Cadherin function probed by laser tweezer and single molecule fluorescence in vascular endothelial cells

Werner Baumgartner[1], Gerhard J. Schütz[2], Johannes Wiegand[1], Nikola Golenhofen[1] and Detler Drenckhahn[1,*]

[1]Institute of Anatomy and Cell Biology, University of Würzburg, Koellikerstr. 6, D-97070 Würzburg, Germany
[2]Institute of Biophysics, University of Linz, Altenbergerstr. 69, A-4040 Linz, Austria
*Author for correspondence (e-mail: anat015@mail.uni-wuerzburg.de)

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Summary

In endothelial monolayers agonist-induced influx of Ca^{2+} and activities of the actin cytoskeleton have been shown to be crucially involved in regulation of barrier properties. By laser tweezer application we demonstrated that the strength of adhesion of VE-cadherin-coated microspheres to the surface of cultured endothelial monolayers is significantly reduced by treatment with two well-established permeability-increasing compounds, cytochalasin D and the Ca^{2+}-ionophore A23187, which shows that both compounds directly affect cadherin-mediated adhesion. Cytochalasin D and A23187 caused considerable decay of F-actin (30-60%). Stabilisation of F-actin by jasplakinolide completely blocked drug-induced weakening of bead adhesion showing that attenuation of cadherin-cadherin trans-interaction induced by cytochalasin D and A23187 depends largely on downregulation of F-actin. Single molecule fluorescence microscopy demonstrated that drug-induced weakening of adhesion is accompanied by an increase in lateral mobility of cadherins as well as by dispersal of cadherin-enriched plasmalemmal microdomains. However, the lifetime (≈700 milliseconds, k_{off}≈1.4 second^{-1}) and apparent on-rate of cadherin trans-interaction (relative frequency of binding) remained unchanged in response to cytochalasin D and A23187 indicating that cadherin-mediated adhesion is not modulated by inside-out changes of the affinity but, rather, appears to be controlled by actin-dependent tethering and compartmentalization of cadherins.

Key words: VE-cadherin, Biophysics, Permeability, Actin

Introduction

A wide spread and functionally important family of cell adhesion molecules are the cadherins, which are Ca^{2+}-dependent single-span transmembrane glycoproteins that are capable of trans-interaction with cadherins of opposing cell surfaces (Yap et al., 1997a; Steinberg and McNutt, 1999; Angst et al., 2001). The ectodomains of classical cadherins such as vascular endothelial (VE)-cadherin form a rod-like structure that extends from the cell surface and associates into parallel cis-dimers (Shapiro et al., 1995; Yap et al., 1998; Takeda et al., 1999; Koch et al., 1999; Baumgartner et al., 2000a). The cytoplasmic domain of classical cadherins is highly conserved and serves as binding site for a family of proteins denoted as catenins, which are critically involved in linkage of cadherins to the actin-based cytoskeleton and also play a role in nuclear signalling (Kemler and Ozawa, 1989; Yap et al., 1997a; Angst et al., 2001; Hübner et al., 2001).

Regulation of adhesion is necessary to allow cells to adapt to changing environmental conditions occurring during morphogenetic cell rearrangement, migration, wound repair and several steps of tumour invasion and metastasis (Christofori and Semb, 1999). A well studied example of rapid agonist-induced modulation of cadherin-based adhesion is the inflammatory response of the vascular endothelium, which is characterised by circumscribed separation of intercellular junctions to form large paracellular gaps through which plasma proteins and blood cells can leave the blood compartment to fulfil a variety of important functions such as attacking invading pathogens (Allport et al., 1997; Dejana, 1997; Michel and Curry, 1999; Hordijk et al., 1999; Petzelbauer et al., 2000; Vestweber, 2000).

A fundamental challenge is to understand how cells can actively regulate adhesive strength in a wide dynamic range. The presently favoured model postulates regulation of adhesive strength by alteration of catenin-mediated tethering of the cytodomains to the cytoskeleton (Angres et al., 1996; Yap et al., 1997a; Hordijk et al., 1999; Angst et al., 2001; Baumgartner and Drenckhahn, 2002a; Vasioukhin and Fuchs, 2001). Direct evidence for this hypothesis has been difficult to obtain because correlation between catenin modification (e.g. by phosphorylation) and concomitant cytoskeletal disconnection is mostly indirect. Moreover, inhibition of adhesive contact formation in the presence of cytochalasins that inhibit actin polymerisation and induce fragmentation of F-actin (Theodoropoulos et al., 1994; Urbanik and Ware, 1989; Sampath and Pollard, 1991) does not distinguish between direct cytoskeletal effects on cadherin function and more general cellular consequences of cytochalasins (from Greek *cytos*: cell; *chalasis*: collapse, relaxation) including destabilisation of plasma membrane structure, cell shape and contractile tonus (Kolega et al., 1991; Van Deurs et al., 1996). Even if these experiments are taken as evidence for adhesive strengthening by cytoskeletal tethering of cadherins, it still remains to be shown whether this effect is caused by

cytoskeleton- and catenin-induced inside-out modulation of the affinity of cadherins [similar to the mechanism involved in affinity regulation of some integrins (Calderwood et al., 2000)] or by other more indirect effects, such as cytoskeleton-mediated clustering of cadherins at sites of cumulative adhesive strength (Angres et al., 1996; Yap et al., 1997a; Yap. et al., 1997b) and damping of the lateral mobility of cadherins. In view of the apparent low adhesive affinity of purified trans-interacting cadherin ectodomains (millimolar range determined for VE-cadherin) (Baumgartner et al., 2000a; Baumgartner et al., 2000b) linkage of cadherins to the cytoskeleton should facilitate rapid rebinding of cadherins after dissociation, thereby increasing the number of bonds and overall adhesive strength between the interacting cell surface [for theoretical evaluation of this aspect, see Baumgartner and Drenckhahn (Baumgartner and Drenckhahn, 2002a)].

In the present study we provide direct evidence that two permeability-increasing compounds known to cause gradual opening of endothelial junctions, that is, the inhibitor of actin polymerisation, cytochalasin D, and the Ca^{2+}-ionophore A23187 (Suttorp et al., 1989; Schnittler et al., 1990; Kuhne et al., 1993; Drenckhahn and Ness, 1997; Dejana, 1997; Hordijk et al., 1999; Michel and Curry, 1999), have a strong negative impact on the adhesion of VE-cadherin-coated microbeads to the surface of cultured endothelial cells. Drug-induced reduction of adhesion was not caused by changes of the affinity for trans-interaction but depended on the decay of F-actin, and correlated with the increase of the lateral mobility of cadherins and dispersal of cadherin-enriched plasmalemmal microdomains. Reduction of cytoskeletal tethering is assumed to be the main mechanism responsible for drug-induced weakening of cadherin-mediated intercellular adhesion.

Materials and Methods

Recombinant VE-cadherin-Fc

A VE-cadherin-Fc fusion protein was generated and purified as described recently (Moll and Vestweber, 1999; Baumgartner et al., 2000a). Briefly, a DNA fragment coding for the complete extracellular part of mouse VE-cadherin (EC1-EC5), including the membrane proximal glutamine was placed in front of a cDNA fragment coding for the Fc part of human IgG1, including the hinge region and Ig domains CH2 and CH3. VE-cadherin-Fc (secreted by stably transfected Chinese hamster ovary (CHO) cells) was purified from the culture supernatants by affinity chromatography using protein A agarose (Oncogene, Cambridge, MA).

