assays (Härtel et al., 2007). This supports the assumption that the increase in phosphatase activity is due to an increase in the MLCP specific phosphatase PP1, and excludes the recruitment of other phosphatases.

As a specific substrate of MLCP, MLC co-immunoprecipitates with MYPT1 and PP1. However, in this study it remains unclear as to what extent this is due to a direct interaction of MYPT1 with MLC or with the catalytic subunit PP1. The co-immunoprecipitation of MLC gives rise to the question whether the immunoprecipitates could contain even other phosphatases attached to the actin part of actomyosin. The close correspondence between the recruitment of PP1 into the complex and the rec. I-2-sensitive inhibition of its activity, however, renders these possible contaminants of minor importance.

4.3 Forskolin induced MLCP complex formation is independent of RhoA/Rock pathway

Activation of the RhoA/Rock pathway is one of the most important regulatory steps in basal, and agonist-mediated inhibition of MLCP. RhoA, a member of the

Ras superfamily, has been implicated as the Ca^{2+}-independent regulator of non-muscle and smooth muscle cell contraction. RhoA cycles between its inactive (GDP-bound) and active (GTP-bound) form (Nobes and Hall, 1995). Activation of RhoA leads to its translocation to the cell membrane (Takaishi et al., 1996). The GTP-bound form of RhoA then interacts with the downstream effectors and transmits the signals. RhoA regulates cellular tension through its effector kinase, RhoA-dependent kinase (Rock). Active Rock catalyzes the phosphorylation of MYPT1 and is assumed to cause inactivation of MLCP. In smooth muscle cells it was also shown that Rock can directly phosphorylate the regulatory MLC (Somlyo and Somlyo 2003, Fukata et al 2001). Phosphorylation of either substrate results in a net increase in MLC phosphorylation, activation of the contractile machinery and contraction.

It is well known that the phosphorylation state of MLC is regulated antagonistically by the cAMP/PKA and RhoA/Rock pathways (Essler et al., 2000). Activation of cAMP/PKA pathway leads to a decrease while activation of RhoA/Rock pathway leads to an increase in phosphorylation of MLC in smooth muscle cells as well as in endothelial cells. Studies both *in vitro* and *in vivo* (Ellerbroek et al., 2003; Lang et al., 1996; Essler et al., 2000) have shown that PKA can phosphorylate RhoA and this phosphorylation has been proposed as a molecular mechanism by which cAMP directly inactivates RhoA or indirectly RhoA effectors. In a recent study Goeckeler and Wysolmerski (2005) have shown that in endothelial cells cAMP/PKA activation led to RhoA phosphorylation accompanied by a reduction in its activity. Qiao et al., (2003) showed that cAMP inhibited thrombin-induced RhoA activation in microvascular endothelial cells. Consistent to these previous reports, in the present study it was confirmed that exposure of endothelial cells to FSK resulted in a 50% inhibition of RhoA translocation to the membrane under basal conditions. FSK completely blocked RhoA translocation to the membrane in thrombin stimulated cells.

Therefore, in context of the study, the question was analyzed whether cAMP/PKA mediates its effect on MLCP complex formation via inhibition of the RhoA/Rock pathway and/or via other, yet unknown mechanisms. For that reason Rock was blocked by a specific pharmacological inhibitor Y27632 (Fu et al., 1998), and the effect of cAMP/PKA on MLC phosphorylation and assembly of the MLCP was determined. Inhibition of Rock alone led to a partial reduction of MLC

phosphorylation. Stimulation of cAMP/PKA pathway in Rock inhibited cells reduced MLC phosphorylation to almost zero. Immunoprecipitation experiments in the presence of Rock inhibitor showed that Rock inhibition does not lead to recruitment of PP1 to MYPT1. This indicates that RhoA/Rock inhibition does not enhance MLCP complex formation and that cAMP/PKA-induced assembly of MLCP complex most probably is independent of Rock inhibition. Recently, we have shown that extracellular ATP can also induce MLCP holoenzyme complex formation in endothelial cells (Härtel et al., 2007). Interestingly, ATP does not inhibit RhoA activation in endothelial cells (Gündüz et al., 2003), indicating that induction of MLCP complex formation by ATP is also independent of RhoA inhibition. In another study Ruegg et al., (1981) have shown that in smooth muscle cells, cAMP and cGMP can activate MLCP independent of RhoA/Rock inhibition. Similarly Surks and co-workers (1999 and 2003) demonstrated that PKG can directly interacts with MYPT1 and activates MLCP. This suggests that RhoA/Rock-independent induction of MLCP complex formation by cAMP/PKA might involve a direct interaction of PKA with MLCP, however, there is no evidence for this interaction untill now and further studies are required to investigate this interaction.

