Journal of Cell Science 107, 1347-1357 (1994)
Printed in Great Britain © The Company of Biologists Limited 1994



# Modulation of tight junction structure in blood-brain barrier endothelial cells

## Effects of tissue culture, second messengers and cocultured astrocytes

**Hartwig Wolburg[1], Jochen Neuhaus[1,*], Uwe Kniesel[1,3], Bodo Krauß[1], Eva-Maria Schmid[1], Mücella Öcalan[2], Catherine Farrell[2,†] and Werner Risau[2,3,‡]**

[1]Institut für Pathologie, Universität Tübingen, D-72076 Tübingen, FRG
[2]Max-Planck-Institut für Psychiatrie, Abteilung Neurochemie, D-82152 Martinsried, FRG
[3]Max-Planck-Institut für physiologische und klinische Forschung, W.G. Kerckhoff Institut, Abteilung Molekulare Zellbiologie, Parkstrasse 1, D-61231 Bad Nauheim, FRG

*Present address: Max-Delbrück-Zentrum für molekulare Medizin, Robert-Rössle Str. 10, D-13125 Berlin, FRG
†Present address: AMGEN Inc., 1900 Oak Terrace Lane, Thousand Oaks, CA 91320, USA
‡Author for correspondence at address 3

## SUMMARY

**Tight junctions between endothelial cells of brain capillaries are the most important structural elements of the blood-brain barrier. Cultured brain endothelial cells are known to loose tight junction-dependent blood-brain barrier characteristics such as macromolecular impermeability and high electrical resistance. We have directly analyzed the structure and function of tight junctions in primary cultures of bovine brain endothelial cells using quantitative freeze-fracture electron microscopy, and ion and inulin permeability. The complexity of tight junctions, defined as the number of branch points per unit length of tight junctional strands, decreased 5 hours after culture but thereafter remained almost constant. In contrast, the association of tight junction particles with the cytoplasmic leaflet of the endothelial membrane bilayer (P-face) decreased continuously with a major drop between 16 hours and 24 hours. The complexity of tight junctions could be increased by elevation of intracellular cAMP levels while phorbol esters had the opposite effect. On the other hand, the P-face association of tight junction particles was enhanced by elevation of cAMP levels and by coculture of endothelial cells with astrocytes or exposure to astrocyte-conditioned medium. The latter effect on P-face association was induced by astrocytes but not fibroblasts. Elevation of cAMP levels together with astrocyte-conditioned medium synergistically increased transendothelial electrical resistance and decreased inulin permeability of primary cultures, thus confirming the effects on tight junction structure and barrier function. P-face association of tight junction particles in brain endothelial cells may therefore be a critical feature of blood-brain barrier function that can be specifically modulated by astrocytes and cAMP levels. Our results suggest an important functional role for the cytoplasmic anchorage of tight junction particles for brain endothelial barrier function in particular and probably paracellular permeability in general.**

Key words: tight junction, endothelium, blood-brain barrier, cAMP, astrocyte, permeability, freeze fracture

## INTRODUCTION

The blood-brain barrier protects the brain from the blood milieu and maintains homeostasis of the brain microenvironment, which is crucial for neuronal activity and function. In mammals and higher vertebrates, the sites of the blood-brain barrier are the complex tight junctions between brain endothelial cells that prevent the passage of hydrophilic molecules from blood to brain and vice versa (Reese and Karnovsky, 1967; Brightman and Reese, 1969). Brain endothelial cells are derived from permeable capillaries of the perineural vascular plexus that invade the embryonic neuroectoderm and form the intraneural capillaries by an angiogenic process (Risau, 1991). Differentiation of blood-brain barrier characteristics takes place later when intraneural capillaries become impermeable to horseradish peroxidase (Wakai and Hirokawa, 1978; Risau et al., 1986a,b; Risau and Wolburg, 1990, 1991; Saunders et al., 1991). The development of the blood-brain barrier is a complex process that leads to endothelial cells with unique permeability characteristics due to high electrical resistance and the expression of specific transporters and metabolic pathways (for review see Risau and Wolburg, 1990; Dermietzel and Krause, 1991). Since the properties of brain endothelial cells are not predetermined but can be induced in non-brain endothelial cells by the neural microenvironment (Stewart and Wiley, 1981; Risau et al., 1986a,b), it is important to elucidate the molecular mechanism that is responsible for the induction and maintenance of blood-brain barrier properties in endothelial cells. There is some evidence that astroglial cells play a role in this process (Janzer and Raff, 1987).

We have previously investigated the expression of blood-brain barrier proteins in vivo and in vitro. While some of these molecules may be markers for blood-brain barrier properties of endothelial cells in vivo (e.g. glucose transporter), the majority

are not useful for in vitro studies because either their function is not known (e.g. the HT7-antigen) or the expressing cell type in brain capillaries is not consistent with a role in the endothelial blood-brain barrier (e.g. gamma-glutamyl-transpeptidase). Furthermore, many of these proteins are upregulated under in vitro culture conditions in endothelial cells, fibroblasts and transformed cells, casting doubt on the functional significance of this expression for blood-brain barrier properties (e.g. see Risau et al., 1986a,b, 1992; Seulberger et al., 1990; Frey et al., 1991; Meyer et al., 1991).

Therefore, we decided to study the tight junctions as the primary structural feature of the blood-brain barrier in endothelial cells in vivo and in vitro. Tight junctions between the endothelial cells of the brain microvessels are more complex than those in other endothelial cells in the body (Nagy et al., 1984) and provide a high electrical resistance that is comparable to that of a tight epithelium. However, the molecular nature of the tight junction is still enigmatic and it is not clear whether it is mainly of a proteinaceous or lipid composition. The expression of tight junction-associated proteins such as ZO-1 was found not to correlate with the 'tightness' of junctions as measured by electrical resistance in MDCK monolayers (Stevenson et al., 1988). Hence, we decided to develop a quantitative freeze-fracture method to analyze directly the structure and complexity of tight junctional strands in primary cultures of bovine brain endothelial cells. We observed a rapid decline in the continuity and branching capacity of tight junctional strands in tissue culture. Most importantly, tight junction particles rapidly switched their position in the plasma membrane from the P-face to the E-face. Astrocytes and elevation of cAMP increased the complexity of tight junctions and were also effective in maintaining the P-face association of the particles. Our results indicate an important functional role for the cytoplasmic anchorage of tight junctions in the P-face of brain endothelial cells for blood-brain barrier permeability, and suggest that astrocyte-derived factors and second messenger levels are important modulators of tight junction structure.

## MATERIALS AND METHODS

### Isolation and culture of bovine brain endothelial cells

The principal procedure has been described previously for rat brain endothelial cells (Risau et al., 1990). Briefly, bovine cerebral cortices, obtained freshly from the local slaughterhouse, were dissected free of meninges and white matter, and the remaining grey matter was minced and digested using collagenase (0.35%, w/v, collagenase; Worthington CLS II in 10 mM HEPES buffered DMEM, 90 minutes at 37˚C). The capillaries were separated from the remaining slurry by BSA density gradient centrifugation. As a modification of the previous procedure we used collagenase/dispase (0.1%, w/v; Boehringer, Mannheim, FRG) for the second digestion (60 minutes, 37˚C) of the capillary pellet. After Percoll gradient purification capillary fragments were seeded onto rat tail collagen-coated dishes. Clonal cell lines were obtained by sparsely plating the capillary fragments (after fractionation in Percoll gradients) on collagen and individual colonies were inspected after 3-6 days. Those that seemed to be free of pericytes were isolated using cloning cylinders (5 mm internal diameter) and brief trypsinization. The cells were transferred to 35 mm dishes and grown to confluency. They could be subcultured at a split ratio up to 1:20 in DMEM medium (4.5 g glucose/l) supplemented with 10% calf serum (Hyclone, Logan, USA), and 1% bovine retinal extract (as a crude source of fibroblast growth factors). All endothelial cell cultures were routinely checked for von Willebrand factor expression.

