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Jens Waschke · Nikola Golenhofen
Teymuras V. Kurzchalia · Detlev Drenckhahn

Protein kinase C-mediated endothelial barrier regulation is caveolin-1-dependent

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Abstract Protein kinase C (PKC) is activated in response to various inflammatory mediators and contributes significantly to the endothelial barrier breakdown. However, the mechanisms underlying PKC-mediated permeability regulation are not well understood. We prepared microvascular myocardial endothelial cells from both wild-type (WT) and caveolin-1-deficient mice. Activation of PKC by phorbol myristate acetate (PMA) (100 nM) for 30 min induced intercellular gap formation and fragmentation of VE-cadherin immunoreactivity in WT but not in caveolin-1-deficient monolayers. To test the effect of PKC activation on VE-cadherin-mediated adhesion, we allowed VE-cadherin-coated microbeads to bind to the endothelial cell surface and probed their adhesion by laser tweezers. PMA significantly reduced bead binding to 78 ± 6% of controls in WT endothelial cells without any effect in caveolin-1-deficient cells. In WT cells, PMA caused an 86 ± 18% increase in FITC-dextran permeability whereas no increase in permeability was observed in caveolin-1-deficient monolayers. Inhibition of PKC by staurosporine (50 nM, 30 min) did not affect barrier functions in both WT and caveolin-1-deficient MyEnd cells. Theses data indicate that PKC activation reduces endothelial barrier functions at least in part by the reduction of VE-cadherin-mediated adhesion and demonstrate that PKC-mediated permeability regulation depends on caveolin-1.

J. Waschke (✉) · D. Drenckhahn
Institute of Anatomy and Cell Biology,
Julius-Maximilians-University,
Koellikerstr. 6, 97070 Würzburg, Germany
E-mail: jens.waschke@mail.uni-wuerzburg.de
Tel.: +49-931-312384
Fax: +49-931-312712

N. Golenhofen
Department of Anatomy and Cell Biology, University of Ulm,
Albert-Einstein Allee 11, 89081 Ulm, Germany

T. V. Kurzchalia
Max Planck Institute for Molecular Cell Biology and Genetics,
Pfotenhauerstr. 108, 01307 Dresden, Germany

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Introduction

It is well established that inflammatory mediators increase permeability in postcapillary venules by formation of intercellular gaps (Michel and Curry 1999). This requires opening of several types of endothelial intercellular junctions such as the barrier-sealing tight junction and the mechanical strength-providing adherens junction (Baldwin and Thurston 2001). However, the mechanisms underlying the regulation of intercellular junctions which finally lead to the formation of inflammatory gaps are not well understood.

Protein kinase C (PKC) represents a family of serin/threonine kinases which are activated in response to several inflammatory mediators like bradykinin, platelet-activating factor or thrombin (Yuan 2002). PKC has been shown to significantly contribute to the endothelial barrier breakdown induced by these agents (Murray et al. 1991; Ramirez et al. 1996; Sandoval et al. 2001). Moreover, direct stimulation of PKC by phorbol esters also reduced barrier functions in several studies (Johnson et al. 1990; Wolf et al. 1990; Mehta et al. 2001; Moy et al. 2004). However, the mechanisms by which PKC regulates endothelial barrier functions are not well characterized at present (Yuan 2002). It has been shown that PKC is associated with caveolae (Lisanti et al. 1994; Liu et al. 2002). Caveolae are specialized invaginations of the plasma membrane which are stabilized by caveolins and thought to be important scaffolding structures involved in the localization and regulation of several signalling cascades (Drab et al. 2001; Frank et al. 2003; Minshall et al. 2003). In endothelial cells, caveolae contain caveolin-1 and caveolin-2. Caveolin-1 is essential for generation and maintenance of endothelial caveolae (Drab et al. 2001). Previously, caveolin-1 has been shown to stabilize endothelial barrier functions by binding and inactivation of endothelial NO synthase

(eNOS) (Bucci et al. 2000; Curry 2002; Bauer et al. 2005; Bernatchez et al. 2005).

Most studies so far have been performed using macrovascular cell lines like HUVECs. However, inflammatory mediators increase permeability primarily in venules of the microvasculature. Therefore, to characterize the role of caveolae in PKC-mediated endothelial barrier regulation, we prepared microvascular myocardial endothelial (MyEnd) cells from both wild-type (WT) and caveolin-1-deficient (Cav-1 −/−) mice. We have demonstrated previously that MyEnd cells express all typical endothelial marker proteins and respond to all mediators tested so far (Golenhofen et al. 2002; Waschke et al. 2004b). Particularly, in studies investigating the role of Rho GTPases in microvascular barrier regulation, we found that MyEnd cells responded similar to Rho protein-inhibiting and -stimulating toxins like endothelial cells of intact mesenteric microvessels (Waschke et al. 2004a, b). This indicates that MyEnd cells, despite of their origin from the specialized vascular bed of heart muscle, are a useful model to investigate general mechanisms participating in microvascular barrier regulation. Using these cultured endothelial cells, we studied the effect of PKC on VE-cadherin-mediated adhesion by laser tweezers. This approach allowed us to quantify binding of VE-cadherin-coated beads to VE-cadherin molecules on the endothelial cell surface and has been used previously in our laboratory to investigate the role of cytoskeletal anchorage and small Rho family GTPases in the regulation of VE-cadherin-mediated adhesion (Baumgartner et al. 2003; Waschke et al. 2004a).