4.4 MYPT1 dephosphorylation

A second level of regulation of MLCP activity is the phosphorylation state of MYPT1 at two threonine residues, T696 and T850. It is generally accepted that in smooth muscle cells the phosphorylation of these two sites is regulated mainly by Rock and to some extent by other kinases causing MLCP inactivation and/or dissociation of MLCP from myosin (Muranyi et al., 2002; Feng et al., 1999; McDonald et al., 2001; Velasco et al 2002). It has been shown recently, that stimulation of endothelial cells with thrombin induces a Rock-mediated increase in phosphorylation of MYPT1 at T850 and T696 (Birukova et al., 2004, Härtel et al., 2007). The results of the present study demonstrate that activation of the cAMP/PKA pathway antagonizes the effect of thrombin at these strategic sites of MYPT1, since FSK could completely abolish the effect of thrombin on T696 and T850 phosphorylation. Furthermore RhoA translocation studies and pulldown assays show that FSK effectively blocks RhoA translocation and activation under basal conditions as well as thrombin induced RhoA translocation and activation.

This indicates that cAMP/PKA induced MYPT1 dephosphorylation involves inhibition of RhoA/Rock pathway.

4.5 cAMP/PKA signaling inactivates CPI-17

Data from smooth muscle cells shows that in addition to MYPT phosphorylation, the activity of the MLCP complex can be controlled by variety of other mechanisms. Today several small molecular weight endogenous inhibitory proteins are known to regulate MLCP activity by direct interaction with PP1 catalytic subunit. One of the most prominent and specific endogenous inhibitor of PP1 is a small protein CPI-17 (Eto et al., 1995), which initially was assumed to be expressed only in smooth muscle cells. However, a recent report by Kolosova and co-workers (2004) shows the expression of CPI-17 also in endothelial cells. CPI-17 can inhibit MLCP holoenzyme complex as well as PP1 catalytic subunit (Eto et al., 1995; 1999). Most of our understanding about CPI-17-induced inhibition of MLCP and PP1 are from studies in smooth muscle cells. It has been proposed that inflammatory mediators (e.g. histamine and thrombin)-induced phosphorylation of CPI-17 at threonine 38 (T38) potentiates its MLCP/PP1-inhibitory activity both in smooth muscle cells and endothelial cells (Kitazawa et al., 2000; Kolosova et al., 2004) but precise mechanism is still unknown. Structure activity study (Ohki et al., 2001) shows that phosphorylation of CPI-17 at T38 leads to conformational changes which may expose an inhibitory motif to the PP1 catalytic subunit. Accumulating evidence shows that cAMP and cGMP exert stimulating effects on MLCP by acting on more than a single target (Somlyo and Somlyo, 2003). In the present study, it was analyzed whether cAMP/PKA signaling mechanism can exert its effect on MLCP via direct influence on the PP1 catalytic subunit were examined. Co-immunoprecipitation experiments show for the first time the existence of CPI-17/PP1 complexes in endothelial cells. Activation of cAMP/PKA pathway reduced the interaction of CPI-17 with PP1 and led to dissociation of the CPI-17/PP1 complex, with a corresponding increase in PP1 activity. Stimulation of endothelial cells with thrombin induced CPI-17/PP1 complex formation and a corresponding decrease in PP1 activity. This complex formation and MLCP inactivation was effectively blocked by FSK.

Phosphorylation of CPI-17 has been reported to induce its inhibitory activity

towards PP1 (Kitazawa et al., 2000). In vitro studies indicate that CPI-17 can be phosphorylated at T38 and hence be activated by multiple kinases including PKC (Eto et al., 1995) and Rho kinase (Koyama et al., 2000). In intact endothelial and non-endothelial cells, only PKC and Rock have been reported to phosphorylate CPI-17 (Kolosova et al., 2004; Pang et al., 2005; Sakai et al., 2006). In the present study it was shown by western blot analysis that FSK reduces basal CPI-17 phosphorylation by 50%. This dephosphorylation corresponds with a decrease in CPI-17/PP1 complex to the same extent and a 60% 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 40% reduction in phosphatase activity.

To this point, the results left the question open whether the effect of the cAMP/PKA pathway on CPI-17 phosphorylation is due to an interference with the RhoA/Rock pathway or not. This question also can be addressed for the “basal” conditions of this study, since these imply also a basal activity of the latter pathway as indicated by some membrane binding of RhoA and the ability of the Rho-kinase inhibitor Y27632 to lower MYPT1 phosphorylation under basal conditions. To answer this question, the effect of Rho kinase inhibitor Y27632 was studied on CPI-17 phosphorylation. At maximal effective concentrations, Y27632 induced dephosphorylation of CPI-17 to 60% of the basal level. Copresence of Y27632 and FSK lead to nearly complete CPI-17 dephosphorylation. This indicates the existence of at least two mechanisms regulating the phosphorylation state of CPI-17. One is Rock-dependent and the other is -independent.

A recent study in smooth muscle cells has shown that activation of integrin linked kinase (ILK) via PI3K pathway can cause phosphorylation of CPI-17 in a Rock independent manner (Huang et al., 2006). Furthermore Stevens et al., (2004) have shown in human platelets that thrombin induced activation of ILK in a PI3K dependent manner. However, there is no study showing that cAMP/PKA inhibits ILK.

Taken together, this study indicates that in endothelial cells cAMP/PKA pathway can activate myosin phosphatase by at least two mechanisms: inhibition of Rock-dependent phosphorylation of MYPT1, CPI-17 phosphorylation and as shown here for the first time, by inhibition of CPI-17 interaction with PP1.