Coating of polystyrene beads

After extensive vortexing, 10 μl solution of protein A-coated superparamagnetic 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 Na-phosphate, pH 8.1). Washing was performed by sedimenting the beads via application of a magnetic field for ~1 minute using a magnetic tube holder (MPC-E-1, Dynal) and reuptake in the corresponding buffer. The washed beads were suspended in 100 μl buffer A containing 10 μg of either VE-cadherin-Fc or of the Fc-portion of human IgG (for control experiments) and allowed to react for 30 minutes at room temperature (RT) under permanent slow overhead rotation of the reaction tube to avoid sedimentation and aggregation of the beads. After washing three times in 100 μl of buffer B (200 mM triethanolamine, pH 9.0) beads were incubated for 45 minutes in 100 μl buffer B containing 0.54 mg dimethyl pimelimidate dihydrochloride (DMP, Pierce, Rockford, USA) at RT to covalently crosslink protein A and bound Fc-portions. Free DMP was blocked by two washes for 30 minutes at 37°C in 100 μl 100 mM Tris pH 8.0. Finally, the beads were washed three times in Hanks Balanced Salt Solution (HBSS, Gibco, Karlsruhe, Germany) and stored in HBSS at 4°C for up to 5 days under permanent slow overhead rotation. Concentration of VE-cadherin-Fc bound to the bead surface (24.6 μm^2/bead) was determined by ELISA as described previously (Baumgartner and Drenckhahn, 2000a).

Cell culture

The microvascular endothelial cell line used (MyEnd) was generated from mouse myocardium by immortalisation with polyoma middle T oncogene (PymT) as described and characterised previously (Golenhofen et al., 2002). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Eggenstein, Germany) supplemented with 50 U/ml penicillin-G/streptomycin and 10% fetal calf serum (Biochrom) in a humidified atmosphere (95% O_2/5% CO_2) at 37°C. For experiments cells were grown on coverslips coated with gelatine crosslinked with glutaraldehyde (Schnittler et al., 1993). The culture was split once a week and used for experiments between passages 5 and 20. MyEnd cells formed monolayers of highly elongated cells frequently organised into whirl-like formations. The overall cell shape and growth pattern resembled primary cultures of microvascular endothelial cells from brain and skin (Karasek, 1989; Rubin et al., 1991) and differed significantly from the typical cobblestone pattern formed by macrovascular endothelial cells from various sources (Schnittler et al., 1997). MyEnd cells were immunopositive for three endothelial marker proteins tested: von Willebrand factor (Fig. 1A), VE-cadherin (Fig. 1B) and PECAM-1 (Fig. 1C).

Laser tweezer

The home-built laser tweezer setup consisted of a Nd:YAG laser (1064 nm) the beam of which was expanded to fill the back aperture of a high NA-objective (63×1.2 oil, Zeiss), coupled through the epiillumination port of an Axiovert 135 microscope (Zeiss, Oberkochen, Germany) and reflected to the objective by a dicroic mirror (FT510, Zeiss). The laser intensity was adjusted to 20 mW up to 200 mW in the focal plane. Beads resisting displacement at 20 mW also resisted detachment at higher laser intensities. This all-or-nothing behaviour was also observed under various drug conditions. Therefore we decided to perform all tweezer experiments at 20 mW. Protein-coated polystyrene microbeads were allowed to interact with the cell surface of MyEnd monolayers for 15 minutes. Then 100-300 beads were probed during the following 2-10 minutes by the laser tweezer.

Cytochalasin D, colchicine, the Ca^{2+}-ionophore A23187 (all purchased from Sigma, St Louis, MO) and jasplakinolide (Calbiochem, Bad Soden, Germany) were used at 10 μM in DMEM. Jasplakinolide was applied for 60 minutes, whereas the other drugs were applied for 30 minutes under cell culture conditions as described above. Ca^{2+}-free conditions were achieved by addition of 5 mM EGTA to the culture medium.

Cytochemistry

For visualisation of endogenous VE-cadherin, β-catenin, and F-actin in association with adhering VE-cadherin-Fc-coated beads, Dynabeads could not be used because of strong autofluorescence. Therefore, immunolocalisation studies were performed with latex-sulfate beads (Interfacial Dynamics, Portland, OR). 125 μl bead solution (5 μl packed beads) were washed twice in 1 ml MES buffer (2-morpholino-ethane sulfonic acid, 25 mM, pH 6.0) by resuspension and centrifugation at 3000 ***g*** for 10 minutes. Beads were resuspended in 500 μl MES including 2.5 μg VE-cadherin-Fc and incubated overnight at RT under permanent slow overhead rotation. After

centrifugation at 3000 ***g*** for 10 minutes beads were washed three times in 1 ml PBS containing 0.1% glycine. Beads were stored for up to 5 days under permanent overhead rotation at 4°C. Monolayers with adhering beads were fixed at RT with 2% formaldehyde in phosphate buffered saline (PBS, pH 7.4) for 5 minutes and permeabilised using 0.1% (v/v) Triton X-100 (Sigma) in PBS for 3 minutes. After preincubation of the cells with 10% normal goat serum (NGS) and 1% bovine serum albumin (BSA) (both from Sigma) in PBS for 30 minutes at RT, monolayers were incubated overnight at 4°C with rabbit polyclonal VE-cadherin antibody directed against the cytoplasmic domain of mouse VE-cadherin (kindly provided by D. Vestweber, Münster, Germany) or mouse monoclonal antibody against β-catenin (Transduction Laboratories, San Diego, CA) (dilution 1:100 and 1:300, respectively). For visualisation of F-actin coverslips were incubated with Alexa-phalloidin (1 U/μl, Molecular Probes, Eugene, OR) for 1 hour at 37°C. For characterisation of MyEnd cells immunostaining was performed with rabbit polyclonal antibodies against von Willebrand factor (Sigma, dilution 1:200) or rat monoclonal antibody against PECAM-1 (kindly provided by B. Nieswandt, Würzburg, Germany; dilution 1:100). After washing with PBS (3×5 minutes) cells were incubated with one of the following: Cy3-labelled goat anti-rabbit, goat anti-rat or goat anti-mouse IgG (Dianova, Hamburg, Germany) at RT for 30 minutes. After final rinsing with PBS cells were mounted in 60% glycerol in PBS containing 1.5% *N*-propyl gallate as an antifading compound.

For scanning electron microscopy endothelial cell monolayers with adhering VE-cadherin-Fc coated beads were fixed for 24 hours with 4% glutaraldehyde in HBSS. After dehydration with graded aceton series, critical point drying and sputtercoating with palladium-gold (CPD 030, Bal-Tec, Schalksmühle, Germany) cells were examined with a DSM-962 scanning electron microscope (Zeiss, Germany).

Quantification of F-actin and VE-cadherin

For determination of the relative F-actin contents of endothelial cells, monolayers were fixed at RT with 3% formaldehyde in PBS for 15 minutes and then permeabilised with 0.1% (v/v) Triton X-100 in PBS for 5 minutes. Afterwards each coverslip was incubated with 500 μl (1 μg/ml) phalloidin covalently labelled with tetramethyl-rhodamine isothiocyanate (TRITC) for 1 hour at 37°C (Faulstich et al., 1983; Franke et al., 1984). Series of experiments with changing concentrations of TRITC-phalloidin (0.1-10 μM) were conducted to show that the conditions chosen allow saturation of binding. After washing three times for 5 minutes in PBS, TRITC-phalloidin was extracted from the cells by two subsequent 1 hour incubation steps with 1 ml of methanol at 37°C. Methanol supernatants were pooled, centrifuged at 100,000 ***g*** for 20 minutes and quantified in a fluorescence spectrometer at an excitation wavelength of 540 nm and an emission wavelength of 563 nm.