In the present study, we provide evidence that PKC-induced endothelial barrier breakdown requires caveolin-1. In WT cells, activation of PKC by phorbol myristate acetate (PMA) caused intercellular gap formation, loss of VE-cadherin-mediated adhesion and increased FITC-dextran permeability across the monolayer. In contrast, in Cav-1(−/−) cells activation of PKC had no effect on the endothelial barrier.

Materials and methods

Cell culture and test reagents

The immortalized mouse MyEnd cells were generated from Cav-1(−/−) mice and WT mice (both C57BL/6) as described before in detail (Golenhofen et al. 2002). In brief, myocardial tissue of newborn mice was minced, digested with 0.05% trypsin (Biochrom, Berlin, Germany) and 0.02% collagenase (Boehringer, Mannheim, Germany), and seeded onto gelatine-coated culture dishes. One day after plating, adherent cells were transfected with Polyoma virus middle T antigen (PymT). Pym T transfection causes growth advantage of endothelial over non-endothelial cells leading to a homogenous monolayer of cells with endothelial morphology after 4–6 weeks of culture. MyEnd cell lines were characterized by immunoblotting for expression of von Willebrand factor as well as for several junctional proteins, such as VE-cadherin, α-, β-, γ-catenin, ZO-1 and claudin 5. The localization of these junctional markers was similar in both cell lines as verified by immunostaining. The cultures were grown in Dulbeccos modified Eagles medium (DMEM, Life Technologies, Karlsruhe, Germany) supplemented with 50 U/ml penicillin-G, 50 µg streptomycin and 10% foetal calf serum (FCS) (Biochrom, Berlin, Germany) in a humidified atmosphere (95% air/5% CO_2) at 37°C and used for experiments when grown to confluent monolayers (day 3 up to day 7).

PMA and staurosporine were obtained from Sigma (Taufkirchen, Germany). PMA was used as the sole mediator to inhibit PKC, because it induced consistent effects in both cell lines used in this study. The optimal dose for both reagents has been tested by dose–response experiments. In some monolayers, PMA also induced gap formation when applied for 15 min or at concentrations of 50 nM. However to get consistent results, application of PMA for 30 min at a concentration of 100 nM was required. Higher doses or prolonged incubation periods did not change the effects observed. Similarly, 50 nM of staurosporine was used because this concentration was found to inhibit PMA-induced gap formation in MyEnd cells within 30 min of incubation (not shown).

Cytochemistry

MyEnd cells were grown on cover slips coated with gelatine cross-linked with glutaraldehyde (Schnittler et al. 1993). Following incubation with PMA or staurosporine as indicated above, culture medium was removed and monolayers fixed for 10 min at room temperature (RT) with 2% formaldehyde (freshly prepared from paraformaldehyde) in PBS. Afterwards, monolayers were treated with 0.1% Triton X-100 in PBS for 5 min. After rinsing with PBS at RT, MyEnd cells were preincubated for 30 min with 10% normal goat serum (NGS) and 1% bovine serum albumin (BSA) at RT and incubated for 16 h at 4°C with rat monoclonal antibody 11D4.1 (undiluted hybridoma supernatant) directed to the ectodomain of mouse VE-cadherin (Gotsch et al. 1997) or with a mouse monoclonal caveolin-1 antibody (1:200 in PBS, BD Biosciences, Heidelberg, Germany). After several rinses with PBS (3×5 min), monolayers were incubated for 60 min at RT with Cy3-labelled goat anti-rat IgG or a Cy3-labelled goat anti-mouse IgG (both from Dianova, Hamburg, Germany, diluted 1:600 in PBS). For visualization of filamentous actin (F-actin), monolayers were incubated with ALEXA-phalloidin (Mobitec, Göttingen, Germany; diluted 1:60 in PBS, 1 h at RT). Cells incubated with antibodies or ALEXA-phalloidin were rinsed with PBS (3×5 min). Cover slips were mounted on glass slides with 60% glycerol in PBS, containing 1.5% *n*-propyl

gallate (Serva, Heidelberg, Germany) as anti-fading compound. For visualization of cell surface VE-cadherin in association with bound VE-cadherin-coated beads, Dynabeads could not be used because of strong autofluorescence. Therefore, immunolocalization studies were performed with latex sulphate beads (Interfacial Dynamics, Portland, OR). The 125 µl bead solution (5 µl packed beads) were washed twice in 2-morpholinoethane sulphonic acid, (25 mM, pH 6.0) (MES) buffer by centrifugation and resuspension at 3,000*g* for 10 min. Beads were resuspended in 500 µl MES including 10 µg VE-cadherin-Fc and incubated over night at RT under permanent slow overhead rotation. After centrifugation at 3,000*g* for 10 min beads were washed three times in 1 ml PBS containing 0.1% albumine. Beads were stored for up to 10 days under permanent overhead rotation at 4°C. After incubation of MyEnd cells with VE-cadherin-coated microbeads for 30 min, culture medium was removed and monolayers were treated as described above. For specific labelling of cellular VE-cadherin underneath attached beads, a rabbit polyclonal antibody directed against the cytoplasmic domain of VE-cadherin (kindly provided by D. Vestweber, Münster, Germany) was used. Monolayers were examined using a LSM 510 (Zeiss).

Transmission electron microscopy

MyEnd monolayers were fixed in 2.5% glutaraldehyde containing 0.01% ruthenium red in 0.1 M sodium cacodylate, pH 7.35 for 1 h at 4°C. After rinsing in 0.1 M cacodylate (3×5 min) cells were postfixed in 2% OSO_4 in 0.1 M cacodylate for 1 h at RT. Cells were rinsed again in cacodylate and dehydrated in ascending concentrations of methanol (25, 50, 70, 80, 2×95 and 2×100% 10 min each). Following incubation in 70% methanol, monolayers were placed for 1 h in saturated uranyl acetate in 70% methanol for 1 h at dark. After dehydration cells were equilibrated in propylene oxide (2×10 min) and embedded in Epon 812. Semithin sections (1 µM) were stained with toluidine blue, and ultrathin sections were contrasted with uranyl acetate and lead citrate and examined with a LEO AB 912 electron microscope.