4.6 CPI-17 as mediator of thrombin induced barrier failure

Finally, experiments using siRNA directed against CPI-17, illustrated a functional role of CPI-17 and PP1 activation in thrombin induced barrier failure. Specific siRNA down-regulated CPI-17 protein by approximately 70%. This down-regulation attenuated the thrombin effect on endothelial barrier function by 35%. This is in accordance with a 50% increase in CPI-17 phosphorylation, a 1.5-fold increase in CPI-17/PP1 complex formation and a 40% decrease in PP1 activity.

Changes in macromolecular permeability result from complex changes in the contractile activation and cell-cell and cell matrix adhesion. The fact that downregulation of an endogenous inhibitor of the catalytic subunit of the MLCP complex, as only one element in complex mechanism can lead to a one-third-reduction of the effect, demonstrates the relative importance of this regulatory protein. In a previous report Kolosova and colleagues (2004) showed that in endothelial cells the effect of thrombin had no profound effect on CPI-17 phosphorylation and depletion of CPI-17 in these cells could not attenuate the thromin effect significantly. This might have been due to the use of higher passage endothelial cells in the study. In this study either primary endothelial cells or cells of passage one were used.

In summary, activation of the cAMP/PKA pathway protects the endothelial barrier function mainly via activation of MLCP. The novel findings are that cAMP/PKA pathway targets the MLCP complex at multiple sites. These include: (i) inhibition of upstream inactivating pathway e.g. Rho/Rock, thus shielding from inactivation (ii) induction of MLCP holoenzyme complex formation, thus providing quantitively increased levels of active MLCP to the contractile machinery, and (ii) activation of the catalytic subunit PP1 mainly by inactivating the inhibitory CPI-17. This activation of MLCP overrides the inhibitory pathways induced by inflammatory mediators.

