

A Cell Culture Model of the Blood-Brain Barrier

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Abstract. Endothelial cells that make up brain capillaries and constitute the blood-brain barrier become different from peripheral endothelial cells in response to inductive factors found in the nervous system. We have established a cell culture model of the blood-brain barrier by treating brain endothelial cells with a combination of astrocyte-conditioned medium and agents that elevate intracellular cAMP. These cells form

high resistance tight junctions and exhibit low rates of paracellular leakage and fluid-phase endocytosis. They also undergo a dramatic structural reorganization as they form tight junctions. Results from these studies suggest modes of manipulating the permeability of the blood-brain barrier, potentially providing the basis for increasing the penetration of drugs into the central nervous system.

THE blood-brain barrier (BBB),¹ interposed between the circulatory system and the central nervous system (CNS), is relatively impermeable to ions, many amino acids, small peptides, and proteins. In vertebrates, the BBB exists at the level of the endothelial cells (ECs) that make up brain capillaries (Brightman, 1989). These ECs differ from those in most peripheral capillaries in two important respects. First, tight junctions with extremely high electrical resistance are present between brain capillary ECs (Crone and Olesen, 1982; Butt et al., 1990). This limits the amount of paracellular flux. Second, brain ECs undergo a relatively slow rate of fluid-phase endocytosis, as assayed by the uptake of tracer molecules, such as HRP (Reese and Karnovsky, 1967). This limits the amount of transcellular flux. In short, it is the combination of the limited paracellular and transcellular movement that accounts for the existence of the BBB.

In recent years, the manner in which ECs in the brain become different from those in the periphery has been examined. Stewart and Wiley (1981) found that ECs derived from brain capillaries that are then permitted to vascularize peripheral tissue become relatively leaky. Conversely, ECs originally derived from peripheral capillaries become less leaky when they vascularize brain tissue. They concluded that tissue environment influences EC phenotype. Janzer and Raff (1987) showed further that type 1 astrocytes, which normally project to brain capillary ECs in vivo, are able to provide some of the inductive influences of brain tissue, again

making ECs less leaky than they are when present in contact with peripheral tissues.

These studies were both based on in vivo measurements of cationic dye (e.g., Evan's blue) penetration, a procedure that, in effect, assays albumin permeability since these dyes generally bind tightly to albumin when introduced into the circulation. Hence, they introduced some important questions. For instance, how does the CNS microenvironment (or, more specifically, astrocytes) influence EC permeability? Also, what factors found in the CNS cause ECs to become brain-like?

In terms of the manner in which astrocytes affect EC permeability, there is no direct evidence that astrocytes cause endothelial cells to form tight junctions of extremely high electrical resistance. The available evidence indicates that it seems likely that paracellular movement of albumin (and, therefore, Evan's blue-albumin) across ECs will be limited even when ECs are connected by tight junctions with resistances much lower than 1,000 ohm-cm² (Madara and Dharmasathaporn, 1985). Moreover, pial vessels, which are not contacted by astrocytes in vivo (Bundgaard, 1982) are able to form high resistance junctions (Crone and Olesen, 1982; Butt et al., 1990). Although several studies have shown some influence of astrocytes on EC tight junction structure assessed by microscopic techniques (Arthur et al., 1987; Tao-Cheng et al., 1987; Tao-Cheng and Brightman, 1988; Shivers et al., 1988), there is again no certainty that those ECs had formed junctions of high electrical resistance. A reasonable conclusion is that while astrocytes clearly have an effect on EC permeability to large molecules in vivo, they may not be the sole modulators of tight junction resistance. Clearly, it is crucial to determine the nature of the changes induced in ECs by astrocytes and to identify other factors that might influence EC permeability properties.

To understand how such properties of brain ECs are regu-

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Abbreviations used in this paper: BBB, blood-brain barrier; CNS, central nervous system; CPT-cAMP, 8-(4-chlorophenylthio)-adenosine-3':5' monophosphate, cyclic; CNTF, ciliary neurotrophic factor; EC, endothelial cell.

lated and also how they might be manipulated, a variety of studies have focused on perfecting *in vitro* models of the BBB. With few exceptions, these models have been based on isolating and culturing brain ECs that appear to rapidly lose at least some of their specialized properties. Occasional exceptions have been based on the use of clonal populations of cells (Rutten et al., 1987; Dehouck et al., 1990). That brain ECs should become less "brain-like" when removed from their normal environment is not total unexpected, given the observations of Stewart and Wiley (1981) discussed above.