Immunoblotting

Samples (60 µg of total endothelial protein) were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Western blotting and immunodetection with a mouse monoclonal IgG directed against caveolin-1 (1:1,000, BD Biosciences) using horseradish peroxidase-labelled goat anti-mouse IgG (1:3,000, Dianova, Hamburg, Germany) and the enhanced chemiluminescence technique (ECL, Amersham, Braunschweig, Germany).

Recombinant VE-cadherin-Fc

As described before, we used the VE-cadherin-Fc fusion protein consisting of the complete extracellular domain of mouse VE-cadherin (EC1–EC5) fused to the Fc portion of human IgG1, including the hinge region and Ig domains CH2 and CH3 (Gotsch et al. 1997; Baumgartner et al. 2003). The protein was expressed by stably transfected Chinese hamster ovary (CHO) cells and purified from culture supernatants by affinity chromatography using protein A agarose (Oncogene, Cambridge, MA).

Coating of polystyrene beads

After vortexing, 10 µl of protein A-coated super-paramagnetic polystyrene microbeads (Dynabeads, diameter 2.8 µm, Dynal, Oslo) containing 2×10^9 beads/ml were washed three times using 100 µl of buffer A (100 mM sodium phosphate buffer, pH 8.1). Washing was performed by immobilization of beads for 1 min in a magnetic tube holder (MPC-E-1, Dynal) and reuptake in the corresponding buffer. Washed beads were suspended in 100 µl of 100 mM sodium phosphate buffer, pH 8.1 in Hanks Balanced Salt Solution (HBSS, Gibco, Karlsruhe) containing 10 µg of either VE-cadherin-Fc or of the Fc part of human IgG (for control experiments) and allowed to react for 16 h at 4°C under permanent slow overhead rotation to avoid aggregation. After washing 3×5 min in 100 µl of buffer A and 3×5 min in buffer B (100 mM sodium borate, pH 9.0) beads were incubated for 45 min at RT in 100 µl buffer B containing 0.54 mg dimethyl pimelimidate dihydrochloride (DMP, Pierce, Rockford, USA) to covalently cross-link protein A and bound Fc parts. After washing 2×5 min in buffer C (100 µl 0.2 M ethanolamine, pH 8.0) beads were incubated in buffer C for 2 h at RT. Finally, beads were washed 3×5 min in HBSS and stored in HBSS at 4°C for up to 8 days under permanent slow overhead rotation to avoid aggregation of beads. The concentration of beads in these stocks was about 1.6×10^8 beads/ml.

Laser tweezer

As described previously (Baumgartner et al. 2003), the home-built laser tweezer set-up consisted of a Nd:Yag laser (1,064 nm), the beam of which was expanded to fill the back aperture of a high NA-objective (100×1.3 oil, Zeiss), coupled through the epi-illumination port of an Axiovert 135 microscope (Zeiss, Oberkochen, Germany) and reflected to the objective by a dichroic mirror (FT 510, Zeiss). Through all experiments, the laser intensity was 42 mW in the focal plane. Coated beads (10 µl of stock solution) were suspended in 500 µl of culture medium and allowed to interact with MyEnd monolayers for 30 min at 37°C before initiation of experiments. Beads were considered tightly bound when

resisting laser displacement at 42°mW setting. For every condition 100 beads were counted. Percentage of beads resisting laser displacement under various experimental conditions was normalized to control values.

Measurement of FITC-dextran flux across monolayers of cultured endothelial cells

Experiments were performed as described previously (Förster et al. 2005). MyEnd cells were seeded on top of gelatine-coated transwell chambers for six-well plates (0.4 μM pore size) (Falcon, Heidelberg, Germany) and grown to confluence. After rinsing with PBS, cells were incubated with fresh DMEM (Sigma) without phenol red to avoid absorption of FITC fluorescence containing 1 mg/ml FITC-dextran (molecular weight 4 kDa, Sigma) in the presence or absence of PMA (100 nM) or staurosporine (50 nM). Paracellular flux was assessed by taking 100 μl aliquots from the outer chamber over 4 h of incubation. Fluorescence was measured using a Wallac Victor2 fluorescence spectrophotometer (Perkin-Elmer, Überlingen, Germany) with excitation and emission at 485 and 535 nm, respectively. The FITC-dextran clearance through the monolayer was correlated to clearance of MyEnd monolayers under control conditions and the initial amount.

Statistics

Values throughout are expressed as mean ± standard error. Differences in bead binding and FITC-dextran flux between groups were assessed using unpaired Students' t test. Statistical significance is assumed for $P < 0.05$.