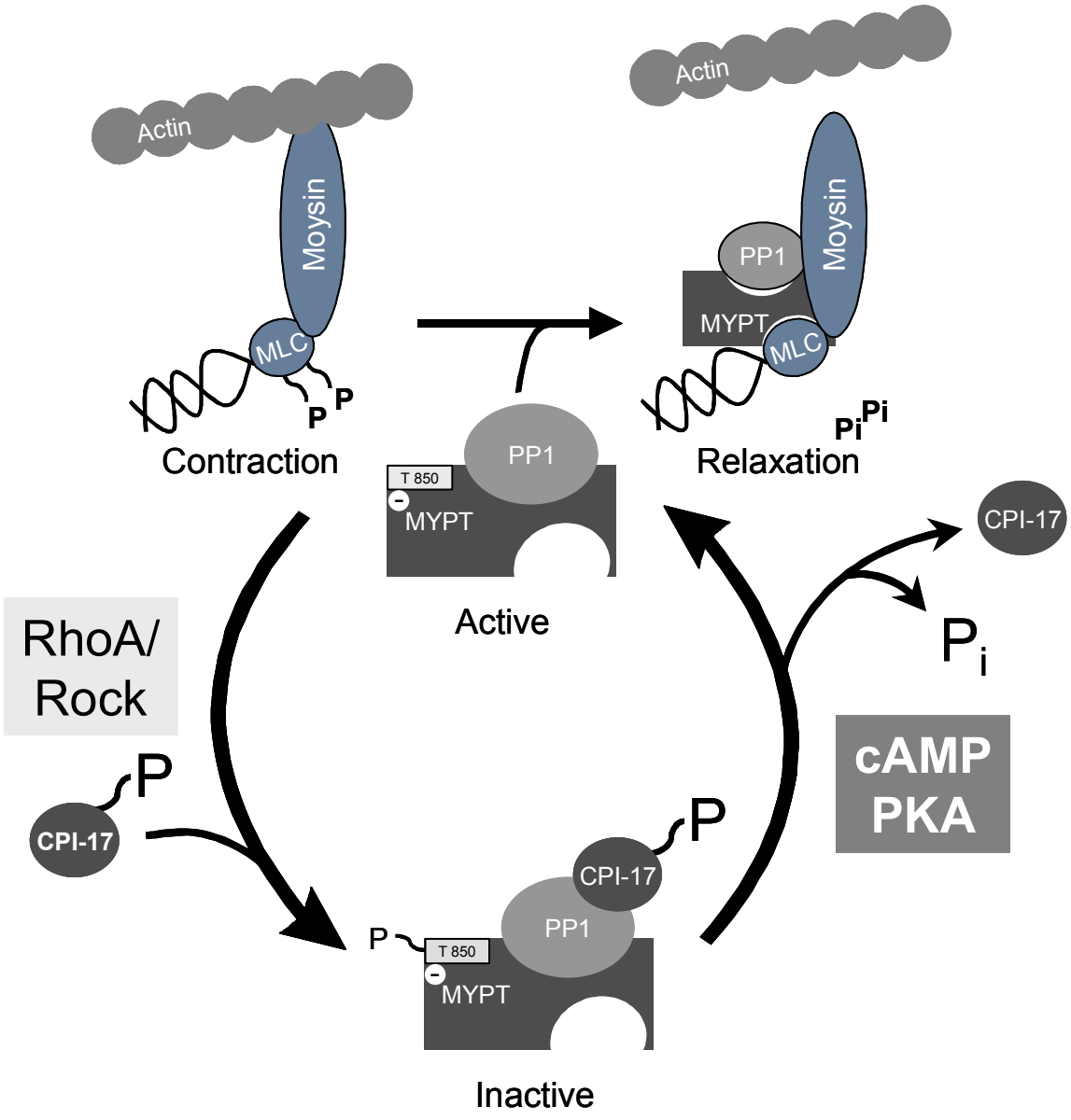

Fig. 4.1 Schematic presentation of inactivation of contractile machinery via activation of myosin light chain phosphatase (MLCP) by cAMP/PKA pathway. Activation RhoA/Rock pathway by thrombin leads to phosphorylation of myosin phosphatase targeting subunit (MYPT1) at threonine 850(T850). It also leads to phosphorylation of CPI-17 and its increased interaction with PP1 catalytic subunit. This causes the inactivation of MLCP, an increas in phosphorylation of myosin light chain (MLC), actin myosin interaction and contraction. Activation of cAMP/PKA pathway leads to dephosphorylation of MYPT1 and an increase in recruitment of PP1 catalytic subunit and MYPT1 to myosin. Dephosphorylation of CPI-17 facilitates its dissociation from PP1 catalytic subunit. All these events cause activation of MLCP, dephosphorylation of MLC and endothelial cell relaxation.

4.7 Future perspective

Endothelial barrier dysfunction is a frequent cause of vascular leakage during inflammation. Elevation of cAMP can effectively block this leakage and is already being used in clinical situations with some limitations. β-adrenergic agonists in combination with phosphodiesterase inhibitors are applied to increase the cellular contents of cAMP. However, therapeutic use of β-adrenergic agonists and phosphodiesterase inhibitors leads to rapid desensitization of endothelial cells to these agents i.e. tachyphylaxis or tolerance, that leads to shortening the time of their effective application to reduce vascular leakage (Doorenbos et al., 1989; Droder et al., 1992). Secondly, endothelial cells from different vascular provenience do not respond similarly to cAMP elevating agents agents. Endothelial cells from microvasculature of the coronary and brain circulation do not respond with a reduction in permeability, but rather with an increase (Hempel et al., 1996; Palmer et al., 1986). Similarly, cAMP increasing agents have different effects on different cell types. It has been shown that in endothelial cells, β-adrenergic agonists lead to protetection against barrier failure, however, in the same animal increased cAMP levels only in smooth muscle cells leads to increased vascular leakage (Warren et al., 1993). These unwanted side effects of cAMP elevating agents limit their use in clinical conditions. The knowledge of the targets of cAMP/PKA pathway can harnessed for the development of more specific therapeutic strategies targeting endothelial cells. One such target could be CPI-17. Knockdown of this protein leads to a 1/3 reduction in the effect of thrombin. Development of specific pharmacological inhibitors against CPI-17 might be an important innovation to block vascular leakage during pathophysiological conditions.

5 References

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6 Summary

Endothelial barrier dysfunction is often the underlying cause of capillary leakage during pathophysiological conditions like inflammation and ischemia reperfusion. Maneuvers increasing intracellular levels of cAMP protect against imminent failure of endothelial barrier function induced by inflammatory mediators or ischemia-reperfusion. This protective effect is mainly due to an inactivation of the contractile machinery, a primary determinant of endothelial barrier function. The activation of the contractile machinery is regulated by the phosphorylation state of the regulatory myosin light chains (MLC), which is controlled by balanced but antagonistic activities of myosin light chain phosphatase (MLCP) and myosin light chain kinase (MLCK). Here the molecular mechanisms by which cAMP/PKA induced the activation of the MLCP leading to inactivation of the contractile machinery were analyzed. In cultured human umbilical vein endothelial cells activation of adenylyl cyclase by forskolin (FSK) reduced basal macromolecule permeability and caused dephosphorylation of MLC. It also antagonized thrombin-induced increase of both parameters. FSK stimulated the formation of MLCP holoenzyme complex, i.e. it induced the recruitment of the protein phosphatase 1 (PP1) catalytic subunit and myosin phosphatase targeting subunit (MYPT1) to myosin, and activation of the PP1 catalytic subunit. FSK inhibited the RhoA/Rock pathway leading to dephosphorylation of MYPT1. It caused dephosphorylation of the PP1 inhibitory protein CPI-17 at threonine 38 and its detachment from the PP1 catalytic subunit. FSK also blunted the thrombin-induced effect on MYPT1 and CPI-17 phosphorylation. Down regulation of CPI-17 by siRNA attenuated the thrombin effect on endothelial permeability by 35 %. The data of the present study show that stimulation of adenylyl cyclase causes assembly and activation of the MLCP holoenzyme complex and thereby stabilizes endothelial barrier function.