Our goal was to develop a cell culture system in which monolayers of ECs with high resistance tight junctions and low rates of fluid-phase endocytosis could be grown reliably on permeable filters. Our approach to this problem was somewhat different than had been used in the past in that we didn't rely solely on electrical resistance or molecular flux measurements to characterize the cells. We reasoned that ECs induced to form high resistance tight junctions in cell culture would be similar in morphology to MDCK cells, an epithelial cell line that forms high resistance monolayers (Gumbiner, 1987). In particular, MDCK cells show a characteristic belt-like distribution of several proteins that are associated with the junctional complex including ZO-1 (Stevenson et al., 1986), filamentous actin (Gumbiner et al., 1988), and E-cadherin (Behrens et al., 1985; Boller et al., 1985; Gumbiner et al., 1988). E-cadherin, a Ca^{2+} -dependent cell adhesion molecule, has been shown to function in an essential way in tight junction formation between epithelial cells (Behrens et al., 1985; Gumbiner et al., 1986, 1988). Vascular ECs also express cell adhesion molecules, including cadherins (Heimark et al., 1990; Liaw et al., 1990) and at least one member of the Ig superfamily (PECAM; Newman et al., 1990). Recently, we have also observed E-cadherin-like immunoreactivity in brain capillaries (F. Bard and L. L. Rubin, unpublished observations). In this paper, we describe a method that utilizes a combination of astrocyte conditioned medium (CM) and elevated cAMP to produce brain ECs that form high resistance tight junctions and adopt other properties characteristic of the BBB.

Materials and Methods

Bovine Brain ECs

Bovine brains were obtained fresh from a slaughterhouse and cleared of meninges. Cortical grey matter was collected in cold L-15 medium, and capillary fragments were prepared by homogenization and filtration through a 155- μ m nylon mesh. Fragments were digested in 0.2% collagenase and 0.04% trypsin for 60 min at 37°C and plated on tissue culture flasks in a medium consisting of 50% astrocyte CM (see below) and 50% MEM containing 10% plasma-derived horse serum (Hyclone Laboratories, Logan, UT). Flasks had previously been coated overnight with rat tail collagen (diluted to ~100 μ g/ml with 1 mM acetic acid), rinsed with PBS, and coated with human fibronectin (50 μ g/ml in PBS; New York Blood Center, New York). Control cells were maintained in the same medium except that astrocyte CM was replaced by MEM with 10% FCS. Both media were supplemented with 10 ng/ml basic FGF (R & D Systems Inc., Minneapolis, MN) and 123 μ g/ml heparin (Sigma Chemical Co., St. Louis, MO). When ECs had reached confluence, they were trypsinized briefly to remove them from the tissue culture flasks and plated at 20,000 cells/filter on 6.5 mm collagen-fibronectin coated Costar Transwell filters (0.4- μ m pore size; Costar Corp., Cambridge, MA) in a medium consisting of 50% astrocyte CM and 50% N2 medium (Bottenstein and Sato, 1979). After an additional 2–3 d, cells were either left untreated or incubated with 250 μ M 8-(4-chlorophenylthio) cAMP (CPT-cAMP; Boehringer Mannheim, Indianapolis, IN) plus 17.5 μ M of the phosphodiesterase inhibitor RO20-1724 (BioMol, Plymouth

Meeting, PA). Typically, cells were used 2–3 d later. At that point, cells were treated for certain experiments with either sodium nitroprusside (Sigma Chemical Co.), staurosporine (Kyowa Hakko USA Inc., Costa Mesa, CA) or H-89 (generously provided by Drs. H. Hidaka, Department of Pharmacology, Nagoya University School of Medicine, Nagoya, Japan and V. John, Athena Neurosciences, Inc., South San Francisco, CA).