Results

Effects of PMA and staurosporine on morphology of WT and Cav-1(−/−) microvascular endothelial monolayers

To investigate the mechanisms involved in PKC-regulated endothelial barrier function and to test the requirement of caveolin-1 in PKC signalling, MyEnd cells were prepared from both WT and Cav-1(−/−) mice as described before (Golenhofen et al. 2002). The Cav-1(−/−) mouse model has been characterized in detail elsewhere (Drab et al. 2001). Immunostaining revealed that caveolin-1 was present in WT cells and enriched within the Golgi area and along intercellular junctions (Fig. 1a). Similar to WT cells, Cav-1(−/−) cells formed regular monolayers as revealed by ALEXA-phalloidin staining of F-actin (Fig. 1b, d). However, caveolin-1 was not detectable in Cav-1(−/−) cells (Fig. 1c). Western blot analysis confirmed expression of caveolin-1 in WT MyEnd cells whereas no expression was found in Cav-1(−/−) cells (Fig. 2). Accordingly, WT cells displayed numerous caveolae on the cellular surface as revealed by transmission electron microscopy (Fig. 3a). Moreover, caveolae were also observed at intercellular clefts (arrows in Fig. 3b). In contrast, no caveolae were detected in Cav-1(−/−) cells (Fig. 3c).

Under control conditions, in WT cells VE-cadherin was distributed continuously along cell junctions (Fig. 4a). Activation of PKC was achieved by incubation with PMA under conditions outlined above because PMA induced consistent effects in both cell lines. Treatment of WT cells with PMA resulted in formation of intercellular gaps and fragmentation of VE-cadherin staining (arrows in Fig. 4b). When WT cells in the absence of PMA were challenged with staurosporine to inhibit PKC, neither gap formation nor fragmentation of VE-cadherin staining was observed (Fig. 4c, $n = 5$). Cell borders appeared ruffled under these conditions.

In contrast, in Cav-1(−/−) cells, PMA had no effect on monolayer integrity and distribution of VE-cadherin compared to controls (Fig. 4d, e, $n = 5$). Following treatment with staurosporine cell borders became slightly ruffled similar to the effects of staurosporine on WT endothelium (Fig. 4f). Taken together, these results demonstrate that PKC activation causes formation of intercellular gaps, a hallmark of impaired endothelial barrier function. Moreover, the data show that PKC-induced gap formation is strictly dependent on caveolin-1.

Effects of PMA and staurosporine on VE-cadherin-mediated adhesion in WT and Cav-1(−/−) endothelium

The mechanisms underlying PKC-mediated endothelial barrier breakdown as well as intercellular gap formation are largely unknown. To test the effect of PKC on VE-cadherin-mediated adhesion, we applied laser tweezers using microbeads labelled with recombinant VE-cadherin. For controls, coated beads were allowed to settle on the surface of MyEnd cells for 30 min before binding was probed by laser displacement. Under these conditions, specific cell-to-bead junctions containing cell surface VE-cadherin were observed underneath VE-cadherin-coated microbeads (arrows in Fig. 5a, b) but not underneath uncoated beads (arrows in Fig. 5c, d). Further characterization of cell-to-bead junctions and detection of other key components typical for adherens junctions have been demonstrated previously (Baumgartner et al. 2003; Waschke et al. 2004a). Moreover, in our previous studies we have performed parallel experiments to measure microvascular permeability in single-perfused mesenteric postcapillary venules and VE-cadherin-mediated adhesion under same experimental conditions. We found that adhesion of VE-cadherin-coated microbeads was reduced by conditions under which microvascular permeability was increased (Waschke et al. 2004a, b). This indicates that the

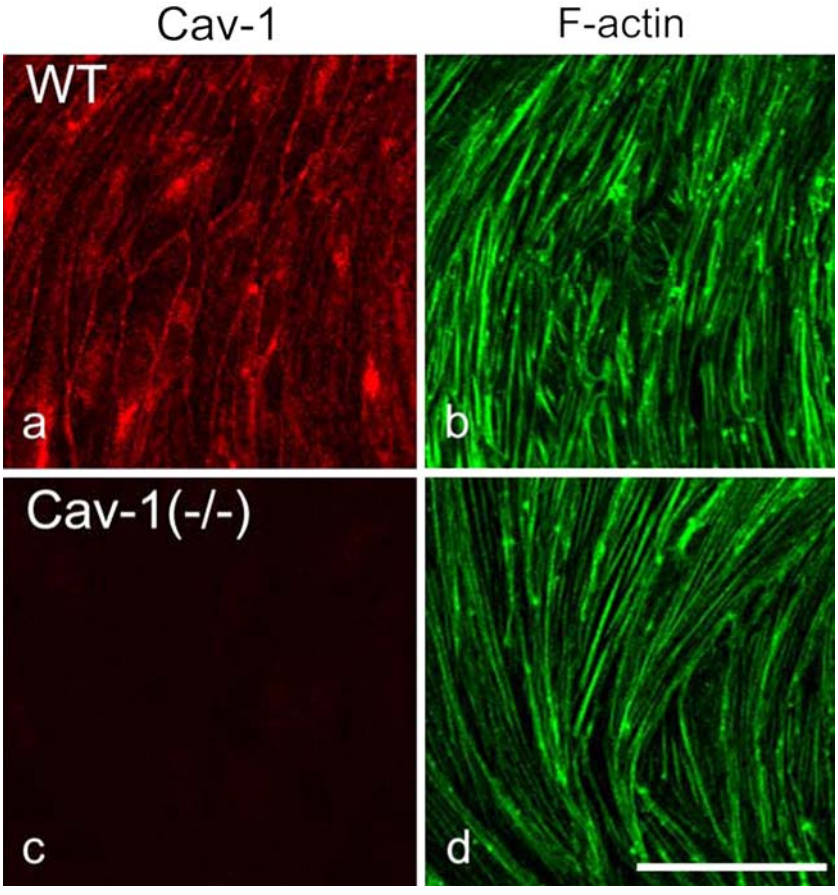

Fig. 1 Characterization of WT and Cav-1(−/−) endothelial cells by immunostaining. Cells were immunostained for caveolin-1 (**a**, **c**) or stained by ALEXA-phalloidin to label F-actin (**b**, **d**). Caveolin-1 is enriched along intercellular junctions in WT cells but is not observed in Cav-1(−/−) cells (**a**, **c**). Both cell lines show formation of intact monolayers (**b**, **d**). Scale bar is 20 μm for all panels ($n = 5$)

mechanisms regulating VE-cadherin-mediated adhesion at the cell surface are comparable to the mechanisms inside the intercellular clefts.