7 Zusammenfassung

Das Versagen der endothelialen Schrankenfunktion ist eine häufige Ursache für die Entstehung eines „*Capillary leakage*“ im Rahmen einer Entzündung oder Ischämie/Reperfusion. Manöver, die die intrazelluläre cAMP-Konzentration steigern, schützen vor einem drohenden Verlust der Schrankenfunktion. Dieser Schutzeffekt beruht hauptsächlich auf einer Inaktivierung des kontraktilen Apparates, der ein wesentlicher Faktor der endothelialen Schrankfunktion ist. Der Aktivierungszustand des kontraktilen Apparates wird durch den Phosphorylierungsgrad der regulatorischen leichten Kette des Myosins (MLK) geregelt, welche durch die antagonistische Wirkung der MLK-Phosphatase (MLKP) und MLK-Kinase (MLKK) kontrolliert wird.

In kultivierten humanen Endothelzellen der Nabelschnurvene reduziert die Aktivierung der Adenylylzyklase durch Forskolin (FSK) die basale Makromolekülpermeabilität und löst eine Dephosphorylierung der MLK aus. FSK antagonisiert auch die Thrombin-induzierte Steigerung beider Parameter. FSK stimuliert die Bildung des MLCP Holoenzymkomplexes, d.h. es induziert die Rekrutierung der Proteinphosphatase (PP1)-Untereinheit und der Myosin-Phosphatase-bindenden Untereinheit (MYPT1) an Myosin und aktiviert die PP1-Untereinheit. FSK hemmt den RhoA/Rock weg, was zu einer Dephosphorylierung von MYPT1 führt. Es induziert die Dephosphorylierung des PP1-inhibitorischen Proteins CPI-17 an Threonin 38 und seine Lösung von der PP1-Untereinheit. FSK vermindert auch die Thrombinwirkung auf die MYPT1 und CPI-17-Phosphorylierung. Die Herunterregulation von CPI-17 durch siRNA verminderte die Thrombin-induzierte Zunahme der endothelialen Permeabilität.

Die Daten der vorliegenden Untersuchung zeigen, dass die Stimulation der Adenylylzyklase die Assemblierung und Aktivierung des MLKP-Komplexes auslöst und dadurch die endotheliale Schranke stabilisiert und vor einem drohenden Versagen schützt.

8 Erklärung

„Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

Gießen, den..... .....

Muhammad Aslam

9 Appendix

9.1 Chemicals and consumables

6-amino hexanoic acid	Merck, Darmstadt, Germany
Acrylamide solution (40%; wt/vol)	Amersham Pharmacia, UK
Acrylamide	Carl Roth, Karlsruhe, Germany
Ammonium persulfate	SERVA, Heidelberg, Germany
Ammonium sulfate	Merck, Darmstadt, Germany
ATP	Roche, Mannheim, Germany
Benzonase®	Merck, Darmstadt, Germany
bFGF	PromoCell, Heidelberg, Germany
Bisacrylamide solution (2%; wt/vol)	Amersham Pharmacia, UK
Bisacrylamide	Carl Roth, Karlsruhe, Germany
Bovine serum albumin	Sigma-Aldrich, Steinheim, Germany
Bromophenol blue	Sigma-Aldrich, Steinheim, Germany
Caffein	Sigma-Aldrich, Steinheim, Germany
Calcium chloride	Merck, Darmstadt, Germany
Calyculin A	Calbiochem, Bad Soden, Germany
Cantharidin	Calbiochem, Bad Soden, Germany
Collagenase II	PAA Labs., Pasching, Austria
Complete® inhibitor cocktail	Roche, Mannheim, Germany
Culture dishes	BD, Heidelberg, Germany
Diethyl ether	Merck, Darmstadt, Germany
Dimethyl sulfoxide	Sigma-Aldrich, Steinheim, Germany
Di-sodium hydrogen phosphate	Carl Roth, Karlsruhe, Germany
Dithiothreitol	Amersham Pharmacia, UK
EDTA	Carl Roth Karlsruhe, Germany
EGTA	Boehringer, Mannheim
Endothelial cell basal medium® kit	PromoCell, Heidelberg, Germany
Endothelial cell growth supplement	PromoCell, Heidelberg, Germany
Eppendorf tubes (0.5, 1.5, 2 ml)	Eppendorf, Hamburg, Germany
Falcon tubes (50 ml, 12 ml)	BD, Heidelberg, Germany
FCS	PAA, Pasching, Austria
Filter papers	Biotech-Fischer, Reiskirchen, Germany