The relative amount of endogenous VE-cadherin exposed on the cell surface of MyEnd cells was determined by western blotting of cell cultures treated for 7 minutes with 0.05% trypsin (Serva) in DMEM in either the presence or the absence of 5 mM EGTA at 37°C. After removal of the trpysin-containing supernatant, monolayers were washed three times with PBS containing a mixture of protease inhibitors (leupeptin, aprotinin, pepstatin, 20 μg/ml each, Sigma). Cells were then removed from the culture dish by a rubber policeman and dissolved immediately in 10% sodium docecylsulfate (SDS)-containing sample buffer at 95°C for 3 minutes. Afterwards, samples were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent western blotting and immunodetection with hybridoma supernatant containing rat monoclonal VE-cadherin antibody [mAb 11D4.1, specific for mouse VE-cadherin ectodomain (Gotsch et al., 1997)] using horseradish-peroxidase labelled goat anti-rat IgG (Dianova, Hamburg, Germany) and the enhanced chemilumeniscence technique (ECL, Amersham, Braunschweig, Germany).

For determination of the amount of VE-cadherin exposed on dorsal cell surface cells were fixed with 2% formaldehyde for 10 minutes at RT without permeabilisation. After blocking non-specific binding sites for 1 hour with PBS containing 10% NGS and 1% BSA, monolayers were incubated overnight with mAb 11D4.1 at 4°C. Under these conditions antibodies have no access to lateral and basal cell surface as assayed by immunostaining. After several washes with PBS monolayers were subjected to SDS-PAGE and western blotting. Bound mAb 11D4.1 (rat IgG1) was detected by horseradish-peroxidase-labelled goat anti-rat IgG and ECL-technique. Confluent monolayers of human umbilical cord venous endothelial cells (HUVEC) (Schnittler et al., 1997) served as controls (mAb 11D4.1 is specific for mouse VE-cadherin).

Single molecule optical microscopy

The experimental setup including data acquisition and automatic data analysis has been described in detail elsewhere (Schmidt et al., 1995). In brief, samples were illuminated for 5 milliseconds by 514 nm light from an Ar^+ laser (C306, Coherent, CA) using a 100× objective (PlanNeofluar, NA=1.3, Zeiss) in an epi-fluorescence microscope (Axiovert 135TV, Zeiss). The laser beam was defocused to an area of ~500 μm^2 at a mean intensity of 1.2 kW/cm^2. Rayleigh scattered light was effectively blocked by appropriate filter combinations (515DRLPEXT02, Omega; 605DF50, Omega; 2xGG530; Schott, Mainz, Germany). Images were obtained by a liquid-nitrogen-cooled, back-illuminated CCD-camera system (Roper Scientific, Trenton, NJ; Micro Max 1300-PB) and stored on a PC. Consecutive images were obtained at an illumination time of 5 milliseconds and a constant delay of either 140, 170, 200, 250 or 500 milliseconds. During the delay time, the laser was turned off by an accoustooptic modulator (1205 c-1 Isomet, Springfield, VA).

Single molecule fluorescence imaging was applied to both VE-cadherin-Fc-coated glass slides and to MyEnd cells. Glass slides (width 25 mm; Knittel, Berlin, Germany) were cleaned by sonification for 5 minutes in chloroform, subsequently dried in a stream of nitrogen and immediately incubated with 1 ml HBSS containing 0.01 mg/ml VE-cadherin-Fc and 1 mg/ml BSA (to block unspecific protein-glass interaction) for 45 minutes. Cells were treated as described above. Coated glass slides or glass slides with MyEnd cells were mounted in a home-built sample stage with temperature controller (20/20 Technology, Whimington, NC). For these experiments VE-cadherin was fluorescently labeled in solution (HBSS containing 1.8 mM Ca^{2+} and 1% w/v BSA) by addition of a tenfold molar excess of Cy3-labelled anti-human F(ab).

Single molecule microscopy data analysis

The position of each fluorescently labeled molecule was obtained with an accuracy of ~50 nm by an automatic analysis program (Schmidt et al., 1995). Correlation of consecutive images allowed to reconstruct the 2-dimensional trajectory of each molecule observed. The length of a trajectory is directly related to the lifetime of a single cadherin-cadherin bond. The probability for a trajectory of length n, i.e. n consecutive observations given the underlying lifetime t is as follows:

$$p(n|t) = \begin{cases} \dfrac{t-(n-1)\cdot\vartheta}{\vartheta} & \text{for} \quad (n-1)\cdot\vartheta < t \le n\cdot\vartheta \\ \dfrac{(n+1)\cdot\vartheta - t}{\vartheta} & \text{for} \quad n\cdot\vartheta < t \le (n+1)\cdot\vartheta \end{cases}, \qquad (1)$$

with ϑ denoting the time between two observations (sampling time). To obtain the distribution of the number of consecutive observations one has to integrate over the lifetime t:

$$p(n) = \int_{(n-1)\cdot\vartheta}^{n\cdot\vartheta} f(t)\cdot\frac{t-(n-1)\cdot\vartheta}{\vartheta}\,dt + \int_{n\cdot\vartheta}^{(n-1)\cdot\vartheta} f(t)\cdot\frac{(n+1)\cdot\vartheta - t}{\vartheta}\,dt\,. \qquad (2)$$

If the lifetimes are exponentially distributed, i.e. $f(t) = \tau^{-1}\cdot\exp(-t/\tau)$, with τ denoting the time constant, we obtain the distribution of the number of consecutive observations to be as follows:

$$p(n) = \theta\cdot[e^{-(n-1)/\theta} + e^{-(n+1)/\theta} - 2e^{-n/\theta}]\,, \quad (3)$$

with $\theta = \tau/\vartheta$ denoting the characteristic time in units of the sample time.

Results

Characterisation of adhesion of microbeads coated with VE-cadherin-Fc to endothelial cells

In the present study, we used polystyrene microbeads, 2.8 μm in diameter, covalently coated with cis-dimeric ectodomains of recombinant mouse VE-cadherin (VE-cadherin-Fc) to study various aspects of cadherin-mediated adhesion on endothelial cells by application of the laser tweezer technique. The average concentration of VE-cadherin bound to the bead surface ranged between 500 and 1000 cadherin-dimers/μm^2. Within this range bead adhesion was highly reproducible and consistent. At surface densities far below this value (i.e. 100 molecules/μm^2) adhesion became inconsistent showing that a critical concentration of cadherins is required for tight bead adhesion. VE-cadherin-Fc-coated beads were allowed to settle on the cell surface for 15 minutes. Immunostaining and staining with Alexa-phalloidin showed that during this time course, typical adherens-like junctions were formed between the majority of beads (70-80%) and the endothelial cell surface as indicated by recruitment of endogenous VE-cadherin (immunolabelled with antibody to the cytoplasmic domain), β-catenin and F-actin to the site of bead attachment (Fig. 1D-F). That staining is not caused by unspecific association of antibodies (phalloidin) with beads (coated with VE-cadherin-Fc) is demonstrated by 20-30% non-reactive bead sites in direct vicinity to reactive beads (Fig. 1) and by experiments with poly- and monoclonal antibodies to vimentin that stained the endothelial intermediate filament system but not adhering beads (not shown). In addition, examination of adhering beads by scanning electron microscopy showed specialisations of the endothelial cell surface at sites of bead contacts (Fig. 1J,K). These sites were characterised by small cellular protrusions abutting on the bead surface. Beads were not endocytosed during the time course of experiments (20-60 minutes).

Typically ~70-80% of the beads suspended in DMEM (containing 1.8 mM Ca^{2+}) were tightly bound to the cell surface and resisted displacement (detachment) by laser tweezers. Specificity of binding of VE-cadherin-coated beads to the endothelial cell surface was confirmed by the following control experiments: beads coated with BSA or human IgG instead of VE-cadherin-Fc displayed strongly reduced frequency of binding (Fig. 2, Table 1) with ~9-12% of beads resisting detachment by laser tweezer. In the presence of 5 mM EGTA binding of beads was reduced to 23%. Preincubation of both VE-cadherin-coated beads as well as cell cultures with hybridoma supernatant containing rat mAb 11D4.1 to external domain of mouse VE-cadherin reduced binding by ~40%.