Under control conditions, 56 ± 4% of beads were tightly bound to the MyEnd cell surface and could not be displaced by the laser beam ($n = 6$). These values were set to 100% (Fig. 6). In Cav-1(−/−) cells, baseline numbers of bead adhesion were slightly higher (115 ± 6% of WT control values). However, immunoblotting experiments revealed no difference in the amount of total VE-cadherin (not shown).

When WT MyEnd cells were treated with PMA, the number of bound beads was significantly reduced to 78 ± 6% of controls indicating that PKC negatively regulates VE-cadherin-mediated adhesion ($n = 6$). In contrast, PMA did not reduce bead adhesion to Cav-1 (−/−) cells under same conditions (107 ± 6% bound beads). Similarly, inhibition of PKC by staurosporine had no significant effect on bead adhesion in both cell lines suggesting that PKC was not activated under resting conditions ($n = 6$).

Effects of PMA and staurosporine on FITC-dextran flux in WT and Cav-1(−/−) endothelium

We demonstrated that activation of PKC induced intercellular gap formation and reduction of VE-cadh-

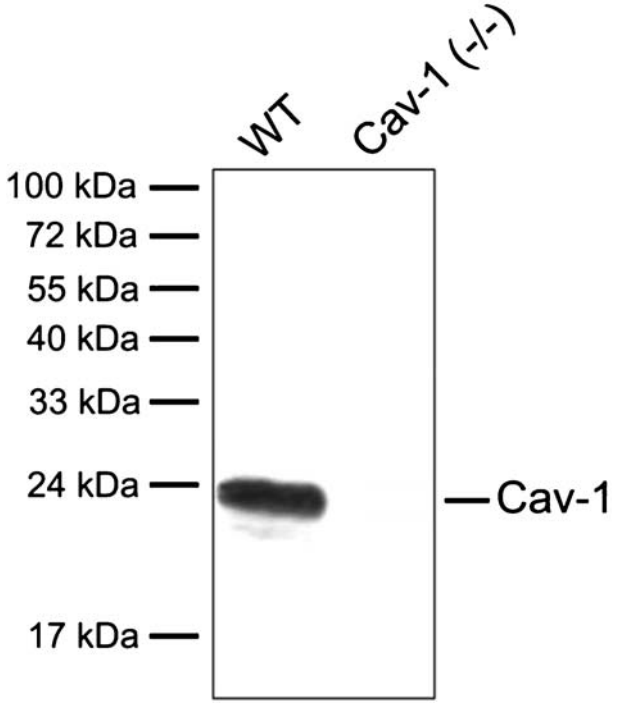

Fig. 2 Western blot analysis demonstrates expression of caveolin-1 in WT but not in Cav-1(−/−) cells. Caveolin-1 was detected in WT cells as single band of 22 kDa but not in Cav-1(−/−) cells. Experiment shows a representative out of three

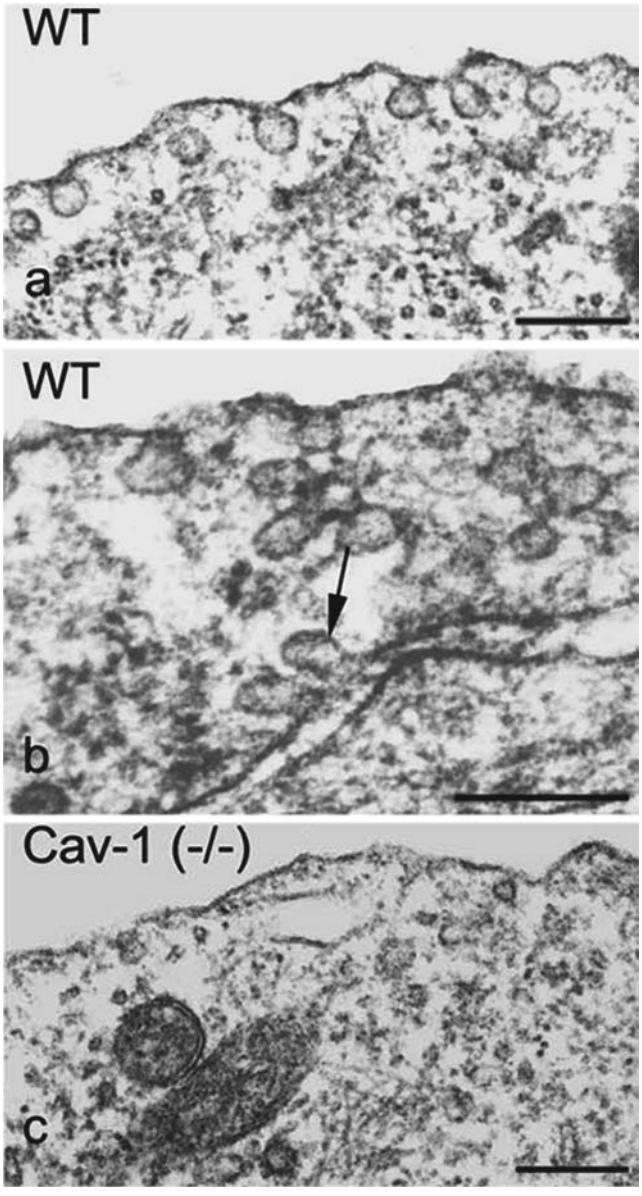

Fig. 3 Caveolae are present at the cell surface and occasionally at intercellular clefts in WT cells. Transmission electron microscopy confirmed that WT cells displayed caveolae on the cellular surface (**a**) and also at intercellular clefts (*arrow* in **b**). In contrast, no caveolae were present in Cav-1 (−/−) cells (**c**). Scale bar is 200 nm for all panels