Forskolin	Calbiochem, Bad Soden, Germany
FuGENE® 6	Roche, Mannheim, Germany
Gentamycin	Gibco BRL, Eggenstein, Germany
Glass cover slips	Menzel, Braunschweig, Germany
Glycerol (100%)	Sigma-Aldrich, Steinheim, Germany
Glycerol (87%)	Amersham Pharmacia, UK
Glycine	Carl Roth, Karlsruhe, Germany
HBSS	PAA, Pasching, Austria
hEGF	PromoCell, Heidelberg, Germany
HEPES	Sigma-Aldrich, Steinheim, Germany
Human recombinant inhibitor 2	New England Biolabs, Frankfurt, Germany
Hydrocortisone	PromoCell, Heidelberg, Germany
Imidazole	Merck, Darmstadt, Germany
Magnesium chloride	Fluka, Switzerland
Magnesium sulfate	Merck, Darmstadt, Germany
Manganese chloride	Merck, Darmstadt, Germany
β-mercaptoethanol	Merck, Darmstadt, Germany
Methanol	Merck, Darmstadt, Germany
Millipore water	Millipore, Eschborn, Germany
Molecular weight marker	Sigma-Aldrich, Steinheim, Germany
Nitrocellulose membrane	Schleicher und Schuell, Dassel, Germany
Non-fat milk powder	Applichem, Darmstadt, Germany,
Nonidet P-40	Sigma-Aldrich, Steinheim, Germany
Okadaic acid	Calbiochem, Bad Soden, Germany
γ-[^{32}P]-ATP	Amersham Pharmacia, UK
Penicillin/streptomycin	Gibco BRL, Eggenstein, Germany
Phosphorylase-b	Sigma-Aldrich, Steinheim, Germany
Phosphorylase-kinase	Sigma-Aldrich, Steinheim, Germany
Pipette tips	Eppendorf, Hamburg, Germany
Pipettes	Eppendorf, Hamburg, Germany
PKI	Calbiochem, Bad Soden, Germany
PMSF	Sigma-Aldrich, Steinheim, Germany
Ponceau S solution	SERVA, Heidelberg, Germany
Potassium chloride	Merck, Darmstadt, Germany

Potassium dihydrogen phosphate	Merck, Darmstadt, Germany
Protein G-coated magnetic beads	Dynal, ASA, Oslo, Norway
Recombinant PP1	New England Biolabs, Frankfurt, Germany
Rhotekin-RBD	Upstate, USA
Rubber policeman	BD, Heidelberg, Germany
Scalpal (disposble)	Feather, Japan
See Blue® (pre-stained marker)	Invitrogen GmbH, Karlsruhe, germany
siRNA	Qiagen, Hilden, Germany
Sodium azide	Merck, Darmstadt, Germany
Sodium bicarbonate	Carl Roth, Karlsruhe, Germany
Sodium chloride	Carl Roth, Karlsruhe, Germany
Sodium di-hydrogen phosphate	Carl Roth, Karlsruhe, Germany
Sodium dodecyl sulfate	SERVA, Heidelberg, Germany
Sodium fluoride	Sigma-Aldrich, Steinheim, Germany
Sodium hydroxide	Carl Roth, Karlsruhe, Germany
Sodium orthovanadate	Sigma-Aldrich, Steinheim, Germany
Sodium thioglycolate	Sigma-Aldrich, Steinheim, Germany
Sterile filters (0.22 µm)	Sartorius, Goettingen, Germany
Sterile pipettes	BD, Heidelberg, Germany
Sucrose	Sigma-Aldrich, Steinheim, Germany
Super signal-west® (ECL solution)	Pierce biotech, Bonn, Germany
Syringes (20 ml, 2 ml)	BD, Heidelberg, Germany
TEMED	Sigma-Aldrich, Steinheim, Germany
Thrombin	Behring, Marburg, Germany
Transwell® membrane filters	Corning, NY, USA
Tricholoroacetic acid	Merck, Darmstadt, Germany
Tris base	Carl Roth, Karlsruhe, Germany
Tritone X-100	SERVA, Heidelberg, Germany
Trypsin-EDTA	Gibco-BRL, Eggenstein, Germany
Tween 20	Amersham Pharmacia, UK
Urea	Amersham Pharmacia, UK
Whatman® 3 MM filter paper	Millipore, Eschborn, Germany
Y27632	Calbiochem, Bad Soden, Germany

9.2 Antibodies

Primary antibodies

Antibodies	Source
CPI-17 (Rabbit IgG polyclonal)	Upstate, Charlottesville, VA, USA
Phospho CPI-17 (Rabbit IgG polyclonal)	Upstate, Charlottesville, VA, USA
MLC (Mouse IgM monoclonal)	Sigma, Steinheim, Germany
MYPT1 (Sheep IgG polyclonal)	Upstate, Charlottesville, VA, USA
Phospho MYPT696 (Rabbit IgG polyclonal)	Upstate, Charlottesville, VA, USA
Phospho MYPT850 (Rabbit IgG polyclonal)	Upstate, Charlottesville, VA, USA
PP1δ (Rabbit IgG polyclonal)	Upstate, Charlottesville, VA, USA
RhoA (Mouse IgG monoclonal)	Santa Cruz Biotechnology, Heidelberg, Germany
Vinculin (Clone hVIN-1) (Mouse IgG monoclonal)	Sigma, Steinheim, Germany

Secondary antibodies (HRP-labeled)

Antibody	Source
Anti Mouse IgG	BD Bioscience, Heidelberg, Germany
Anti Mouse IgM	Sigma, Steinheim, Germany
Anti Rabbit IgG	Upstate, Charlottesville, VA, USA
Anti Sheep IgG	Santa Cruz Biotechnology, Heidelberg, Germany