Role of actin filaments

Vascular endothelial cells have been shown to respond to cytochalasin D

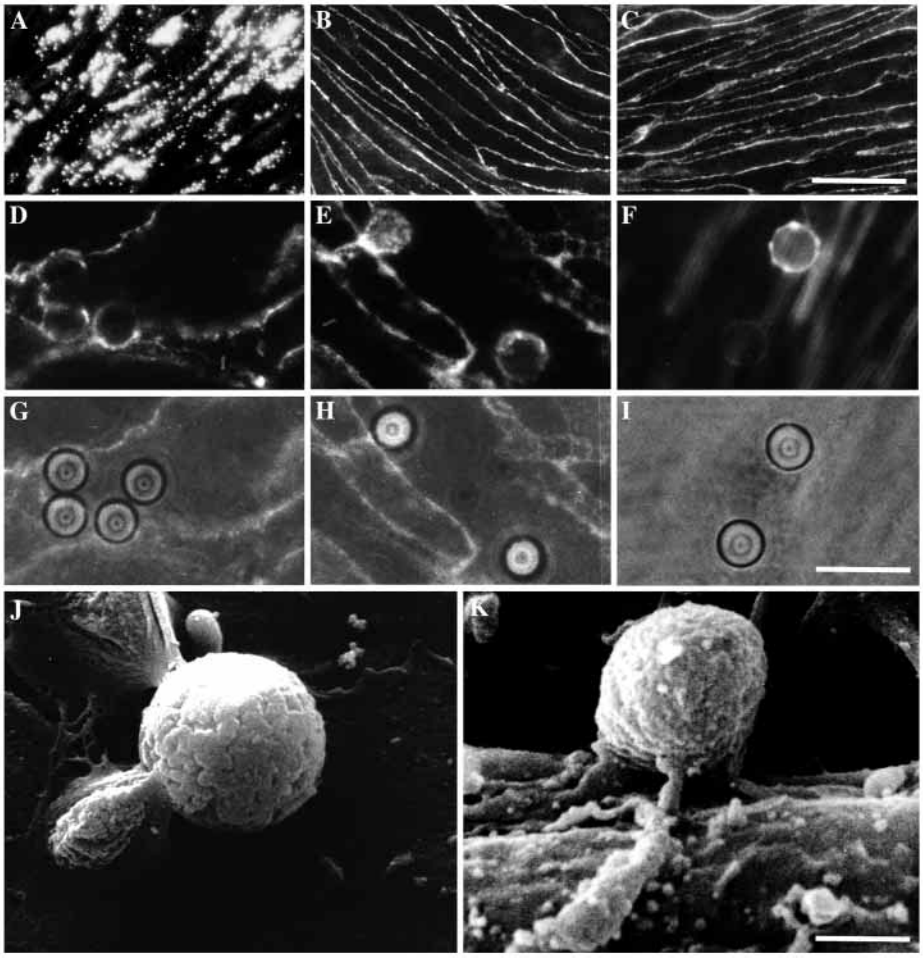

Fig. 1. Characterisation of MyEnd monolayers and sites of cellular adhesion of VE-cadherin-coated microbeads by immunofluorescence (A-E), Alexa-phalloidin-staining (F), phase contrast microscopy (G-I) and scanning electron microscopy (J,K). MyEnd monolayers display a typical granular immunoreactivity for von Willebrand factor (A) and junctional immunostaining for VE-cadherin (B) and PECAM-1 (C). Adhering beads (D-F) are characterised by cellular recruitment of VE-cadherin (D, localised with antibody to cytoplasmic domain), β-catenin (E) and F-actin (F). Staining of junctions (D,E) and stress fibers (F) is blurred because the optical plane is focussed on beads at the dorsal cell surface. Beads not associated with VE-cadherin, β-catenin and F-actin probably represent the population of 20% of beads not firmly attached to cells. Scanning electron micrographs show small cellular protrusions abutting on the bead surface (J,K). Bars, 20 μm (A-C); 10 μm (D-I); 2 μm (J,K).

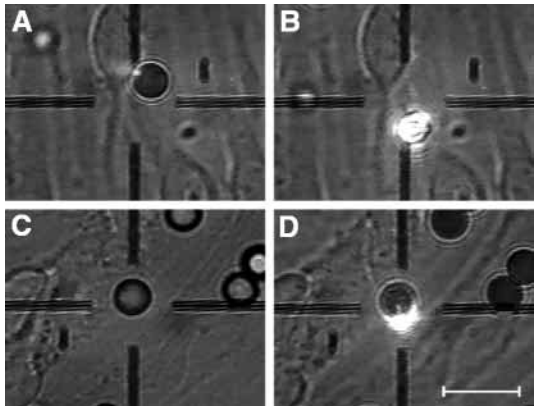

Fig. 2. Example of albumin-coated (A,B) and VE-cadherin-Fc-coated (C,D) beads on dorsal surface of MyEnd monolayers probed with laser tweezer. Albumin-coated beads are displaced by laser tweezer, whereas VE-cadherin-Fc-coated beads resist displacement. Phase contrast images. Bar, 5 μm.

treatment by local dissociation of cell-to-cell junctions (intercellular gap formation) and concomitant breakdown of the permeability barrier for macromolecules (Drenckhahn and Ness, 1997; Michel and Curry, 1999; Hordijk et al., 1999). Treatment of monolayers and microperfused capillaries with the Ca^{2+}-ionophore A23187 (1-10 μM) resulted in similar changes (gap formation, increase in permeability), which was explained by Ca^{2+}-stimulated contraction of the cellular actomyosin system, which was assumed to be strong enough to pull the cells apart by overcoming cadherin-mediated adhesion (Suttorp et al., 1989; Schnittler et al., 1990; Goeckeler and Wysolmerski, 1995). However, another model for explaining A23187-induced junctional dissociation implies Ca^{2+}-gelsolin-induced severing of F-actin as a major cause for junctional barrier breakdown (Kuhne et al., 1993).

Table 1. Percentage of VE-cadherin-Fc-coated beads tightly bound to endothelial cell surface at various conditions (laser tweezer assay)

Conditions (DMEM, 1.8 mM Ca^{2+})	Bound beads (% of control)
Beads coated with VE-cadherin-Fc:	
EGTA (5 mM)	23.4±3.4 (*n*=5)
Cytochalasin D preincubation	33.3±1.3 (*n*=3)
A23187 preincubation	34.4±1.9 (*n*=3)
Colchicine preincubation	102.3±4.5 (*n*=3)
Ethanol (0.1%) preincubation	97.1±2.0 (*n*=4)
Cytochalasin D postincubation	52.1±7.1 (*n*=3)
A23187 postincubation	43.4±5.8 (*n*=4)
Jasplakinolide preincubation	59.3±6.3 (*n*=4)
Jasplakinolide postincubation	121.5±7.2 (*n*=3)
Jasplakinolide postincubation followed by cytochalasin D	95.3±8.3 (*n*=3)
Jasplakinolide postincubation followed by A23187	93.6±6.1 (*n*=3)
Beads coated with Fc of human IgG	12.3±3.7 (*n*=5)

Control values in DMEM containing 1.8 mM Ca^{2+} were normalized to 100%. Preincubation: drug treatment for 30 minutes (jasplakinolide 60 minutes) prior to bead assay. Postincubation: drug treatment for 30 minutes (jasplakinolide 60 minutes) after beads were allowed to settle and attach to monolayers. All drugs were applied at a concentration of 10 μM if not indicated otherwise. Beads coated with Fc of human IgG served as a negative control.