erin-mediated adhesion and that these processes required expression of caveolin-1 in MyEnd cells. To investigate whether these effects correlated with changes in endothelial barrier functions of MyEnd cells, we measured the flux of FITC-dextran across monolayers of WT and Cav-1(−/−) cells. Under resting conditions, permeability of FITC-dextran in WT and Cav-1(−/−) cell monolayers was not significantly different (Fig. 7). However, when WT cells were treated with PMA, permeability was significantly increased by 86 ± 18% of control values ($n = 6$) whereas no change of FITC-dextran permeability was detected in Cav-1(−/−) monolayers (flux was 100 ± 8% of controls). Similar to the other assays, staurosporine did not affect permeability in both cell lines. These results indicate that the mechanisms by which PKC increased permeability required caveolin-1 and likely implied reduction of VE-cadherin-mediated adhesion followed by formation of intercellular gaps.

Discussion

It is well established that the PKC family of serine/threonine kinases is important for the permeability increase induced by several inflammatory mediators (Yuan 2002). The mechanisms involved in endothelial barrier regulation by PKC are not completely understood. In this study, we investigated the requirement of caveolin-1 in PKC signalling and the role of VE-cadherin-mediated adhesion in the regulation of endothelial barrier functions by PKC. We focussed on VE-cadherin because it is well known to be important for endothelial barrier integrity (Gotsch et al. 1997; Hordijk et al. 1999; Corada et al. 2001) and in our previous studies increased microvascular hydraulic conductivity in vivo correlated with inhibition of VE-cadherin-mediated adhesion in cultured endothelial cells (Waschke et al. 2004a, c). We provide evidence that activation of PKC reduces VE-cadherin-mediated adhesion in microvascular endothelium. Moreover, our data demonstrate that PKC-induced endothelial barrier breakdown is dependent on caveolin-1 because the effects of PKC activation on barrier properties were completely abolished in MyEnd cells lacking caveolin-1. In the following, we will first discuss the mechanisms underlying endothelial barrier regulation by PKC and then address the role of caveolin-1 in permeability regulation.

Activation of PKC by PMA induced formation of intercellular gaps as well as disassembly of VE-cadherin containing junctions as revealed by immunostaining of MyEnd monolayers. The opening of intercellular gaps was sufficient to significantly increase the flux of FITC-dextran across the endothelial monolayer. It has to be emphasized that intercellular gaps in monolayers of cultured endothelial cells are not necessarily identical to the morphologic changes in endothelium of intact microvessels. This is at least in part due to the greater contribution of contractile forces to cell dissociation under culture conditions when compared to the situation in intact microvessels in vivo (Waschke et al. 2004b). However, these data support previous studies from cultured cells showing that disassembly of VE-cadherin junctions caused by inflammatory mediators was prevented by inhibition of PKC (Sandoval et al. 2001). To demonstrate that disintegration of adherens junctions could contribute to intercellular gap formation, we studied the effect of PKC activation on VE-cadherin-mediated adhesion using laser tweezers to quantify binding of VE-cadherin-coated beads to the surface of MyEnd cells. This assay allows one to exclude secondary

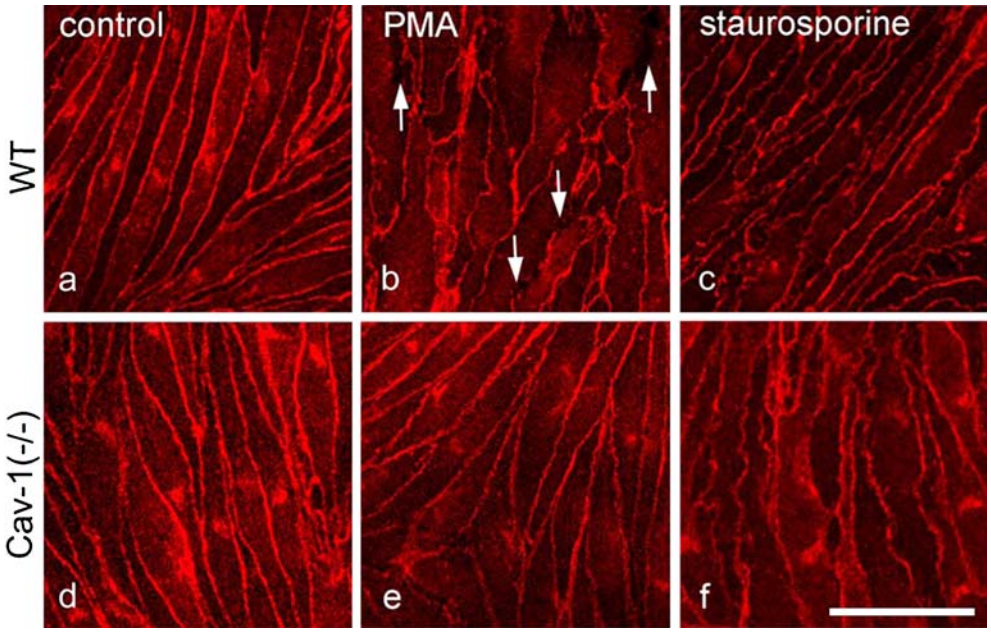

Fig. 4 Activation of PKC induces intercellular gap formation in WT-MyEnd cells but not in Cav-1(−/−) cells. MyEnd cells were immunostained for VE-cadherin which was distributed continuously along cell junctions in both cell lines (**a**, **d**). In WT cells, activation of PKC by PMA (100 nM, 30 min) induced intercellular gap formation (*arrows* in **b**) whereas inhibition of PKC by staurosporine (50 nM, 30 min) had no effect (**c**). In Cav-1(−/−) cells, neither PMA nor staurosporine resulted in cell dissociation (**e**, **f**). Scale bar is 20 μm for all panels ($n = 5$ in each group)