### Isolation and culture of rat astroglial cells

Cerebral cortex from newborn Lewis rats was dissected free of meninges, minced and incubated in a solution containing 0.5% trypsin in PBS at 37˚C for 10 minutes. Cells were allowed to settle over 5 ml of culture medium (DMEM supplemented with 10% FCS) and were then resuspended and plated in 75 $cm^2$ flasks. Medium was changed after 4 hours. After 6 days the contaminating neurons and oligodendrocytes were removed by shaking on a horizontal shaker at 37˚C for 6-8 hours. The remaining cells were treated with cytosine arabinoside (AraC; $2\times10^{-5}$ M; Sigma, Deisenhofen, FRG) for 2 days; split 1:2 and treated again with AraC as described above. This procedure routinely yielded cultures of more than 95% positive cells for glial fibrillary acidic protein (GFAP) (Noble et al., 1984).

### Isolation and culture of other cells

Rat meningeal fibroblasts were derived by trypsin treatment (0.05% trypsin, 0.02% EDTA; at 37˚C for 15 minutes) of the dissected meninges from newborn rat brains. NIH-3T3 fibroblasts and C6 glioma cells were from the American Type Tissue Culture Collection. MDCK cells (high resistance strain) were kindly provided by Dr Kai Simons (EMBL, Heidelberg, FRG).

### Coculture and treatments

Cellagen™ filter discs (ICN Biomedicals, Cleveland, USA) were used for cocultures. The day before the isolation of capillary fragments, cultures of rat astrocytes were trypsinized and the cells were plated onto the underside of the filter in a drop of 100 μl culture medium containing $10^5$ cells. The filter was inverted 2 hours later, fresh medium was added and the next day purified capillary fragments were plated on the other side of the filter (approx. $10^5$ cells). The cells were cocultured for the indicated length of time, fixed and processed for electron microscopy. Conditioned medium was collected from cells cultured for 2-4 days in DMEM medium supplemented with 2% FCS and was added as a 1:1 mixture with regular culture medium, the FCS concentration was then adjusted to 10% and the cells were incubated for the indicated length of time. Cells were treated with forskolin (Sigma, Deisenhofen, FRG) at a concentration of 50 μM together with the phosphodiesterase inhibitor Ro-201724 (BIOMOL, Hamburg, FRG; final concentration 50 μM) or with the phorbol ester TPA (phorbol-12-myristate-13-acetate; Sigma, Deisenhofen, FRG) at a concentration of 10 ng/ml (16.2 nM) for 2 days.

### Freeze-fracturing and electron microscopy

Freshly isolated bovine brain capillary fragments and cells cultured for various time periods on collagen-coated tissue culture plastic or on Cellagen™ filters were immersion-fixed in 2% glutaraldehyde, buffered in 0.1 M cacodylate buffer for 1 hour and then washed in the same buffer without the fixative. For ultrathin sectioning, pieces of filter were dissected out, postfixed in 1% buffered $OsO_4$, washed several times in pure buffer and dehydrated in a graded ethanol series. The 70% ethanol was saturated with uranyl acetate for contrast enhancement. The dehydrated filter segments were embedded in Araldite (Serva, FRG) and sectioned on an LKB Nova ultramicrotome.

For freeze-fracturing, small rectangles of glutaraldehyde-fixed filters were treated in 30% glycerol for half an hour and then placed between two gold specimen holders. They were shock-frozen in nitrogen-slush (–210˚C), transferred into a Balzers freeze-fracturing apparatus (BAF 400 D) and fractured at –150˚C and $5\times10^{-6}$ mbar (1 bar=$10^5$ Pa). The fracture faces were immediately shadowed with platinum/carbon (2 nm, 45˚) and carbon (20 nm, 90˚) for stabilization. The replicas were cleaned with 12% sodium hypochlorite, washed several times in double-distilled water and mounted on Pioloform-coated copper grids (Wacker Chemie, München). Ultrathin sections and replicas were observed in a Zeiss EM10 electron microscope. For quantitation of

**Table 1. Number of experiments and analyzed pictures**

| | Types of cultured cells | | | |
|---|---|---|---|---|
| | BBE prim. | | BBE clon. | |
| Treatment | Filters | Pictures | Filters | Pictures |
| None | 63 | 587 | 24 | 264 |
| A | 18 | 198 | 6 | 62 |
| MF | 6 | 64 | 3 | 33 |
| 3T3 | 6 | 66 | 3 | 32 |
| ACM | 42 | 329 | 12 | 141 |
| MFCM | 12 | 112 | 3 | 33 |
| 3T3CM | 6 | 60 | 9 | 96 |
| Fl | 24 | 238 | 18 | 187 |
| TPA | 24 | 211 | 9 | 91 |
| Fl/TPA | 15 | 159 | 9 | 95 |
| ACM/Fl | 9 | 73 | 3 | 33 |
| ACM/TPA | 9 | 64 | – | – |

Abbreviations: BBE, bovine brain endothelial cells; A, astrocytes; MF, meningeal fibroblasts; 3T3, 3T3 fibroblasts; CM, conditioned medium from the respective cell type; Fl, forskolin plus phosphodiesterase inhibitor; TPA, phorbol ester.

tight junction complexity, both tight junction length (*L*) in μm and the number of branching points (*B*) in approximately 25 micrographs per experiment (Table 1) were evaluated at a final magnification of 140,000:1. *L* and *B* were measured in a defined area of 3.15 $\mu m^2$ using a digitizer tablet (Summagraphics). The complexity index (CI) was calculated as (*B*/*L*) in 1/μm. Studies on the CI were done on E-faces only in order to get a more reliable determination of branching points of the tight junction network. The degree of P-face association (PFA) was determined by analyzing an average of 18 micrographs per experiment (Table 1) at a final magnification of 220,500:1 of an area of 1 $\mu m^2$. The PFA was calculated as (P-face associated strands ×100)/*L* in %. Statistical analysis was performed using the one-way Anova and the Scheffe test for determination of significance.

### Permeability assays

After Percoll purification bovine brain capillary fragments were directly plated onto collagen-coated Transwell™ filters (6.5 mm diameter; Costar, Bodenheim, FRG). All other cells were plated after trypsinization. Cells were grown to confluency, which was assessed by staining a parallel filter using hematoxylin. The permeability to $^3$H-labeled inulin was measured by adding 100 μl (5 μCi/ml) of $^3$H-labeled inulin (1 Ci/mmol; Amersham, UK) in culture medium to the upper compartment. The amount of radioactivity that had diffused into the lower compartment was determined after 30 minutes. Transendothelial resistance was measured using microelectrodes (World Precision Instruments; Spechbach, FRG). MDCK cells were grown and measured as controls.

### Immunocytochemistry

Immunocytochemistry of cells and frozen sections was performed as described previously (Risau et al., 1990). Antibodies and dilutions were rabbit anti-von Willebrand factor (vWF:FVIIIrAG Clotimmune; Behringwerke, FRG), 1:100; mouse monoclonal anti-smooth muscle actin (Renner GmbH, Dannstadt, FRG), 1:100; rabbit anti-GFAP (gift from Dr L. Eng, Stanford University, USA), 1:200. Controls included preimmune serum, omission of primary antibodies and unrelated class-matched monoclonal antibodies.

## RESULTS

### Modulation of the complexity of tight junctions in cultured brain endothelial cells

Since the probability is very low that tight junctions of brain capillary endothelial cells in situ will be exposed in freeze-fracture replicas, we used freshly isolated bovine brain capillaries to analyze endothelial tight junctions (Fig. 1a). The tight junctional strands were observed to be largely associated with the P-face. The E-face of the tight junctions consists of linear grooves occupied discontinuously by differently sized particles, which are consistently found to be smaller than the P-face-associated ones. These E-face-associated particles most likely correspond to the spaces between the P-face-associated strands.

The tight junctions in cultured endothelial cells derived from these capillary fragments showed a different morphology. They were discontinuous at the P-face leaving particle-free ridges between them (Fig. 2). At the E-face of cultured endothelial cells, junctional particles form chains that occupy the grooves and are more or less continuous. Therefore, we only used E-faces for the quantitative evaluation of tight junctional complexity (Fig. 3). We defined the complexity index (CI) as the density of branch points on tight junctional strands.