To obtain direct insight in the effect of cytochalasin D and A23187 on cadherin-mediated adhesion, we determined binding of VE-cadherin-coated beads to MyEnd-cells preincubated for 30 minutes with 10 μM cytochalasin D and 10 μM A23187, respectively. In confluent monolayers preincubated with either of these compounds, F-actin contents (assayed by TRITC-phalloidin binding) dropped to 68±7% (cytochalasin D) and 39±6% (A23187) of control cultures treated with the drug solvent ethanol (0.1%). Percentage of VE-cadherin-coated beads resisting detachment by laser tweezer dropped to 33.3±1.3% (*n*=3) (cytochalasin D) and to 34.4±1.9% (*n*=3) (A23187) of control levels (untreated cultures), whereas preincubation for 40 minutes with 0.1% ethanol or 10 μM colchicine (microtubule disrupting compound) did not attenuate adhesion (Table 1).

In a further series of experiments beads were first allowed to settle on monolayers for 15 minutes. Thereafter monolayers were treated with 10 μM cytochalasin D or 10 μM A23187 for another 30 minutes (postincubation protocol). The fraction of beads resisting displacement by laser tweezer dropped to 40-50% of control levels.

To further study the role of the actin filament system on cadherin-mediated adhesion, monolayers were preincubation with the F-actin stabilising compound jasplakinolide (10 μM), which promotes actin-polymerisation and inhibits depolymerisation-repolymerisation cycles. Preincubation for 60 minutes reduced bead adhesion by 40% of control levels showing again that polymerisation of actin is important for establishment of new adherens contacts. However, if jasplakinolide incubation was performed after beads had been allowed to settle and attach to the monolayer for 15 minutes (postincubation protocol) significant strengthening of bead adhesion was observed (20% above control values). These experiments show that stabilisation of F-actin enhances adhesion of established cadherin-cadherin contacts. Moreover these experiments allowed us to directly address the question whether A23187-mediated weakening of bead adhesion is causally related to A23187-induced depolymerisation of F-actin. In monolayers in which bead adhesion was stabilised by postincubation by jasplakinolide A23187 treatment was ineffective and did not cause significant weakening of bead adhesion. Together these experiments show the important role of actin dynamics in cadherin-mediated adhesion.

In order to rule out the possibility that reduction of adhesion induced by cytochalasin D and A23187 is caused by loss (internalisation) of surface-exposed cadherin molecules (Le et al., 1999) or, alternatively, by changes of the affinity of cadherin dimers by some kind of inside-out signalling, we determined both the relative amount of surface-exposed cadherins and their apparent affinity in response to treatment with cytochalasin D and A23187.

Amount of surface-exposed VE-cadherin in cytochalasin D and A23187 treated cells

The amount of VE-cadherin exposed on the total cell surface of MyEnd cells was assayed by extracellular cleavage with trypsin. Previous experiments with E-cadherin (Hyafil et al.,

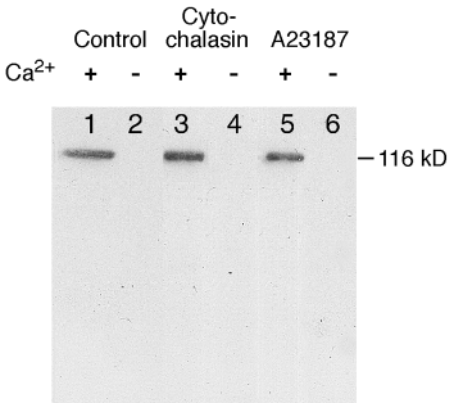

Fig. 3. Determination of relative amount of surface-exposed VE-cadherin in response to cytochalasin D (10 μM) and A23187 (10 μM) assayed by trypsin treatment of MyEnd monolayers in presence and absence of Ca^{2+} and subsequent visualisation of VE-cadherin by western blotting. Virtually all VE-cadherin molecules are exposed on the cell surface and are sensitive to extracellular proteolysis. The total amount of VE-cadherin remains constant as judged from constant immunoblotting signal in the presence of Ca^{2+} that renders cadherins resistant to trypsin cleavage.

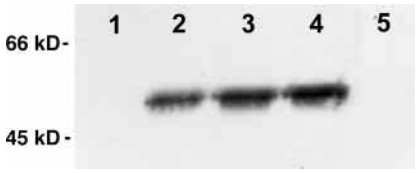

Fig. 4. Determination of relative amount of VE-cadherin exposed on the dorsal surface of MyEnd monolayers assayed by the capacity of the dorsal surface of formaldehyde-fixed monolayers to bind the mAb 11D4.1 antibody specific for VE-cadherin ectodomain. In untreated monolayers (2) surface-bound IgG heavy chain signal is slightly reduced as compared to monolayers treated for 30 minutes with 10 μM cytochalasin D (3) and 10 μM A23187 (4). MyEnd not incubated with mAb 11D4.1 (1) and HUVECs incubated with mAb 11D4.1 (5) served as negative controls.

1981; Pokutta et al., 1994) and VE-cadherin (Baumgartner and Drenckhahn, 2002b) have shown that cadherins are only sensitive to trypsin cleavage in the absence of Ca^{2+}, whereas in the presence of millimolar Ca^{2+} cadherins completely resist cleavage. Treatment of monolayers with 0.05% trypsin for 7 minutes in the presence and absence of Ca^{2+} caused moderate dissociation but still no detachment of cells. In the absence of Ca^{2+} (5 mM EGTA) trypsin treatment resulted in complete disappearance of western blotting signals for VE-cadherin in both control and drug-treated cultures (Fig. 3). This shows that virtually the entire VE-cadherin pool is exposed on the cell surface and that drug treatment did not cause any significant internalisation of VE-cadherins. The total amount of VE-cadherin was not affected by drug treatment as judged by the western blotting signals for VE-cadherin in corresponding cultures treated with trypsin in the presence of 2 mM Ca^{2+}. To determine whether these data obtained for the whole cell surface of MyEnd cells are also valid for the dorsal cell surface of the monolayer (site of bead interaction) we fixed control and drug-treated monolayers with 2% formaldehyde and incubated the monolayers with monoclonal antibody 11D4.1 that has access only to the dorsal cell surface under these conditions as seen by diffuse immunostaining of the dorsal cell surface and absence of any immunosignal in association with the junction-containing lateral cell surface (not shown). The amount of bound mAb 11D4.1 (monoclonal rat antibody) was determined by western blotting of monolayers with antibody to rat IgG. As shown in Fig. 4, there was only a slight increase of the immunoblotting signal in monolayers treated for 30 minutes with A23187 and cytochalasin D. Both compounds do not cause significant opening of intercellular junctions of MyEnd cells during this time interval as judged from continuous non-interrupted immunostaining for VE-cadherin and β-catenin (not shown). Endothelial cells from human umbilical cord (HUVEC) served as a negative control (mAb 11D4.1 is specific for mouse VE-cadherin external domain).

These experiments exclude the possibility that significant reduction of bead adhesion on drug-treated monolayers might be caused by loss (reduction) of VE-cadherin from the dorsal cell surface.

Determination of k_{off} and lateral mobility of VE-cadherin by single molecule fluorescence

The experiments described above showed that significant reduction of VE-cadherin-mediated adhesion in response to reduction of F-actin by treatment with cytochalasin D and A23187 cannot be explained by a decrease of the surface concentration of VE-cadherin molecules. In order to address the question whether changes of translational entropy (lateral mobility), reduction of affinity or a combination of both are responsible for decreased adhesion in response to cytochalasin D or A23187, soluble VE-cadherin-Fc was indirectly labelled by Cy3-tagged F(ab) directed against Fc-portion of human IgG (further denoted as VE-cadherin-Fc*).