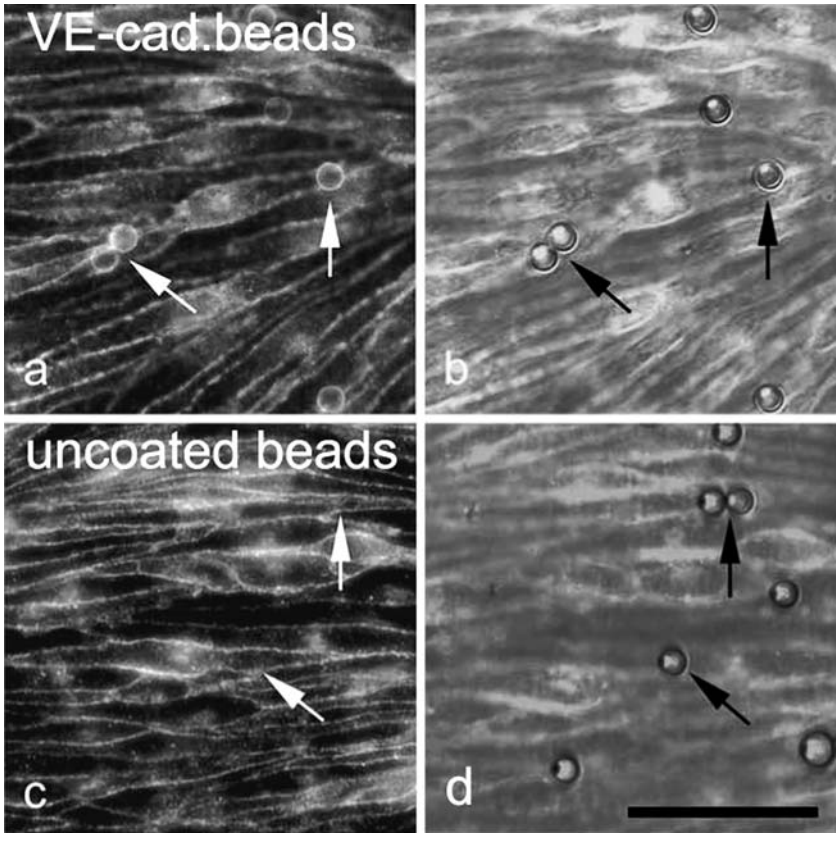

Fig. 5 Characterization of cell-to-bead junctions in MyEnd cells by immunostaining. Monolayers with attached beads were immunostained using an antibody directed to the cytoplasmic domain of VE-cadherin to specifically label cellular VE-cadherin molecules (**a**, **c**). Panels with the respective bright field are shown (**b**, **d**). Following 30 min of bead settlement, specific cell-to-bead junctions containing cell surface VE-cadherin were observed underneath VE-cadherin-coated microbeads (*arrows* in **a, b**) but not underneath uncoated beads (*arrows* in **c**, **d**). Scale bar is 20 μm for all panels ($n = 3$)

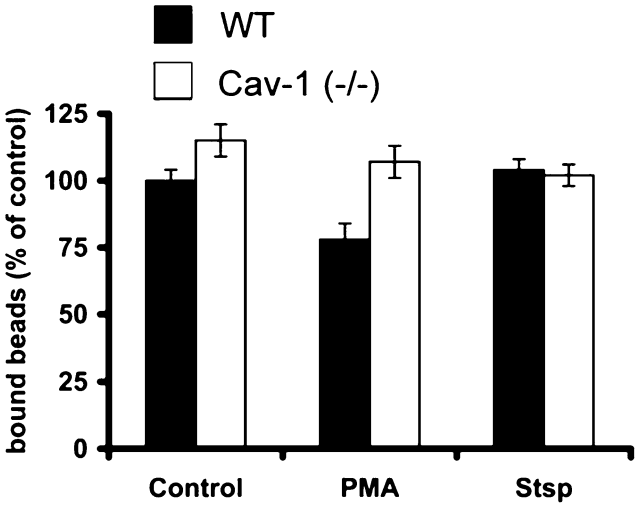

Fig. 6 Activation of PKC reduces VE-cadherin-mediated adhesion in caveolin-1-dependent manner. Bar diagram summarizes the results from the laser tweezer experiments using VE-cadherin-coated microbeads ($n=6$ for each condition). Compared to controls, Cav-1 (−/−) cells exhibited higher baseline values of bound beads (115 ± 6% of WT controls). Activation of PKC by PMA (100 nM, 30 min) reduced bead adhesion to 78 ± 6% of controls in WT cells without any significant effect in Cav-1 (−/−) cells. Inhibition of PKC by staurosporine (50 nM, 30 min) had no effect on bead adhesion in both cell lines

mechanisms like myosin-dependent contraction because contractile forces do not modulate bead binding whereas they contribute to intercellular gap formation in cultured endothelium (Michel and Curry 1999; Curry 2002; Waschke et al. 2004b). Activation of PKC significantly reduced binding of VE-cadherin-coated beads indicating that PKC negatively regulates adhesion of VE-cadherin and that reduction of VE-cadherin might be involved in intercellular gap formation. In contrast, inhibition of