The complexity of tight junctions in capillary fragments appeared to be higher than in the cultured endothelial cells in which anastomoses were rarified, and several strands ended freely in the membrane plane and were not interconnected as continuous belts. Since the cells were derived from intact isolated capillaries, the tight junctions either degenerated in primary nonproliferating cells or formed incompletely in proliferating cells. We were therefore interested in the changes in tight junction complexity over time in culture. Fig. 4 shows that the CI falls very rapidly within the first 5 hours of culture from 4.6/μm in capillary fragments to 3/μm in cultured cells and remained relatively constant until day 11.

Various cell culture models were tested for the capacity to maintain blood-brain barrier characteristics, and tight junction structure and function in the cultured cells. We analyzed the effect on the CI of primary rat brain astrocytes in coculture with brain endothelial cells and of the culture medium conditioned by astrocytes as a source of secreted, soluble factors. Since Rubin et al. (1991) had observed a dramatic effect of the elevation of cAMP on some blood-brain barrier characteristics of cultured bovine brain endothelial cells, we included this treatment in our experiments. Furthermore, phorbol esters were tested to evaluate the role of the protein kinase C signalling system. The effects of the different treatments were studied on cells cultured for 5 hours, 36 hours and 11 days after isolation of capillary fragments. Table 1 summarizes the number of experiments and analyzed pictures thereof. Representative results from the 36 hour experiment are shown in Fig. 5. Forskolin (Fl, which elevates cAMP in these cells; Rubin et al., 1991; and data not shown) and astrocyte-conditioned medium (ACM) together with Fl consistently increased the CI to that of capillary fragments. Phorbol esters consistently decreased the CI, a decrease that could be partly prevented by ACM. Some representative pictures are shown in Fig. 6. We found no significant difference if astrocytes were cocultured or the astrocyte-conditioned medium was used. Meningeal fibroblasts and 3T3 fibroblasts were used as control cells for cocultures and conditioned medium. They had comparable effects to ACM on the CI (data not shown).

### Endothelial tight junctions switch from P-face to E-face in culture

We observed an extreme loss of tight junctional particles at the

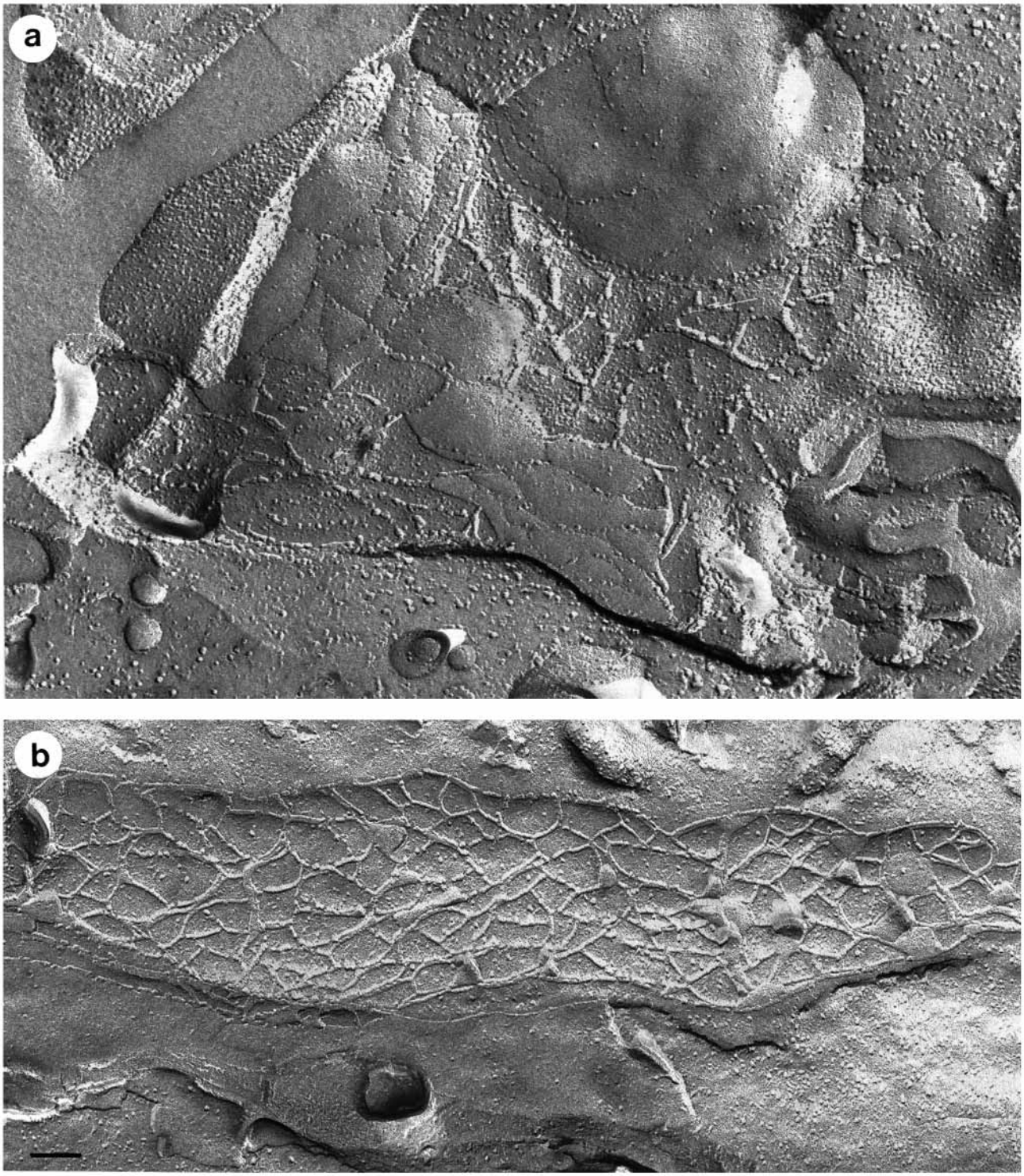


**Fig. 1.** (a) Freeze-fracture replica of a bovine brain capillary fragment showing tight junctions between endothelial cells. These junctions are predominantly P-face-associated. (b) Freeze-fracture replica of MDCK cells showing a dense meshwork of P-face-associated tight junctional strands. Bar, 0.1 μm.

P-face, leading to a complete absence or to remnants of tight junctional networks recognizable only as poorly elaborate, almost particle-free, ridges (Fig. 2). In contrast, the high electrical resistance MDCK cells revealed their typical P-face-associated strands in vitro (Fig. 1b), like epithelial cells of kidney tubules in situ, indicating that the loss of P-face-associated strands is not an obligatory consequence of culture conditions. We have investigated the time course of the loss of P-face association (PFA) during culture (Figs 3, 7). About 60% of the tight junction strands in capillary fragments were found