For some experiments, cells were treated with different growth factors. A preparation enriched in ciliary neurotrophic factor (CNTF) activity was prepared from frozen rat sciatic nerves (Pel-Freez Biologicals, Rogers, AR) according to the procedures of Manthorpe et al. (1986). PDGF (R & D Systems) and insulin-like growth factor-1 (IGF-1; Amgen Biologicals, Thousand Oaks, CA) were also used.

Isolation of Bovine Brain EC Clones

Cloned cells were derived by isolating and passaging individual colonies of primary ECs plated initially at low density in tissue culture dishes (Dehouck et al., 1990), but in the continual presence of astrocyte CM. These clones could be passaged more than 10 times (at a 1:10 split ratio), while maintaining the ability to form monolayers with resistances exceeding 400 ohm-cm² when grown in the presence of astrocyte CM and CPT-cAMP.

Human Brain ECs

Human brain ECs were obtained from freshly biopsied tissue provided by Dr. N. Barbaro (Department of Neurosurgery, University of California at San Francisco School of Medicine, San Francisco, CA). Approximately 1 g of starting material was used, and isolation procedures were similar to those described for bovine brain ECs.

Peripheral Bovine ECs

Bovine aortic ECs were isolated from aorta obtained fresh from the slaughterhouse as described by Liaw et al. (1990). Bovine adrenal microvasculature endothelial cells were the kind gift of Dr. Martha Furie (Department of Pathology, State University of New York, Stony Brook, NY).

Astrocyte-conditioned Medium

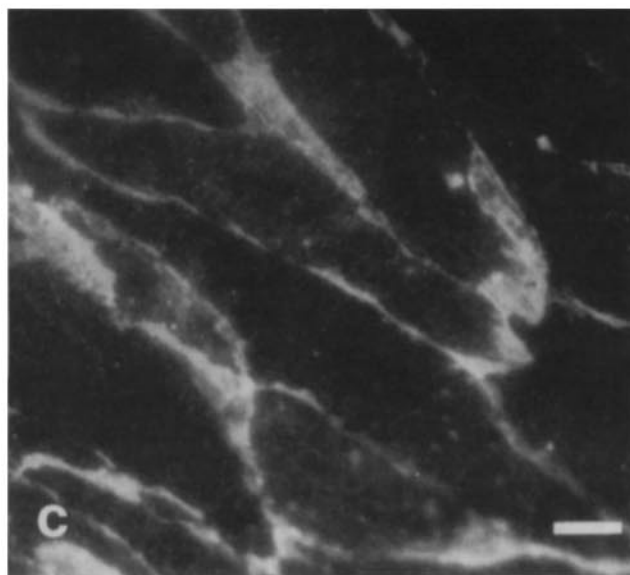
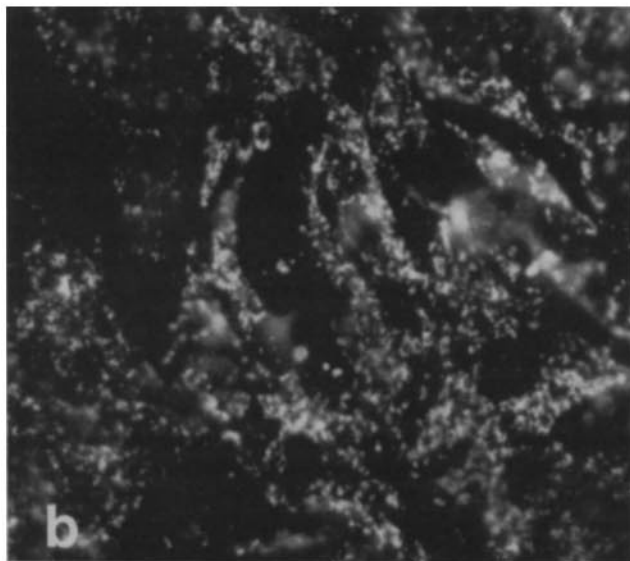
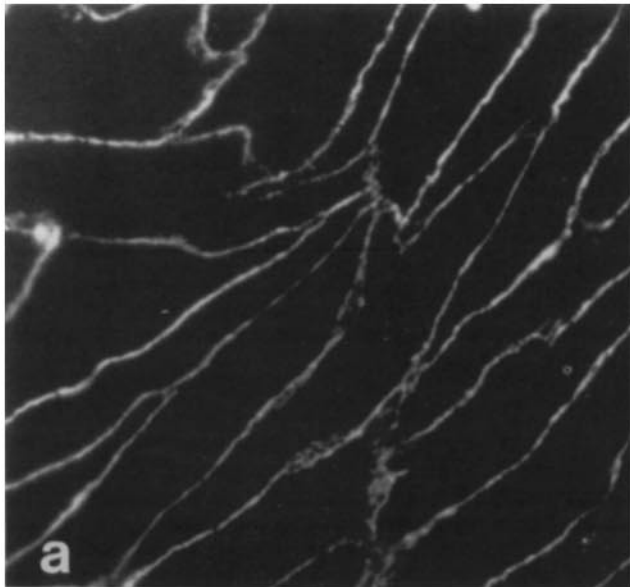
Astrocytes were prepared from 1-d-old rat cortex, essentially as described by Lillien et al. (1988). Briefly, ~7 d after the initial dissociation, flasks containing astrocytes were shaken overnight and the remaining cells (enriched in type 1 astrocytes) were trypsinized and replated on poly-D-lysine-coated flasks. The cells were treated with cytosine arabinoside (10⁻⁵ M) and maintained in serum-free medium as described by Lillien et al. (1988) to limit the growth of more rapidly dividing cells, such as fibroblasts. By immunocytochemical criteria, the cultures were ~95% type 1 astrocytes. For collection of conditioned medium, cultures that were 3–4-wk-old were fed with fresh MEM containing 10% FCS. After 48 h, the medium was removed, sterile-filtered, and either used immediately or stored at -20°C.