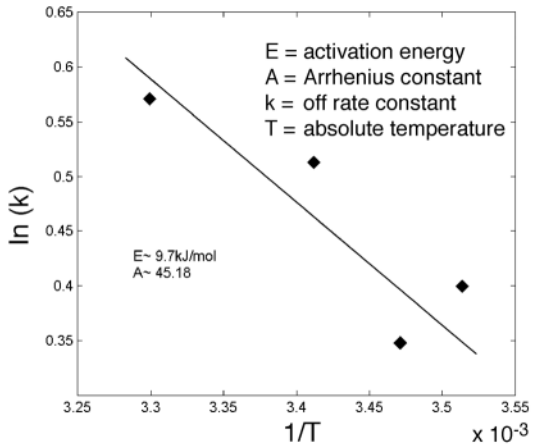

Fig. 5. Determination of activation energy (E) and Arrhenius constant (A) for trans-interaction of VE-cadherin-Fc adsorbed to glass surface and Cy3-F(ab)-labelled VE-cadherin-Fc in soluble phase. Lifetime (k_{off}) of individual trans-interacting events was determined by single molecule fluorescence.

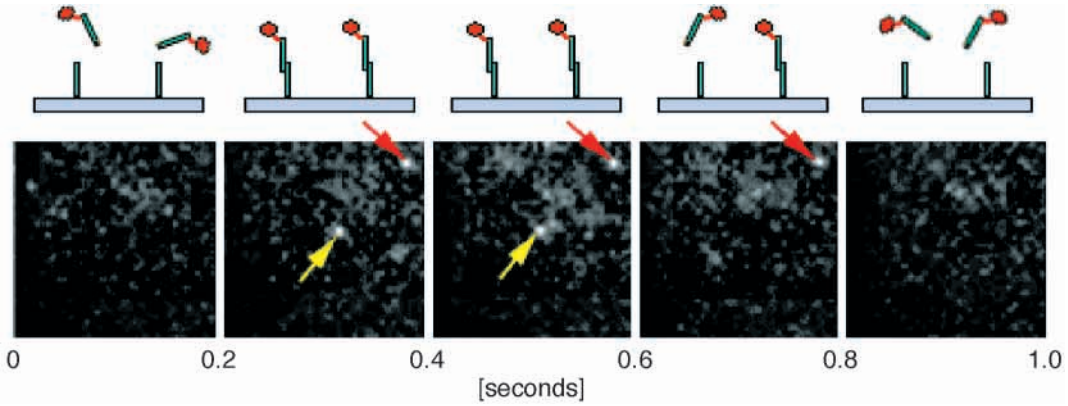

Fig. 6. Video frames (10×10 μm) of MyEnd monolayers exposed to Cy3-F(ab)-labelled VE-cadherin-Fc. Individual events of trans-interaction are marked by arrows. Lifetimes of trans-interaction of the two events shown correspond to ≈400 milliseconds (lower arrow) and ≈600 milliseconds (upper arrow).

Trans-interaction of soluble VE-cadherin-Fc* with immobilised VE-cadherin-Fc

In a first attempt, unlabelled VE-cadherin-Fc was adsorbed to glass surface (solid phase) and then covered with 10 μg/ml VE-cadherin-Fc* in HBSS/BSA (soluble phase) still containing free Cy3-F(ab) directed against human Fc. In a first step, free Cy3-F(ab) molecules were allowed to bind to Fc-portion of adsorbed VE-cadherin-Fc under saturation (30 minutes). This allowed precise focussing of the laser beam to the glass surface and determination of the bleaching characteristics. Complete bleaches at 100 kW/cm^2 within 10 seconds, no significant bleaching at 1 kW/cm^2 within 150 illumination periods of 10 milliseconds each. Surface-bound Cy3-F(ab) was absolutely immobile. After complete bleaching of surface-bound Cy3-F(ab) individual trans-interaction events between soluble VE-cadherin-Fc* to VE-cadherin-Fc on solid phase were recorded at illumination periods of 10 milliseconds. Binding events were seen as individual fluorescent signals (fluorescence peaks) that required binding for ≥5 milliseconds to be detected as specific signal against the background noise of freely diffusing VE-cadherin-Fc* and Cy3-F(ab) in the soluble phase. An example of video frames is shown in Fig. 6 for trans-interaction of VE-cadherin-Fc* with endogenous VE-cadherin expressed on endothelial cells (see paragraph below). The characteristic lifetime for trans-interaction at 21°C between VE-cadherin-Fc* to VE-cadherin-Fc adsorbed to glass surface was τ =670±50 milliseconds, which corresponds to an off-rate constant at 21°C of $k_{off}=\tau^{-1} \approx 1.49$ second^{-1}. No binding events (fluorescence signals) were seen if the glass surface was coated with BSA only or if VE-cadherin-Fc-coated glass surface was covered with Cy3-F(ab) in the absence of VE-cadherin-Fc in soluble phase. Furthermore, trans-interaction was completely abolished by removal of Ca^{2+} from the soluble phase.

In addition to determination of k_{off}, this approach allowed us for the first time to determine activation energy of cadherin trans-interaction. Fig. 5 shows an Arrhenius plot of the temperature dependency of k_{off}. The logarithm of k_{off} is linearly dependent on 1/T (T, absolute temperature) with a slope of 1.33×10^3. This allows determination of the activation energy of E≈9.7 kJ/mol. This value is typical of weak hydrophobic interactions (Mortimer, 1987).

Trans-interaction of soluble VE-cadherin-Fc* with endogeneous VE-cadherin of endothelial cells

Endogenous VE-cadherin exposed on the cell surface of MyEnd monolayers served as solid phase for determination of lifetime (k_{off}) of trans-interaction. Unlike experiments with coated glass surface there was no unspecific binding of Cy3-F(ab) to cell surface as shown in controls in which VE-cadherin-Fc was omitted from the Cy3-F(ab)-containing supernatant. Only in the presence of both VE-cadherin-Fc* and Ca^{2+} (1.8 mM) trans-interaction events (>5 ms) were observed

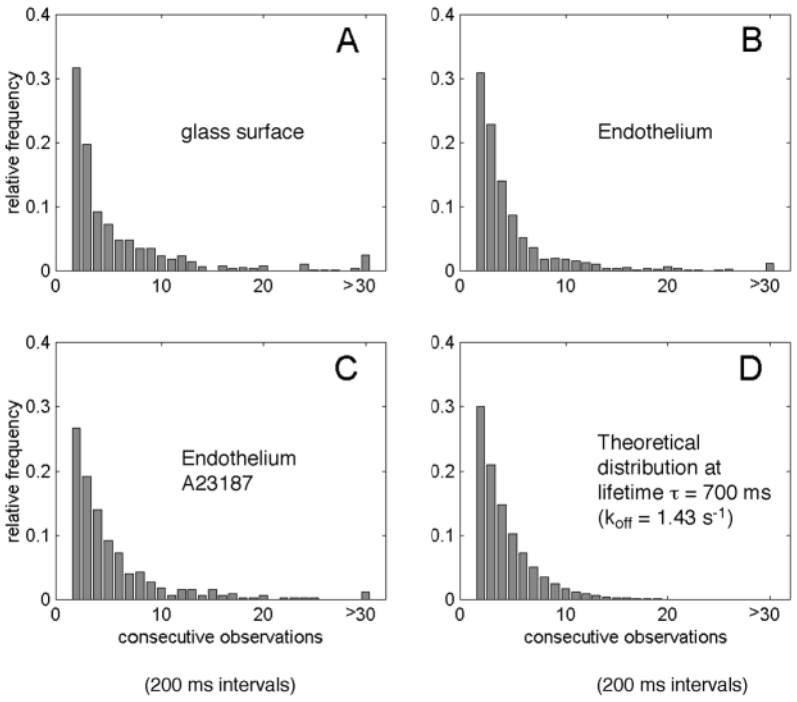

Fig. 7. Determination of lifetimes of trans-interaction of Cy3-F(ab)-labelled VE-cadherin-Fc with VE-cadherin-Fc adsorbed to glass surface and endogenous VE-cadherin exposed to cell surface of MyEnd monolayers. Average lifetime of trans-interaction at various conditions corresponds to the theoretical distribution of an average lifetime of 700 milliseconds.