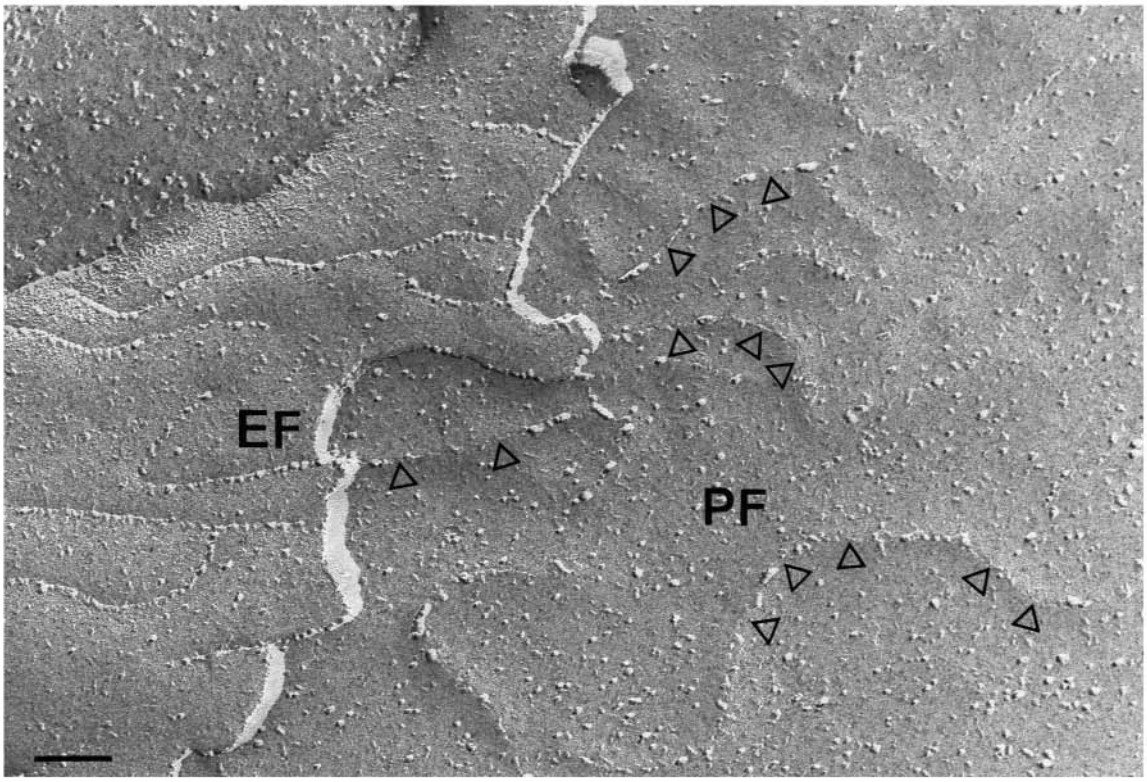


**Fig. 2.** Freeze-fracture replica of cultured endothelial cells, showing the strong E-face association of the tight junctional particles and the P-face ridges largely devoid of particles (arrowheads). Bar, 0.1 µm.

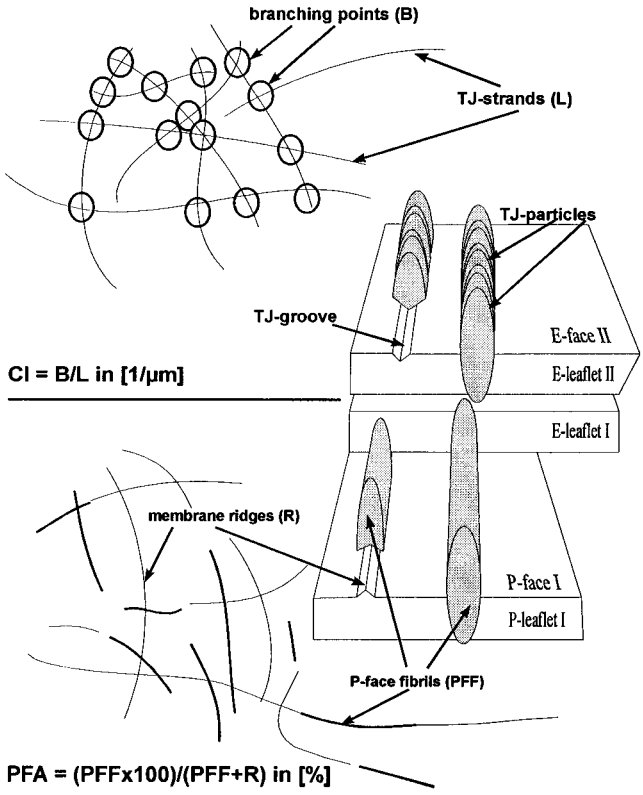


**Fig. 3.** Definition of the complexity index (CI; upper panel) and P-face association (PFA; lower panel) of tight junctions, and a schematic illustration of the tight junction components as visualized in freeze-fracture replicas. The CI was determined on E-faces only. On the P-face, membrane ridges are defined as parts of the tight junction network not occupied by any particles.

to be associated with the P-face. In contrast to the CI, the PFA decreased successively over time and only 17% PFA was measured after 11 days of culture. A major drop is observed between 16 hours and 24 hours. The treatments already described for the analysis of the CI were also studied for their effect on PFA. Figs 8 and 9 show that after 36 hours ACM and forskolin consistently increased the PFA while the phorbol ester TPA had no or very little effect (not shown). There is an additive effect of ACM and forskolin but it did not induce PFA up to the level observed in capillary fragments. After 5 hours (Fig. 10) ACM alone can maintain PFA at the level measured for capillary fragments but after 11 days neither this nor any of the other treatments was capable of reinducing PFA to a similar extent (data not shown). Although the 36-hour and 11-day-old cultures showed a similar degree of PFA, which is, however, significantly smaller than in 5-hour- and 16-hour-old cultures (Fig. 7), the treatments were considerably less effective in the 11-day-old cultures than in the 36-hour-old cultures. This indicates that some of the protoplasmic associations of tight junction particles are irreversibly (at least not reversible by our treatments) lost during culture. This is consistent with our results that cultures of continuously passaged and cloned endothelial cells displayed tight junctions of very low complexity and low PFA, which could not be significantly modulated by any of the above treatments (data not shown). Interestingly, in contrast to the indistinguishable effects on the CI of tight junctions, factors released by meningeal fibroblasts in culture could not substitute for astrocyte factors but apparently even stimulated loss of PFA (Fig. 10).

## Modulation of the permeability of cultured brain endothelial cell monolayers

Tight junctions of brain endothelial cells function as cellular

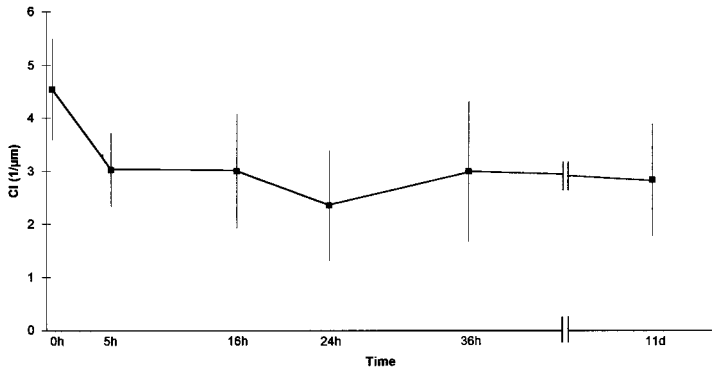


**Fig. 4.** Changes in the complexity index of tight junctions during endothelial cell culture. Means and standard deviations of the mean of triplicate experiments are shown. The CI measured at all time points (5 hours-11 days) is statistically significantly ($P$<0.01) different from the control (0 hours).

barriers to ions and hydrophilic molecules. To test the functional significance of the modulation of tight junction complexity and PFA we analyzed the permeability of endothelial monolayers for ions by transcellular electrical resistance and for inulin, which is a small hydrophilic molecule that is not taken up and metabolized by the cells. The best correlate of junctional permeability is the measurement of electrical resistance, which requires a confluent, tight monolayer of cells. Within 4 days after forming a monolayer, primary brain endothelial cells showed an electrical resistance of 20-800 ohms · $cm^2$. Fig. 11a shows that forskolin increased the electrical resistance of endothelial monolayers about twofold while ACM had very little effect. However, if added together with forskolin, ACM had a strong and synergistic effect, amounting to a tenfold increase compared to the control. Similarly, while ACM only slightly reduced inulin permeability, forskolin strongly decreased the permeability and together with ACM showed an additive effect. In contrast, TPA had no effect on permeability (Fig. 11b).

## DISCUSSION

Since the discovery by Reese and Karnovsky (1967) and Brightman and Reese (1969) that the structural equivalent of the blood-brain barrier in mammals to intravascularly injected dyes and tracers resides in the tight junctions between brain endothelial cells, tight junction structure and formation has been a focus of interest, of in vivo studies (Nagy et al., 1984; Nico et al., 1992). It is clear from the qualitative freeze-fracture and ultrathin section electron microscopic studies that the junctions are more complex in brain capillaries than in other vessels (Nagy et al., 1984) and that they mature during development, as indicated by the direct analysis of the cleft index of individual junctions (Stewart and Hayakawa, 1987; Schulze and Firth, 1992). Qualitative freeze-fracture analysis of cultured brain endothelial cells indicated that cocultured astrocytes increased tight junction complexity (Tao-Cheng et al., 1987; Arthur et al., 1987). However, no quantitative data are available and the dynamic changes of the structure of tight junctions during culture and in response to astrocyte-derived factors and second messengers has not been studied. Our quantitative analysis of the modulation of the complexity and PFA of tight junctions clearly demonstrates that brain endothelial tight junctions are malleable structures that can rapidly change their complexity and their association with the protoplasmic or extracellular leaflet of the lipid bilayer. Fixation conditions are known to alter the fine structure of tight junctions (van Deurs and Luft, 1979) but are not responsible for the differences in PFA of junctional particles because all specimens were treated using identical fixation protocols.