Other Cells

Neonatal rat fibroblasts were derived from limb muscle, as previously described (Anthony et al., 1989). U2-51 human astrocytoma cells were provided by Dr. M. Shelanski (Department of Pathology, College of Physicians and Surgeons, New York). C6-glioma cells were obtained from the American Type Culture Collection (Rockville, MD). These cells were maintained in MEM with 10% FCS, and CM was collected as described for astrocytes.

Immunocytochemistry

For most experiments, immunofluorescence was done essentially as described by Gumbiner et al. (1988). Cells were generally fixed for 20 min in 3% paraformaldehyde and then permeabilized in 0.5% Triton X-100 (Sigma Chemical Co.) for 5 min. Alternatively, cells were permeabilized in 0.5% Triton X-100 before fixation (Heimark et al., 1990). Cells were then incubated for 60 min in primary antiserum diluted in 10% FCS in MEM. After rinsing in PBS, cells were then incubated for 30 min in either FITC-conjugated goat antirabbit, mouse (Accurate Chemical and Scientific Corp., Westbury, NY) or rat Ig (Boehringer Mannheim), as appropriate, diluted in MEM containing 10% FCS and 10% goat serum (Gibco BRL Life Technologies Inc., Grand Island, NY). They were coincubated in rhodamine-phalloidin (Molecular Probes Inc., Eugene, OR) to label



filamentous actin. The cells were rinsed in PBS, mounted in CitiFluor (CitiFluor Ltd., London, U.K.), and examined on a Nikon microscope equipped with epifluorescent illumination and either 20 \times , 40 \times , or 60 \times objectives and photographed with Kodak Tri-X film (ASA 400).

For some of these experiments (e.g., Fig. 3), cells were incubated with a rabbit antibody made against a bacterial fusion protein containing amino acids 9–96 of canine E-cadherin fused to bacterial MS-2 polymerase (Seedorf et al., 1987; Liaw, C. W., K. J. Tomaselli, C. Cannon, D. Davis, K. Bryant, and L. Rubin, manuscript submitted for publication; see also 1990. *J. Cell Biol.* 111:408a.). Before use, antibody was affinity purified on a Sepharose 4B column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) containing bound gel-purified fusion protein and shown to label MDCK epithelial cells and brain capillaries in cryostat section. Some nonspecific nuclear fluorescence was observed with the affinity-purified antibody and is probably related to the presence in the fusion protein of sequences derived from the bacterial polymerase.