9.3 Laboratory instruments

Beckman Allegra 64R centrifuge	Beckman Coulter, USA
Beckman TL 100 ultracentrifuge	Beckman Coulter, USA
Blotting chambers	Biotech-Fischer, Reiskirchen, Germany
Electrophoresis apparatus	Biometra, Goettingen, Germany
Gel documentation system	Quantity One series, Bio-Rad, Munich, Germany
Glass ware	Schott, Mainz, Germany
Hamilton syringe	Hamilton, Bonaduz, Switzerland
Incubators	Heraeus, Hanau, Germany
Laminar flow hood	Heraeus, Hanau, Germany
Tri-Carb 1600 TR liquid scintillation counter	Packard Instrument Company, CT, USA
Magnet stirrer	Jahnke und Kunkel, Staufen, Germany
Magnetic rack	DYNAL, Oslo, Norway
Neubauer chamber	Superior, Marienfeld, germany
Phase contrast microscope	Olympus, Japan
pH-Meter	WTW-Weinheim, Germany
Photometer	Carl Zeiss, Jena, Germany
Power supply	Biometra, Goettingen, Germany
Rocker	Biometra, Goettingen, Germany
Table top (centrifuge)	Eppendorf, Hamburg, Germany
Vortexer	Heidolph, Kelheim, Germany
Water bath	Julabo, Seelbach, Germany
Water demineralisation unit	Millipore, Eschborn, germany

9.4 Solutions

2x-SDS sample buffer

Tris.HCl	250 mM
Glycerol	20 %
SDS	4 % (wt/vol)
DTT	1 mM (wt/vol)
β-mercaptoethanol	5 % (vol/vol)
Cantharidin	10 µM
Bromophenol Blue	0.001 % (wt/vol)

10x TBS

Tris/HCl	100 mM
NaCl	1.6 M
pH 7.4	

TBS Tween (TBST)

1x TBS	1000 ml
Tween 20	1 ml (0.1%, vol/vol)

10X PBS

Na_2HPO_4	0.1 M
KH_2PO_4	17 mM
NaCl	1.37 M
KCl	27 mM

0.1M Sodium Phosphate buffer (pH 7.4)

Soln. A (0.2 M sodium dihydrogen phosphate)

Dissolve 15.6 g sodium dihydrogen phosphate (dihydrate) in 500 mL dH20

Soln. B (0.2 M di sodium hydrogen phosphate)

Dissolve 17.8 g disodium hydrogen phosphate (dihydrate) in 500 mL dH20

Mix 57 ml of soln. A, 243 ml of soln. B with 600 ml of water. The pH would itself be adjusted to 7.4

Urea-glycerol gel buffer

Tris	240 mM
Glycine	276 mM

pH to 8.8 – 8.9

Urea-glycerol gel lysis buffer (10 ml)

Urea	554 mg (8.8 M)
Urea-gel buffer	975 µl
0.5 M DTT	225 µl (10 mM)
1 M Sodium thioglycolate	56.2 µl (5 mM)
100 mM Cantharidin	11.24 µl (10 µM)
6 M Imidazole (pH 8.8)	112.4 µl (60 mM)
Millipore water (qs)	10 ml
Bromophenol blue	0.001% (wt/vol)

Lysis buffer (Immunoprecipitation)

Tris/HCl pH 7.4)	50 mM
NaCl	150 mM
Triton X-100	1 %
Nonidet P - 40	0.5 %
EDTA	1 mM
EGTA	1 mM
NaF	20 mM
Na-orthovanadate	1.5 mM
DTT	10 mM
PMSF	0.5 mM

Complete® protease inhibitor cocktail

RhoA pulldown assay

Lysis buffer

HEPES	25mM
NaCl	150 mM
$MgCl_2$	5 mM
EDTA	1 mM
NaF	10 mM
Na-orthovanadate	2 mM
DTT	5 mM
Tritone-X100	0.5%
Glycerol	2 %
PMSF	0.5 mM

Complete® protease inhibitor cocktail

Wash buffer

Tris/HCl (pH 7.4)	25mM
Triton X-100	1%
NaCl	150 mM
$MgCl_2$	10 mm
PMSF	0.5 mM

Complete® protease inhibitor cocktail

Lysis buffer (Membrane fractionation)

Tris/HCl (pH 7.4)	5 mM
Sucrose	250 mM
EDTA	5 mM
$MgCl_2$	1 mM
NaCl	5 mM
DTT	10 mM
PMSF	0.5 mM

Complete® protease inhibitor cocktail

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Muhammad Aslam

11 Curriculum vitae

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(2001-2003) M.Phil. in Molecular Biology
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(1995-2000) Bachelor of Pharmacy
College of pharmacy, University of the Punjab, Lahore, *Pakistan.*

12 Publications

1. Härtel FV, Rodewald CW, **Aslam M,** Gündüz D, Hafer L, Neumann J, Piper HM, Noll T (2007) Extracellular ATP induces assembly and activation of myosin light chain phosphatase complex in endothelial cells. **Cardiovasc Res;** 74:487-96

2. Gündüz D, Kasseckert SA, Härtel FV, **Aslam M,** Abdallah Y, Schafer M, Piper HM, Noll T, Schäfer C (2006) Accumulation of extracellular ATP protects against acute reperfusion injury in rat heart endothelial cells. **Cardiovasc Res.** 71: 764-73