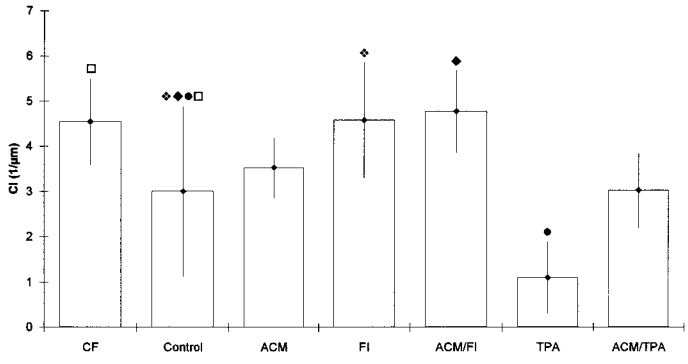


**Fig. 5.** Changes in the complexity of endothelial tight junctions in response to astrocyte-conditioned medium (ACM), elevated cAMP (Fl), phorbol ester treatment (TPA) and combinations thereof compared to capillary fragments (CF). Endothelial cells were cultured from capillary fragments for 36 hours and incubated with the various agents for 48 hours. Means and standard deviations of the mean of triplicate experiments are shown. The values labeled by the same symbols are significantly different from each other ($P$<0.01).

We have defined the complexity index (CI) as the number of branch points per unit length of tight junctional strands. Tight junctions are thought to function as a seal only if they are continuous and branched (Claude and Goodenough, 1973; Claude, 1978; Marcial et al., 1984; Madara and Dharmsathaphorn, 1985; Cereijido et al., 1989). However, the relevance of branching is not clear, because in some species, e.g. elasmobranch fish, which have a glial barrier, just one continuous tight junction strand seems to be sufficient to provide a tight barrier (Bundgaard et al., 1979).

CI and PFA decreased rapidly after capillary fragments were placed in culture. The drop in the CI had occurred already after 5 hours and thereafter remained rather constant, whereas the PFA declined steadily during culture with a major drop between 16 hours and 24 hours. The latter decrease may be related to the onset of cell proliferation whereas the drop in the CI is certainly not. Since cell proliferation is often coupled intracellularly with activation of protein kinase C, it was interesting to study the effect of phorbol esters such as TPA, which

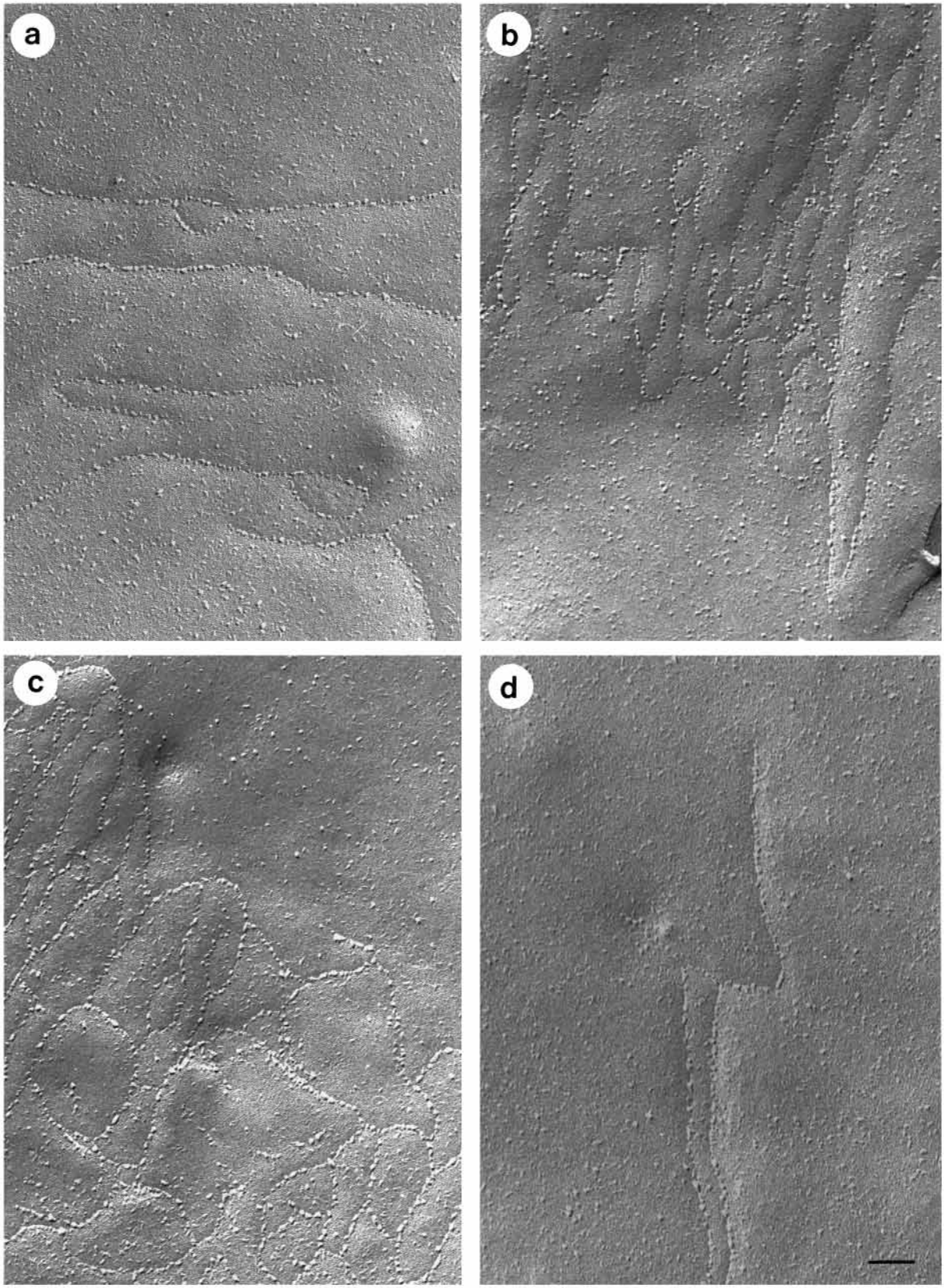


**Fig. 6.** Representative pictures of E-face-associated tight junctions in cultured endothelial cells. Primary cultures of brain endothelial cells (a) cocultured with rat astroglial cells (b) or treated with forskolin plus the phoshodiesterase inhibitor (c): the tight junction complexity is increased. (d) Primary cultures of brain endothelial cells treated with phorbol ester: very low tight junction complexity. Bar, 0.1 μm.

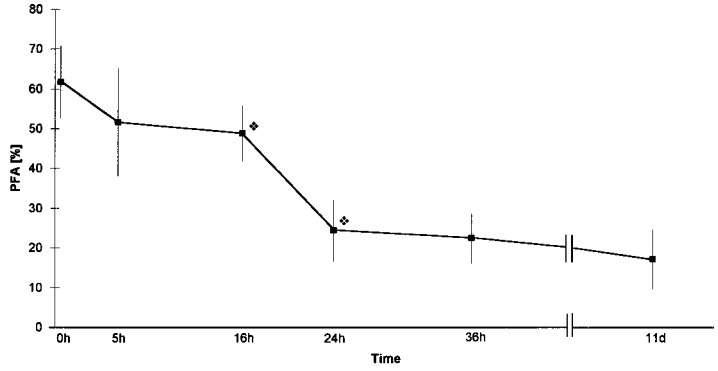


**Fig. 7.** Changes in the P-face association (PFA) of tight junctions during endothelial cell culture. Means and standard deviations of the mean of triplicate experiments are shown. The drop between 16 hours and 24 hours is statistically significant ($P<0.01$).

activate protein kinase C. In all experiments TPA decreased the CI but had no effect on the PFA of endothelial tight junctions. However, ACM and an increase in cAMP were able to counteract this effect. This observation is important in view of the fact that endothelial cells are rapidly proliferating in the postnatal rat and mouse brain but the blood-brain barrier is almost mature at this time (Risau and Wolburg, 1990; Butt et al., 1990).