Primary antibodies used for these experiments include a rat monoclonal anti-ZO-1 (provided by Dr. Bruce Stevenson, Department of Anatomy and Cell Biology, University of Alberta, Edmonton, Canada), a mouse monoclonal antivascular cadherin antibody (provided by Dr. R. Heimark, Icos Corp., Seattle, WA), and a rabbit polyclonal anti-PECAM antibody (provided by Dr. S. Albelda, Wistar Institute, Philadelphia, PA).

Resistance Measurements

Transwells were placed in a chamber containing MEM buffered with 20 mM Hepes. Resistance was measured using an assembly containing current-passing and voltage-measuring electrodes (World Precision Instruments Inc., New Haven, CT). Resistances of blank filters were subtracted from those of filters with cells before final resistances (in ohm-cm²) were calculated.

Measurement of cAMP Levels

cAMP levels were measured by RIA (Dupont-NEN Products, Wilmington, DE) following modification of the protocol of Stelzner et al. (1989). Cultures were rinsed two times with cold PBS and solubilized with 1 M NaOH. Aliquots were taken for protein determination and, after neutralization with 1 M NaOH, for RIA. Occasionally, the cells were rinsed with PBS containing the phosphodiesterase inhibitor isobutyl-methylxanthine (Sigma Chemical Co.) before extraction, but this was found not to influence measured cAMP levels.

Flux Measurements

Approximately 300 nM ¹⁴C-sucrose (New England Nuclear) or 300 nM ³H-propranolol (New England Nuclear) were added to the apical chamber, and the appearance of these compounds in the basolateral chamber was measured at various times thereafter by scintillation counting of small aliquots of the basolateral media. For comparison, flux across cell-free filters was also measured. Transport is expressed as microliters of tracer diffusing from apical to basolateral sides and is calculated from the initial concentration of tracer in the apical and the final concentration of tracer in the basolateral chamber. Care was taken to ensure that fluid levels in the two chambers were equal (200 μ l in the apical and 600 μ l in the basal chambers), and chambers were kept at 37 $^{\circ}$ during the course of the experiment.

Fluid-phase Endocytosis

Brain and peripheral ECs growing on Transwells were incubated for 2 h

Figure 1. Distribution of tight junction-associated proteins in brain ECs. Bovine brain ECs grown on Transwell filters were labeled with antibodies against ZO-1 (a), vascular cadherin (b) or PECAM (c). ZO-1 staining resembled that seen in other cells that form tight junctions, either of high and low resistance types. Vascular cadherin staining was more diffuse, and PECAM staining was junctional, but somewhat broad in regions. The pattern of staining for these cell adhesion molecules was different from that seen with the anti-E-cadherin antibody (Fig. 3). Also, the staining was not altered substantially by the presence of astrocyte CM or elevated cAMP. Bar, 10 μ m.