3. Pfeil U, Paddenberg R, **Aslam M,** Noll T, Yu S, Hsu T, Chang CL, Park J, Weissmann N, Quanz K, Faulhammer P, Goldenberg A, Kummer W (2007) Intermedin – A novel pulmonary peptide upregulated by hypoxia. (submitted)

4. **Aslam M,** Härtel FV, Gündüz D, Piper HM, Noll T cAMP stabilizes endothelial barrier by activation and assembly of the myosin light chain phosphatase holoenzyme and inhibition of CPI-17. (Submitted)

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1. **Aslam M,** Härtel FV, Gündüz D, Pfeil U, Hsu SY, Kummer W, Piper, HM, Noll T (2007) Intermedin induces failure of microvascular endothelial barrier via derangement of actin cytoskeleton and dephosphorylation of focal adhesion proteins. **Circulation;** 116(16):II-204 (American Heart Association-Scientific Session, Nov. 2007, Orlando, FL USA)

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10. Härtel FV, **Aslam M**, Rodewald CW, Gündüz D, Noll T (2006) NO antagonisiert das Thrombin-induziert endotheliale Schrankenversagen durch Hemmung des kontraktilen Apparates. (72nd Annual Meeting of German Cardiac society, April 2006, Mannheim, Germany)

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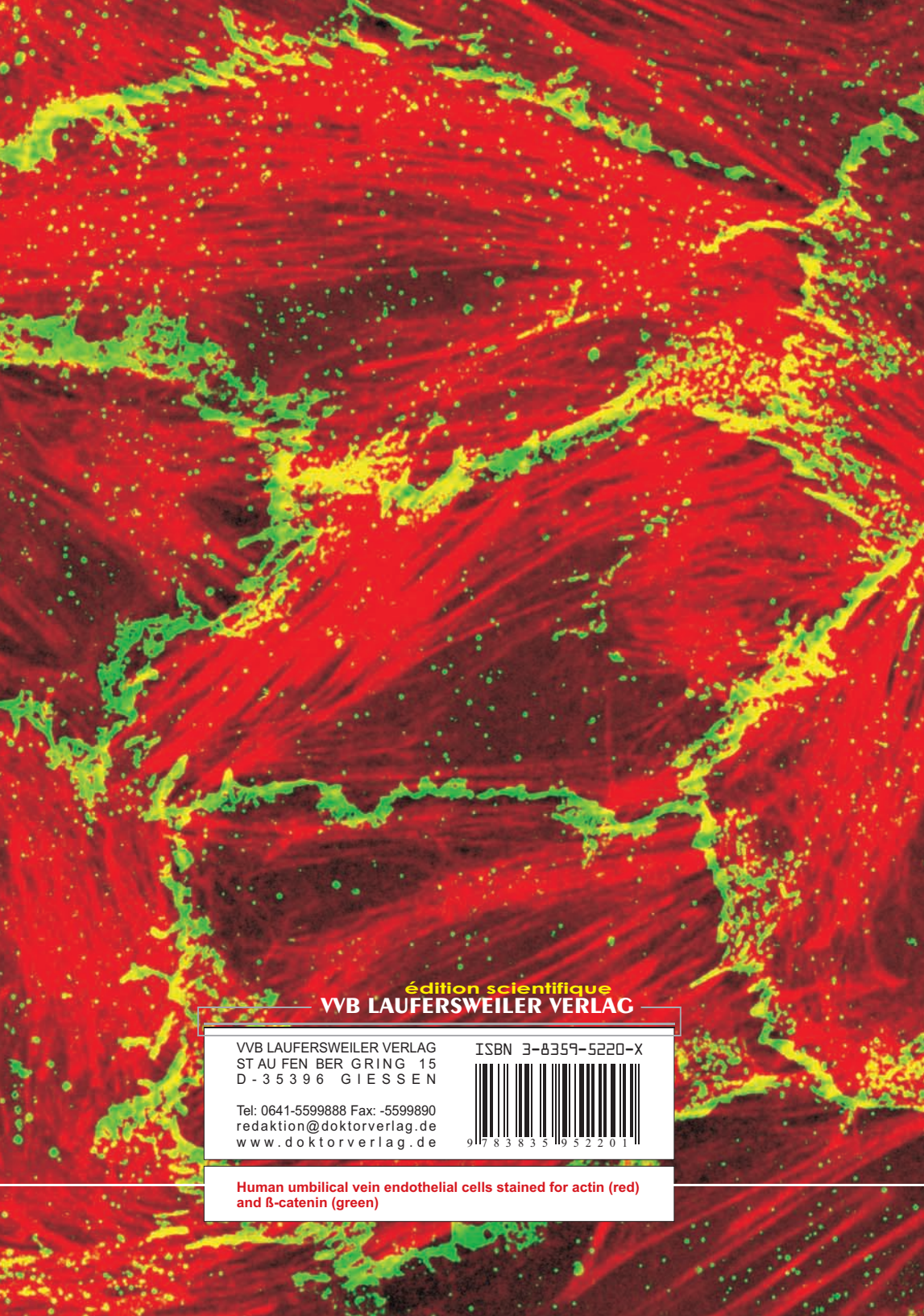

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Human umbilical vein endothelial cells stained for actin (red) and ß-catenin (green)