In agreement with the results of Rubin et al. (1991), we found a strong effect of agents that increase cytoplasmic cAMP levels on the structure and function of endothelial tight junctions. Forskolin by itself, but more substantially if added with ACM, was able to increase the CI (largely due to the increase in the number of branching points of tight junctions) and the PFA. However, in 36-hour and 11-day-old cultures the effects of ACM plus forskolin differed remarkably: whereas the CI could be reinduced up to the same level as is found in capillary fragments, the PFA remained considerably lower at both time points. Since the electrical resistance of cultured cells (20-800 ohms $\cdot$ cm$^2$) is lower than that of capillaries in situ (about 2000 ohms $\cdot$ cm$^2$), these results suggest that the PFA rather than the CI is responsible for this barrier characteristic. This notion is supported by the finding that meningeal fibroblasts did not show any effect on the PFA, whereas astrocytes did, thereby demonstrating the specificity expected for a blood-brain barrier-specific phenomenon. Thus, the association of tight junctional strands with the cytoplasmic leaflet of brain endothelial cells rather than their complexity may be relevant for the barrier properties of the tight junctions. This is a new finding, which is consistent with some recent observations in epithelial cells. First, Mandel et al. (1993) showed that energy-depleted MDCK cells exhibited both decreased electrical resistance and decreased PFA. Second, the different strains of MDCK cells showing high or low electrical resistance seem to differ in PFA but not in CI. This striking difference in junction association of low versus high resistance MDCK cells was not recognized in the study by Stevenson et al. (1989) but was recently suggested to play a crucial role in the determination of electrical resistance (Zampighi et al., 1991; Kniesel and Wolburg, 1993). Since the molecular structure of the tight junction is unknown (for review see Citi, 1993) it is difficult to speculate about the molecular mechanisms that might be involved in the P-face to E-face switch. However, there is evidence that tight junctions are associated with the cytoskeleton, which seems to be important for the regulation of paracellular permeability (Madara, 1987; Madara and Pappenheimer, 1987; Drenckhahn and Dermietzel, 1988). It is conceivable that the interaction between the tight junction and the cytoskeleton is gradually lost in endothelial cells upon withdrawal from the brain environment and it is notable that tight junctions in peripheral, non-barrier endothelial cells are E-face-associated in vivo (Simionescu et al., 1988; Mühleisen et al., 1989). Thus, it is tempting to speculate that tight junction-associated proteins may be involved in the regulation of PFA. However, the tight junction-associated proteins ZO-1 and cingulin in epithelial and endothelial cells are not per se indicators of the tightness of tight junctions (Stevenson et al., 1988; Krause et al., 1991). Therefore, either modifications such as phosphorylation of these proteins may play an important role in this process (Stevenson et al., 1989; Nigam et al., 1991) or some unknown mechanisms and molecules may be involved. E-cadherin (uvomorulin), which is present in adherens junctions, has been shown to influence the permeability of MDCK cells, probably by affecting the tight junctions (Gumbiner and Simons, 1986). The function of E-cadherin can be dominantly inhibited by expressing a truncated cadherin molecule lacking the extracellular domain (Kintner, 1992). Since the cytoplasmic tail of E-cadherin is connected via catenins to the cytoskeleton (Ozawa and Kemler, 1992), it can be concluded that the adhesive function of E-cadherin is dependent on the association to the cytoskeleton. More recently, Itoh et al. (1993) have detected the tight junction-

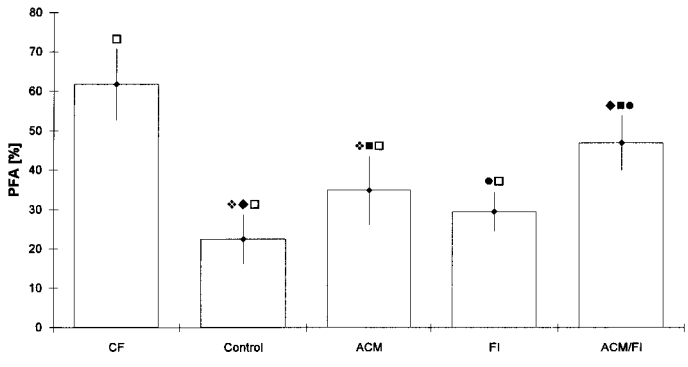


**Fig. 8.** Modulation of the PFA of tight junctions in primary brain endothelial cells in response to astrocyte-conditioned medium (ACM), elevated cAMP (FI), and a combination compared to capillary fragments (CF). Endothelial cells were cultured from capillary fragments for 36 hours and incubated with the various agents for 48 hours. Means and standard deviations of the mean of triplicate experiments are shown. The values labeled by the same symbols are significantly different from each other ($P<0.01$).

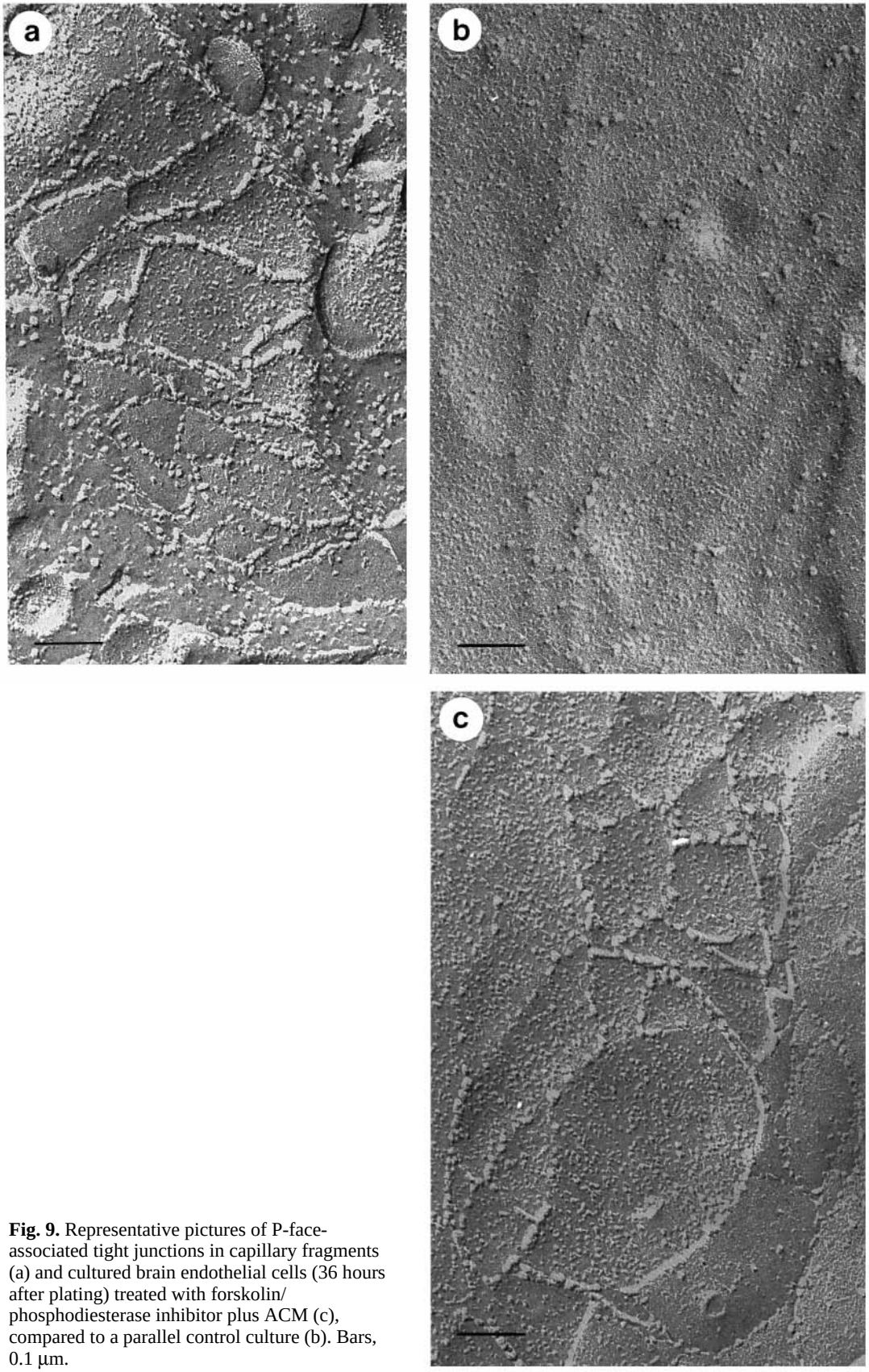


**Fig. 9.** Representative pictures of P-face-associated tight junctions in capillary fragments (a) and cultured brain endothelial cells (36 hours after plating) treated with forskolin/phosphodiesterase inhibitor plus ACM (c), compared to a parallel control culture (b). Bars, 0.1 μm.