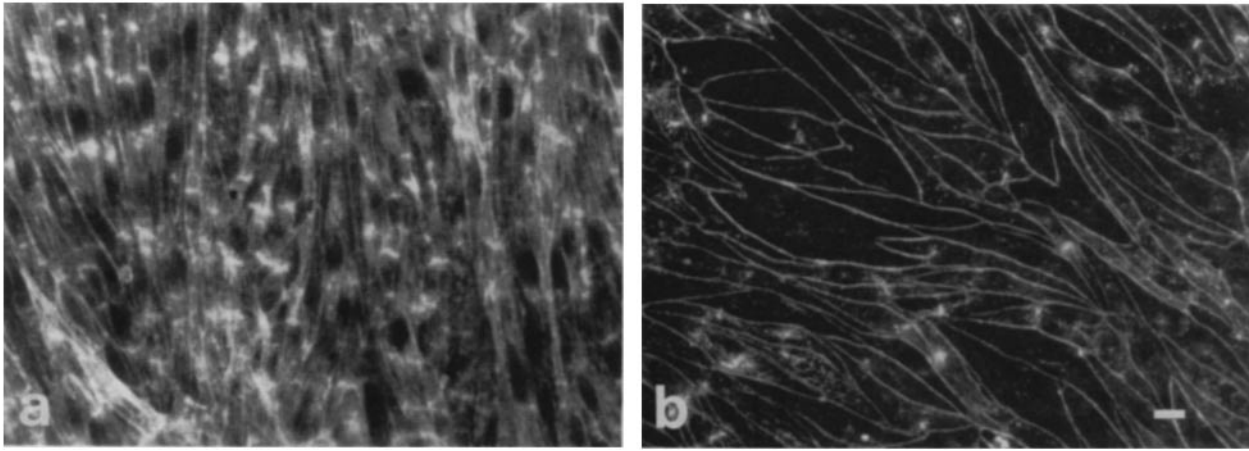


Figure 2. Effect of elevated cyclic AMP on distribution of filamentous actin. Bovine brain ECs grown in the presence of astrocyte CM (a) or astrocyte CM plus CPT-cAMP plus RO20-1724 (b) were fixed, permeabilized, and stained with rhodamine-phalloidin. Filamentous actin was diffusely distributed in cells maintained either in control medium or in astrocyte CM. However, elevation of cAMP in astrocyte CM-treated cells produced a reorganization so that the filamentous actin staining was localized preferentially at cell borders. Bar, 20 μ m.

with 10 mg/ml fluorescein-labeled dextran (Sigma Chemical Co.; approximate molecular weight 70,000; dialyzed extensively against MEM before use) in growth medium, rinsed and fixed in 3% paraformaldehyde. They were then examined under fluorescent illumination. For comparison purposes, photomicrographs of the different types of cells were exposed for the same length of time.

For quantitative studies, fluorescein-dextran (10 mg/ml) was added to growth medium in the apical compartment. After 1-2 h at 37°, the apical medium was aspirated, and the filters were washed three times quickly, and once for 10 min with cold PBS containing 1 mM MgCl₂, 0.1 mM CaCl₂, and 0.3% BSA. Cells were then trypsinized at 4°, microfuged, rinsed, and extracted in PBS containing 1% Triton X-100 and 0.5% SDS. Fluorescence was quantified on a microspectrofluorometer. Results were corrected for fluorescence associated with the cells at 4°; this was typically 10% of that at 37°.

Results

Immunocytochemical Characterization of Brain ECs

The distributions in cultured brain ECs of ZO-1, filamentous

actin, and different cell adhesion molecules were determined. ECs obtained from bovine brain and maintained on filters in cell culture, essentially following previously published procedures (Audus and Borhardt, 1986), retained their EC phenotype, as judged by factor VIII antibody staining, for example. They also stained with an anti-ZO-1 antibody around their borders (Fig. 1 a), as did peripheral ECs derived from bovine aorta and human umbilical vein, both of which form monolayers of low electrical resistance (data not shown). Vascular cadherin and PECAM immunoreactivity were present as well, again just as on peripheral ECs (Fig. 1, b and c). However, filamentous actin, visualized by staining with rhodamine-phalloidin, was distributed in the cells in a diffuse stress fiber-like pattern, as opposed to the belt-like distribution found in MDCK cells (Fig. 2 a). Furthermore, the E-cadherin-like immunoreactivity was weak or absent around cell borders (Fig. 3 a). Importantly, the resistance across monolayers of these cells was quite low, in