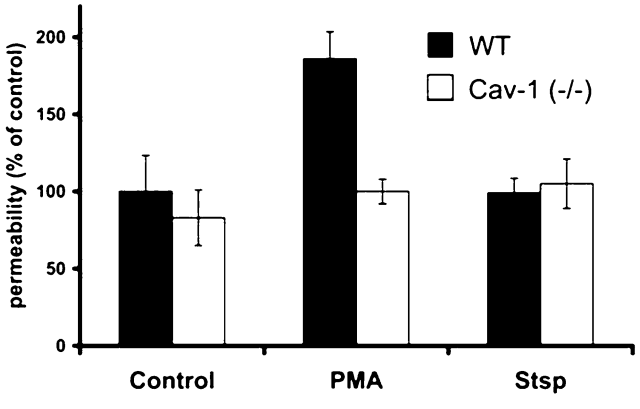

Fig. 7 Activation of PKC increases FITC-dextran flux across WT monolayers but not in Cav-1(−/−) monolayers. Bar diagram summarizes results from transwell filter experiments using FITC-dextran to measure permeability ($n=6$ for each condition). Activation of PKC by PMA (100 nM, 30 min) significantly increased FITC-dextran flux by 86 ± 18% of control values whereas no effect was observed in Cav-1(−/−) cells. Inhibition of PKC by staurosporine had no effect on permeability in both cell lines

PKC by staurosporine affected neither distribution of VE-cadherin along cell junctions nor adhesion of VE-cadherin-coated beads suggesting that PKC does not impair VE-cadherin function in the resting state. The significance of VE-cadherin for the maintenance of endothelial barrier properties is supported by experiments using caveolin 1-deficient endothelial cells. In these monolayers, both VE-cadherin adhesion and endothelial permeability were not modulated by activation of PKC. Taken together, our study supports the hypothesis of Sandoval and colleagues who proposed that PKC regulates endothelial barrier functions at least in part via control of intercellular adhesion mechanisms (Sandoval et al. 2001). These authors showed that thapsigargin as well as thrombin activated PKC and thereby reduced transendothelial resistance. However, only thrombin did induce phosphorylation of the myosin light chain and stress fibre formation indicating that PKC did reduce endothelial barrier functions by mechanisms different from myosin-dependent contraction. Other studies reported contribution of actin-myosin contraction to increased monolayer permeability in response to PKC activation (Stasek et al. 1992; Garcia et al. 1995; Moy et al. 2004).

Our data indicate that PKC signalling requires caveolin-1. Association of PKC isoforms such as PKCα and PKCβ, two of the relevant subtypes of PKC for endothelial barrier regulation (Yuan 2002), with caveolae has been shown (Lisanti et al. 1994; Frank et al. 2003). However, the biologic relevance of this localization for endothelial barrier regulation is unknown at present. In our study, in Cav-1 (−/−) MyEnd cells with no detectable expression of caveolin-1 and no formation of caveolae, PKC completely failed to induce gap formation and to disrupt localization of VE-cadherin to intercellular junction. Moreover, in the functional assays, PKC was found neither to reduce VE-cadherin-mediated adhesion nor to increase permeability in the absence of caveolin-1. This is in contrast to previous studies demonstrating that other enzymes like eNOS are inhibited by caveolin-1 via its scaffolding domain leading to decreased permeability (Bucci et al. 2000; Zhu et al. 2004; Bauer et al. 2005; Bernatchez et al. 2005).

However, our data support recent findings showing that at least some isoforms such as PKCγ translocate to caveolae upon activation and that this event is required for regulation of cell contacts like gap junctions by direct phosphorylation of connexin 43 (Lin and Takemoto 2005). This might also be relevant for the regulation of VE-cadherin-mediated adhesion because activation of PKC has been shown to increase the amounts of threonine-phosphorylated proteins at cell junctions in colocalization with VE-cadherin (Sandoval et al. 2001). Alternatively to direct phosphorylation of adherens junctions components, PKC-signalling could involve GTPases of the Rho family because PKC has been shown to activate Rho A (Mehta et al. 2001) and Rac-1 has been shown to be required for VE-cadherin-mediated adhesion (Waschke et al. 2004a, b). In our study,

caveolin-1 was prominent along cell margins in MyEnd cells and caveolae were also present in plasma membranes facing intercellular clefts. This is consistent with an important role of caveolin-1 in endothelial barrier regulation. However, it is possible that caveolin-1 in this localization is also associated with lipid rafts in addition to caveolae. By now, the exact mechanisms of interaction among the three proteins involved—VE-cadherin, caveolin-1 and PKC—remain unidentified. Our experiments demonstrate that VE-cadherin-mediated adhesion is regulated by PKC and that this is dependent on the expression of caveolin-1. Moreover, because of the intercellular localization of both caveolin-1 and caveolae on one hand and VE-cadherin on the other, it is at least possible that caveolin-1-containing structures provide some kind of scaffolding function for signalling molecules, including PKC, which are involved in the regulation of VE-cadherin-mediated adhesion.

In summary, our study demonstrates that PKC regulates endothelial barrier functions in part by controlling adhesion of VE-cadherin and that PKC signalling is strictly dependent on caveolin-1. However, further investigations are required to completely elucidate the mechanisms by which PKC and caveolin-1 regulate intercellular adhesion in endothelial cells.

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