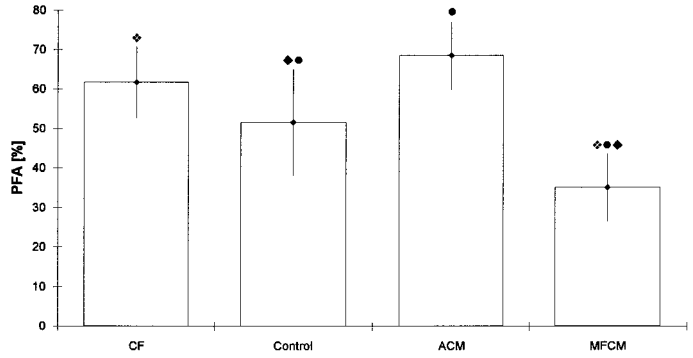


**Fig. 10.** Modulation of the P-face association of tight junctions in primary brain endothelial cells 5 hours after plating incubated with conditioned medium from astrocytes (ACM) or meningeal fibroblasts (MFCM). Means and standard deviations of the mean of triplicate experiments are shown. The values labeled by the same symbols are significantly different from each other ($P$<0.01).

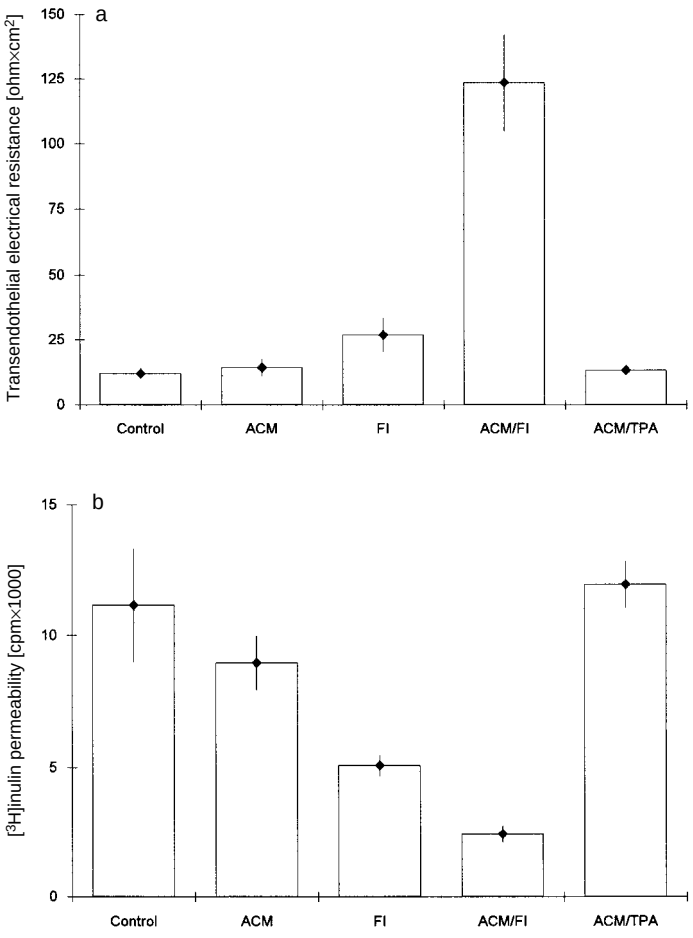


**Fig. 11.** Electrical resistance (a) and [$^3$H]inulin permeability (b) of primary endothelial monolayers 4 days after plating. Control cultures are set at 100%. Transendothelial resistance was measured using microelectrodes and the amount of radioactive inulin that had diffused into the lower compartment was determined after 30 minutes. Means and standard deviations of the mean of quadruplicate experiments are shown. All values except ACM:ACM/TPA in (a) are significantly different ($P$<0.01) from each other.

associated protein ZO-1 also in fibroblasts and shown that it colocalizes with cadherins when these are transfected into fibroblasts. In analogy, PFA of endothelial tight junctions might be regulated in a similar fashion by some unknown mechanisms, possibly mediated by endothelial cadherins (Lampugnani et al., 1992) or putative novel cadherins that are present in tight junctions (Itoh et al., 1993; Citi, 1993). Thus, the regulation of the PFA of complex tight junctions may be an important physiological parameter reflecting the functionality and permeability of blood-brain barrier tight junctions, which might be specifically regulated by astrocytes.

We thank Dr Marc Achen for critically reading the manuscript, Dr Magnus Bundgaard for discussions and the Deutsche Forschungsgemeinschaft for financial support. C.L.F. was supported by a fellowship from the MRC of Canada.

## REFERENCES

**Arthur, F. E., Shivers, R. R. and Bowman, P. D.** (1987). Astrocyte-mediated induction of tight junctions in brain capillary endothelium: an efficient in vitro model. *Dev. Brain Res.* **36**, 155-159.

**Brightman, M. W. and Reese, T. S.** (1969). Junctions between intimately apposed cell membranes in the vertebrate brain. *J. Cell Biol.* **40**, 648-677.

**Bundgaard, M., Cserr, H. and Murray, M.** (1979). Impermeability of hagfish cerebral capillaries to horseradish peroxidase An ultrastructural study. *Cell Tiss. Res.* **198**, 65-77.

**Butt, A. M., Jones, H. C. and Abbott, N. J.** (1990). Electrical-resistance across the blood-brain barrier in anesthetized rats - a developmental study. *J. Physiol.* **429**, 47-62.

**Cereijido, M., González-Mariscal, L. and Contreras, B.** (1989). Tight junction: barrier between higher organisms and environment. *NIPS* **4**, 72-75.

**Citi, S.** (1993). The molecular-organization of tight junctions. *J. Cell Biol.* **121**, 485-489.

**Claude, P. and Goodenough, D. A.** (1973). Fracture faces of zonulae occludentes from "tight" and "leaky" epithelia. *J. Cell Biol.* **58**, 390-400.

**Claude, P.** (1978). Morphological factors influencing transepithelial permeability: a model for the resistance of the zonula occludens. *J. Membr. Biol.* **32**, 219-232.

**Dermietzel, R. and Krause, D.** (1991). Molecular anatomy of the blood-brain-barrier as defined by immunocytochemistry. *Int. Rev. Cytol.* **127**, 57-109.

**Drenckhahn, D. and Dermietzel, R.** (1988). Organization of the actin filament cytoskeleton in the intestinal brush border: a quantitative and qualitative immunoelectron microscope study. *J. Cell Biol.* **107**, 1037-1048.

**Frey, A., Meckelein, B., Weilerguttler, H., Mockel, B., Flach, R. and Gassen, H. G.** (1991). Pericytes of the brain microvasculature express gamma-glutamyl transpeptidase. *Eur. J. Biochem.* **202**, 421-429.

**Gumbiner, B. and Simons, K.** (1986). A functional assay for proteins involved in establishing an epithelial occluding barrier: identification of a uvomorulin-like polypeptide. *J. Cell Biol.* **102**, 457-468.

**Itoh, M., Nagafuchi, A., Yonemura, S., Kitani-Yasuda, T., Tsukita, S. and Tsukita, S.** (1993). The 220-kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. *J. Cell Biol.* **121**, 491-502.

**Janzer, R. C. and Raff, M. C.** (1987). Astrocytes induce blood-brain barrier properties in endothelial cells. *Nature* **325**, 253-257.

**Kintner, C.** (1992). Regulation of embryonic cell adhesion by the cadherin cytoplasmic domain. *Cell* **69**, 225-236.

**Kniesel, U. and Wolburg, H.** (1993). Tight junction complexity in the retinal-pigment epithelium of the chicken during development. *Neurosci. Lett.* **149**, 71-74.