(Fig. 6). Average lifetime for trans-interaction (duration of surface bound fluorescent spots) was τ=710 milliseconds (k_{off}≈1.41 second^{-1}) as calculated by maximum likelyhood fitting of relative frequencies of consecutive observations (Fig. 7) according to Eqn 3 (see Materials and Methods section). After treatment with either cytochalasin D or A23187, frequency of binding events increased 1.6-1.7 fold, whereas the lifetime of trans-interaction events (k_{off}) remained unchanged (τ=690 minutes, k_{off}≈1.45 second^{-1}). Slight increase of binding events (factor below 2) corresponds to the slight increase of VE-cadherin exposed on the dorsal cell surface in response to drug treatment (Fig. 4). This allows us to conclude that the association rate constant (k_{on}) and the overall affinity is not significantly altered by drug treatment (similar level of trans-interaction events at similar surface concentration of VE-cadherin).

Lateral mobility of VE-cadherin

The degree of lateral mobility of VE-cadherin in the plane of the lipid bilayer of the dorsal surface of endothelial cells was determined by mean square displacement (MSD) over time for individual molecules during the lifetime of their trans-interaction with VE-cadherin-Fc* in soluble phase (Fig. 8). Whereas cytochalasin D and A23187 had no effect on lifetime of trans-interaction, both compounds had a strong impact on lateral mobility of endogenous VE-cadherin during trans-interaction with VE-cadherin-Fc*. Correlation of the position of consecutive images allowed to reconstruct the two-dimensional trajectory of each trans-interacting molecule. In control cells the diffusion coefficient (calculated from initial slopes of plots shown in Fig. 8) was D≈0.017 μm^2 second^{-1}, whereas in cells treated with A23187 and cytochalasin D average lateral mobility increased significantly to D≈0.17 μm^2 second^{-1} (cytochalasin D) and D≈0.35 μm^2 second^{-1} (A23187). Barrier free diffusion occurred only at time scales of up to 500 ms. This indicates corralling of trans-interacting complexes into restricted submicron barrier free plasmalemmal areas (BFA), which expanded in response to actin depolymerisation. BFA was calculated according to Edidin et al. (Edidin et al., 1994). In control conditions BFA was≈0.045 μm^2 and increased several fold in response to cytochalasin D (0.18 μm^2) and A23187 (0.37 μm^2) (Fig. 8). Overall lateral mobility of cadherins (apparent D) in response to actin depolymerisation can be concluded from concomitant increase of both, D and BFA.

Distribution of trans-interacting VE-cadherins on endothelial cell surface

In untreated controls, trans-interaction of VE-cadherin-Fc* occurred with high frequency at circumscribed microdomains (up to ~1 μm^2 in size, Fig. 9A). Surface areas between these preferential sites for trans-interaction displayed no or only infrequent binding events. Treatment of cells with either A23187 (Fig. 9B) or cytochalasin D caused significant dispersal of these preformed cadherin microdomains. At the same time overall frequency of trans-interaction increased 1.6-1.7-fold compared with controls (see above) showing that F-actin plays an important role in control of both the size of cadherin microdomains as well as the pool of cadherins present within the free dorsal cell surface.

Discussion

The vascular endothelium is one of the best studied cellular systems in which modulation of cadherin (VE-cadherin)-

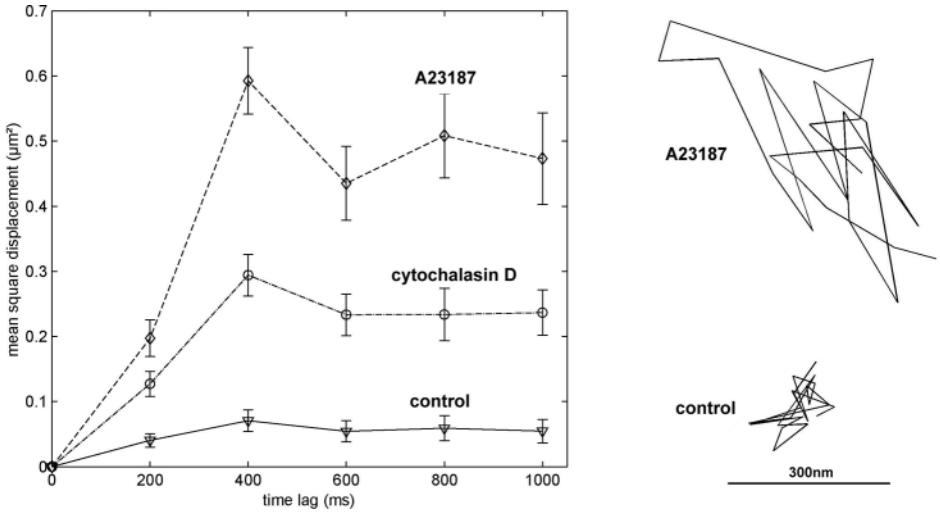

Fig. 8. Effect of cytochalasin D and A23187 on mean square displacement (MSD) vs. time of endogenous VE-cadherin molecules during their trans-interaction with Cy3-F(ab)-labelled VE-cadherin-Fc. The initial slopes allow determination of the diffusion coefficient D≈0.017 μm^2/second (untreated cells), 0.17 μm^2/second (cytochalasin D) and 0.35 μm^2/second (A23187). The saturation value of the MSD allows determination of the barrier free areas to be BFA≈0.045 μm^2 (untreated), 0.18 μm^2 (cytochalasin D) and 0.37 μm^2 (A23187). Typical trajectories of individual cadherin molecules in A23187-treated and untreated cells are shown on the right.

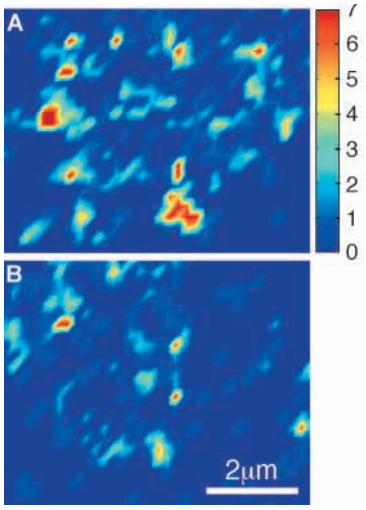

Fig. 9. Visualisation of VE-cadherin binding sites on the dorsal cell surface of MyEnd monolayers. Note preferential sites for trans-interaction before addition of A23187 (A) and dispersal of these sites 10 minutes after addition of A23187 (B). Frames shown in A and B were taken from the same membrane area before and after A23187 application.

mediated adhesion has been shown to play a crucial role in important physiological processes such as the control of macromolecular permeability and transmigration of leukocytes (Dejana, 1997; Allport et al., 1997; Petzelbauer et al., 2000; Vestweber, 2000). Dysregulation of cadherin-mediated barrier properties may cause life-threatening complications resulting from hyperpermeability (edema formation) and hemorrhagia (bleeding diseases). However, the basic molecular mechanisms modulating cadherin ectodomain trans-interactions are far from being understood. Most studies dealing with regulation of cadherin-mediated adhesion have focussed on catenins and on the actin filament system. Although it is clear now that catenins, the cytodomain of cadherins and the actin filament system are targets of signalling pathways modulating intercellular adhesion (Yap et al., 1997a; Angst et al., 2001; Vasioukhin and Fuchs, 2001) it still remains an open question how connection of cadherins to catenins and the actin filament system modifies adhesive properties. In particular, the possibility has so far not been excluded that the catenin/actin system might modulate affinities for adhesive trans-interaction of cadherins by an inside-out signalling mechanism in a similar way to that shown for integrins, which share several basic features of cadherins such as cis-dimeric structure, association of cytodomains with the actin filament system and Ca^{2+}-dependency of adhesion (Calderwood et al., 2000).