**Krause, D., Mischeck, U., Galla, H. J. and Dermietzel, R.** (1991). Correlation of zonula-occludens ZO-1 antigen expression and transendothelial resistance in porcine and rat cultured cerebral endothelial-cells. *Neurosci. Lett.* **128**, 301-304.

**Lampugnani, M. G., Resnati, M., Raiteri, M., Pigott, R., Pisacane, A., Houen, G., Ruco, L. P. and Dejana, E.** (1992). A novel endothelial-specific membrane-protein is a marker of cell-cell contacts. *J. Cell Biol.* **118**, 1511-1522.

**Madara, J. L. and Dharmsathaphorn, K.** (1985). Occluding junction structure-function relationships in a cultured epithelial monlayer. *J. Cell Biol.* **101**, 2124-2133.

**Madara, J. L.** (1987). Intestinal absorptive cell tight junctions are linked to cytoskeleton. *Amer. J. Physiol.* **253**, C171-C175.

**Madara, J. L. and Pappenheimer, J. R.** (1987). Structural basis for physiological regulation of paracellular pathways in intestinal epithelia. *J. Membr. Biol.* **100**, 149-164.

**Mandel, L. J., Bacallao, R. and Zampighi, G.** (1993). Uncoupling of the molecular fence and paracellular gate functions in epithelial tight junctions. *Nature* **361**, 552-555.

**Marcial, M. A., Carlson, S. L. and Madar, J. L.** (1984). Partitioning of paracellular conductance along the ileal crypt-villus axis: a hypothesis based on structural analysis with detailed considerations of tight junction structure-function relationship. *J. Membr. Biol.* **80**, 59-80.

**Meyer, J., Rauh J. and Galla, H. J.** (1991). The susceptibility of cerebral endothelial-cells to astroglial induction of blood-brain-barrier enzymes depends on their proliferative state. *J. Neurochem.* **57**, 1971-1977.

**Mühleisen, H., Wolburg, H. and Betz, E.** (1989). Freeze-fracture analysis of endothelial cell membranes in rabbit carotid arteries subjected to short-term atherogenic stimuli. *Virch. Arch. B Cell Pathol.* **56**, 413-417.

**Nagy, Z., Peters, H. and Hüttner, I.** (1984). Fracture faces of cell junctions in cerebral endothelium during normal and hyperosmotic conditions. *Lab. Invest.* **50**, 313-322.

**Nico, B., Cantino, D., Bertossi, M., Ribatti, D., Sassoe, M. and Roncali, L.** (1992). Tight endothelial junctions in the developing microvasculature - a thin-section and freeze-fracture study in the chick-embryo optic tectum. *J. Submicrosc. Cytol. P* **24**, 85-95.

**Nigam, S. K., Denisenko, N., Rodriguez-Boulan, E. and Citi, S.** (1991). The role of phosphorylation in development of tight junctions in cultured renal epithelial (mdck) cells. *Biochem. Biophys. Res. Commun.* **181**, 548-553.

**Noble, M., Fok-Seang, J. and Cohen, J.** (1984). Glia are a unique substrate for the in vitro growth of central nervous system neurons. *J. Neurosci.* **4**, 1892-1903.

**Ozawa, M. and Kemler, R.** (1992). Molecular-organization of the uvomorulin-catenin complex. *J. Cell Biol.* **116**, 989-996.

**Reese, T. S. and Karnovsky, M. J.** (1967). Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J. Cell Biol.* **34**, 207-217.

**Risau, W., Hallmann, R. and Albrecht, U.** (1986a). Differentiation-dependent expression of protein in brain endothelium during development of the blood-brain barrier. *Dev. Biol.* **117**, 537-545.

**Risau, W., Hallmann, R., Albrecht, U. and Henke-Fahle, S.** (1986b). Brain induces the expression of an early cell surface marker for blood-brain barrier specific endothelium. *EMBO J.* **5**, 3179-3183.

**Risau, W., Engelhardt, B. and Wekerle, H.** (1990). Immune function of the blood-brain barrier: Incomplete presentation of protein (auto-)antigens by rat brain microvascular endothelium in vitro. *J. Cell Biol.* **110**, 1757-1766.

**Risau, W. and Wolburg, H.** (1990). Development of the blood-brain barrier. *Trends Neurosci.* **13**, 174-178.

**Risau, W.** (1991). Vasculogenesis, angiogenesis and endothelial cell differentiation during embryonic development. In *The Development of the Vascular System* (ed. R. N. Feinberg, G. K. Sherer and R. Auerbach), 14th edn, pp. 58-68. Karger, Basel.

**Risau, W. and Wolburg, H.** (1991). The importance of the blood-brain barrier in fetuses and embryos - reply. *Trends Neurosci.* **14**, 15-15.

**Risau, W., Dingler, A., Albrecht, U., Dehouck, M.-P. and Cecchelli, R.** (1992). Blood-brain barrier pericytes are the main source of gamma-glutamyltranspeptidase activity in brain capillaries. *J. Neurochem.* **58**, 667-672.

**Rubin, L. L., Hall, D. E., Porter, S., Barbu, K., Cannon, C., Horner, H. C., Janatpour, M., Liaw, C. W., Manning, K., Morales, J., Tanner, L. I., Tomaselli, K. J., et al.** (1991). A cell-culture model of the blood-brain barrier. *J. Cell Biol.* **115**, 1725-1735.

**Saunders, N. R., Dziegielewska, K. M. and Mollgard, K.** (1991). The importance of the blood-brain barrier in fetuses and embryos. *Trends Neurosci.* **14**, 14-14.

**Schulze, C. and Firth, J. A.** (1992). Interendothelial junctions during blood-brain barrier development in the rat, morphological changes at the level of individual tight junctional contacts. *Dev. Brain Res.* **69**, 85-95.

**Seulberger, H., Lottspeich, F. and Risau, W.** (1990). The inducible blood-brain barrier specific molecule HT7 is a novel immunoglobulin-like cell surface glycoprotein. *EMBO J.* **9**, 2151-2158.

**Simionescu, M., Ghinea, N., Fixman, A., Lasser, M., Kukes, L., Simionescu, N. and Palade, G. E.** (1988). The cerebral microvasculature of the rat: structure and luminal surface properties during early development. *J. Submicrosc. Cytol.* **20**, 243-261.

**Stevenson, B. R., Anderson, J. M., Godenough, D. A. and Mooseker, M. S.** (1988). Tight junction structure and ZO-1 content are identical in two strains of Madin-Darby canine kidney cells which differ in transepithelial resistance. *J. Cell Biol.* **107**, 2401-2408.

**Stevenson, B. R., Anderson, J. M., Braun, D. and Mooseker, M. S.** (1989). Phosphorylation of the tight-junction protein ZO-1 in two strains of Madin-Darby canine kidney cells which differ in transepithelial resistance. *Biochem. J.* **263**, 597-599.

**Stewart, P. A. and Wiley, M. J.** (1981). Developing nervous tissue induces formation of blood-brain barrier characteristics in invading endothelial cells: A study using quail-chick transplantation chimeras. *Dev. Biol.* **84**, 183-192.

**Stewart, P. A. and Hayakawa, E. M.** (1987). Interendothelial junctional changes underlie the developmental 'tightening' of the blood-brain barrier. *Dev. Brain Res.* **32**, 271-281.

**Tao-Cheng, J., Nagy, Z. and Brightman, M. W.** (1987). Tight junctions of brain endothelium in vitro are enhanced by astroglia. *J. Neurosci.* **7**, 3293-3299.

**van Deurs, B. and Luft, J. H.** (1979). Effects of glutaraldehyde fixation on the structure of tight junctions. A quantitative freeze-fracture analysis. *J. Ultrastruct. Res.* **68**, 160-172.

**Wakai, S. and Hirokawa, N.** (1978). Development of the blood-brain barrier to horseradish peroxidase in the chick embryo. *Cell Tiss. Res* **195**, 195-203.

**Zampighi, G., Bacallao, R., Mandel, L. and Cereijido, M.** (1991). Structural differences of the tight junction between low and high resistance MDCK monolayers. *J. Cell Biol.* **115**, 479a; abstract.

(Received 18 November 1993 - Accepted, in revised form, 17 February 1994)