Regulation of adhesion by cytoskeletal tethering

Cadherin-coated microbeads adhere to the free dorsal cell surface of neuronal cells (Lambert et al., 2000) and endothelial monolayers (this study) by recruitment of cellular cadherins, catenins and F-actin. Adhesion of VE-cadherin-coated beads to the endothelial cell surface could be significantly reduced by cytochalasin D and the Ca^{2+}-ionophore A23187. Both compounds reduced cellular F-actin content by 30-60%. Stabilisation of F-actin by pretreatment of cells with jasplakinolide prevented drug-induced weakening of bead adhesion demonstrating that both, cytochalasin D and A23187 mediate anti-adhesive activity via destabilisation of the actin filament system.

These observations are in line with studies reporting disturbance of junctional integrity of endothelial and epithelial monolayers by both, cytochalasin D (Hirano et al., 1987; Stevensen and Begg, 1994; Nybom and Magnusson, 1986; Drenckhahn and Ness, 1997) and A23187 (Suttorp et al., 1989; Kuhne et al., 1993; Schnittler et al., 1997; Michel and Curry, 1999). A23187-induced barrier breakdown was suggested to be caused by Ca^{2+}-calmodulin-mediated myosin-based contractility (Schnittler et al., 1990; Goeckler and Wysolmerski, 1995) and by Ca^{2+}-gelsolin-induced actin depolymerisation (Kuhne et al., 1993). Whereas these studies could not discriminate between an essential facilitative role of cytoskeletal activity on overall monolayer structure [cytochalasin-treated cells undergo shape changes and display contraction of stress fibres and of actin-myosin-based gels (Kolega et al., 1991)] versus a direct modulatory action of the actin-based cytoskeleton on adhesive strength, the laser tweezer experiments performed in the present study allow to conclude that drug-induced depolymerisation of actin exerts a direct inhibitory action on cadherin-mediated adhesion. Importantly, weakening of bead adhesion associated with depolymerisation of F-actin was not caused by reduction of extracellular affinity of VE-cadherin. Thus the possibility of inside-out modulation of affinity can be excluded as a possible mechanism of how cadherin adhesion might be regulated by the actin filament system.

Based on our previous theoretical evaluation of the effects between cytoskeletal linkage and extracellular adhesion we favor a mechanism by which adhesion (transmembrane cooperativity) is primarily controlled by the degree of lateral mobility of cadherins in the plane of the plasma membrane. As shown in the present study on living cells and in our previous study using single molecule atomic force microscopy, the life time of adhesive trans-interaction of cadherins is only 550-700ms and occurs at extremely low affinity [K_D~10^{-4} M (Baumgartner and Drenckhahn, 2002b)]. These very weak binding properties require immobilisation of cadherins within the cytoskeleton in order to guarantee that cadherins can rapidly rebind after dissociation. Without tethering to the cytoskeleton, cadherins would be driven apart by increased lateral mobility (~10- to 20-fold increase, this study) and would require a prolonged time interval for new collision and rebinding. Inhibition of lateral mobility by any kind of immobilisation of cadherins would increase the number of bonds per unit surface area and hence the overall adhesive strength by a factor of 10 to 100 (Baumgartner and Drenckhahn, 2002a). This conclusion is further supported by experiments in which the cytoplasmic domains of cadherins were extensively crosslinked and clustered by the FKBP/FK1012 crosslinking system. Under conditions of crosslinking and immobilisation of cadherins a significant improvement of adhesion to cadherin-coated substrates was observed (Yap et al., 1997b).

Clustering of cadherins at the bead interface will probably further strengthen adhesion by improving immobilisation of cadherins within clusters and, in addition by increasing the local concentration of cadherins at clustered sites. Moreover clusters stabilised by cytoskeletal linkage might allow multivalent zipper-like binding between opposing cadherins which further would increase the strength of adhesion (Shapiro et al., 1995). However such a mechanism is less likely to occur at sites of bead adhesion because the average concentration of cadherins covering the bead surface (up to 10^3 molecules/μm^2) would be too low to allow zipper-like formation (average distance between cadherins on bead surface is about 30-60 nm).

In the present study we show that the diffusion coefficient of actively trans-interacting endogenous VE-cadherin molecules within the free dorsal plasma membrane (D=0.017 μm^2 $second^{-1}$) is in the order of the mobility determined previously for ~90% of transfected E-cadherin-GFP fusion proteins in MDCK-cells by fluorescence recovery after photobleaching (Adams et al., 1998) and for the mobility of ~50% of small particles (40-200 nm) coated with IgG against ectodomain of E-cadherin in transfected L-cells (Sako et al., 1998). Tenfold higher values (0.3 μm^2 $second^{-1}$) were observed for ~50% of E-cadherin-GFP fusion proteins in transfected L-cells by single molecule fluorescence (Iino et al., 2001).

Whereas we have observed that lateral mobility of

endogenous VE-cadherin increased up to 10 fold in response to reduction of F-actin by 10 μM cytochalasin D and A23187, respectively, overall mobility of cadherin antibody-coated particles was reported by Sako et al. to decrease rather than to increase in response to 1 μM cytochalasin D (Sako et al., 1998). These differences between both studies may depend on different drug concentrations applied and on the fact that tracking of antibody-coated particles (Sako et al., 1998) may primarily allow to determine bulk motion of clustered rather than of free single molecules which were recorded in our study.

With respect to the surface distribution of actively trans-interacting VE-cadherin molecules our observations indicate non-homogeneous distribution of VE-cadherin. The size of microdomains enriched in actively trans-interacting VE-cadherin was up to ≈1 μm^2 and these hot spots depended on an intact actin filament system as indicated by their dispersal in response to cytochalasin D and A23187 (Fig. 9). At the same time cytochalasin D and A23187 resulted in an enlargement of submicron domains in which barrier free diffusion (BFA) is possible.

Cadherin microdomains located at the free cell surface may serve as preformed building blocks facilitating rapid formation of new junctions as soon as neighbouring cells approach each other for a limited time interval. The recruitment of freely diffusing single molecules into such initial sites of cell-to-cell contact may be more time consuming and less effective than the involvement of preformed microdomains with high local cadherin concentrations.

Within the cadherin microdomains VE-cadherin molecules were compartmentalised (corralled) to submicron-sized BFA with average size of 0.045 μm^2. Three fold larger BFA (0.16 μm^2) was observed by particle tracking for E-cadherin in transfected L-cells (Sako et al., 1998) whereas class I MHC molecules displayed BFA about twentyfold larger (1.13 μm^2) (Edidin et al., 1994). Cytochalasin D and A23187 caused significant enlargement of BFA for VE-cadherin (0.18-0.37 μm^2) which is in the range of BFA for E-cadherin with truncated cytodomain (0.50 μm^2). Enlargement of BFA for both cadherins can be most readily explained by loss of cytoskeletal tethering caused either by truncation of E-cadherin cytodomain (Sako et al., 1998) or inhibition of actin polymerisation (this study). Similar observations were made with GPI-anchored membrane protein Qa-2 that appears to be indirectly linked via transmembrane proteins to the actin cytoskeleton as the main elastic barrier for diffusion sensitive to cytochalasin D (Suzuki and Sheetz, 2001). Studies by single particle tracking and laser tweezers have shown that barriers (tethers) determining BFA do not represent absolute obstacles because they can be overcome by jumping events, dissociation of tethers or by pulling forces (Kusumi and Sako, 1996; Sako et al., 1998). Although confinement to BFA is a phenomenon now documented for a variety of integral membrane proteins and shown in this study for VE-cadherin to be sensitive to the state of actin polymerisation, the functional significance of BFA is still enigmatic.

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