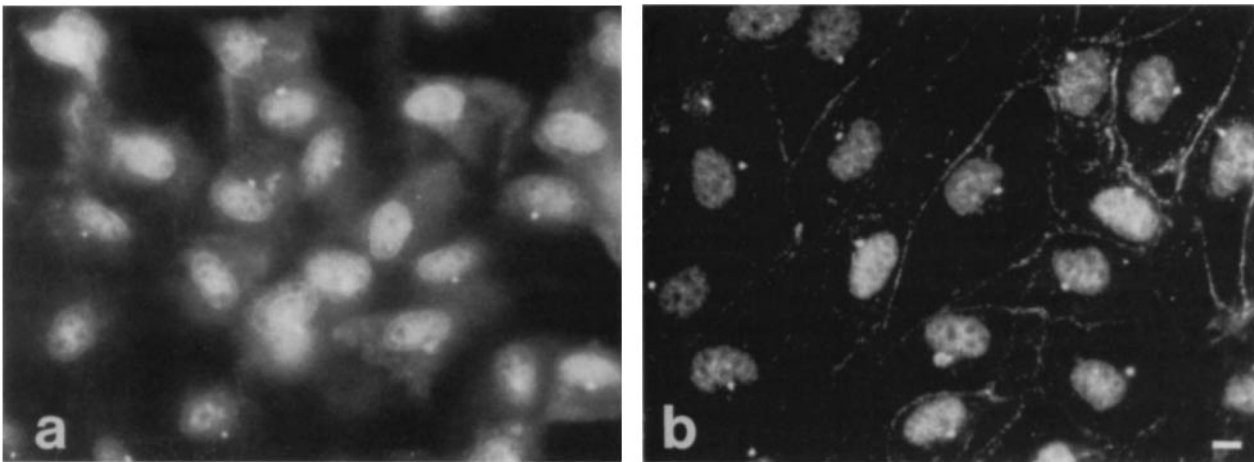


Figure 3. Effect of elevated cAMP on the distribution of E-cadherin immunoreactivity. Cells were grown as in Fig. 2 in either astrocyte CM alone (a) or astrocyte CM plus elevated cAMP (b). They were fixed, permeabilized, and stained with an anti-E-cadherin fusion protein antibody (see Materials and Methods). Increased cAMP produced a marked increase in the amount of staining at cell borders. This increase was noticeable 1 h after addition of CPT-cAMP. Nuclear fluorescence was nonspecific (see Materials and Methods). Bar, 10 μ m.

Table I. Effects of Astrocyte CM and Elevated cAMP on Brain EC Resistance

Condition	Resistance <i>ohm-cm²</i>
Bovine brain	
Control	61 ± 2
Astrocyte CM	115 ± 11
cAMP	305 ± 50
Astrocyte CM+cAMP	625 ± 82
Human brain	
Astrocyte CM	67 ± 12
Astrocyte CM+cAMP	339 ± 107

Bovine brain and human brain ECs were prepared as described in Materials and Methods. For cAMP experiments, cells were treated for 48–72 h with a combination of CPT-cAMP plus RO20-1724. Results in this table are average resistances ± standard deviations. Resistances were measured for three to six filters for each condition and are calculated from four individual experiments for bovine brain ECs and two for human brain ECs.

the range of 10–50 ohm-cm² (Table I), and the leakage of small and large molecules across these monolayers was comparatively high (see Fig. 7, bottom). Thus, these cells failed to constitute a legitimate model of the BBB.

Effects of Astrocyte-conditioned Medium on Brain ECs

It seemed quite possible that astrocytes would be capable of influencing the differentiation of brain ECs in culture, as they do in vivo. Astrocytes do not normally make direct contact with ECs in vivo, but, rather, are separated from them by an extracellular matrix. Therefore, the influence of astrocytes may be mediated by a secreted factor.

To test for the presence of such a factor, we compared ECs cultured in the presence or absence of conditioned medium (CM) from type 1 astrocytes prepared from neonatal rat brain as described by Janzer and Raff (1987) for use in their in vivo transplant studies. We found that treating ECs with astrocyte CM produced somewhat sharper ZO-1 staining (not shown) and approximately a twofold increase in transmonolayer electrical resistance (Table I). However, even in CM-treated cultures, we did not obtain cell monolayers that had electrical resistances higher than ~200 ohm-cm² (with the average being substantially less). Nor did we find that this treatment produced the belt-like pattern of filamentous actin staining and cadherin-like immunoreactivity that we expected (Figs. 2 and 3).

Effect of Increased cAMP Levels on the Distribution of Junctional Components

Since astrocyte CM by itself was not sufficient to fully induce brain EC differentiation, we examined other factors that might induce these cells. Numerous descriptions of nerve endings on cerebral vasculature have been provided (Harik et al., 1981; Goldstein and Betz, 1986), and neurotransmitter–target cell interactions might involve changes in second messengers. It has also been observed that second messengers cause some changes in epithelial and peripheral EC permeability (Duffey et al., 1981; Smirnov et al., 1989; Stelzner et al., 1989; Balda et al., 1990; Yamada et al., 1990), so we investigated the effects of second messenger manipulation on brain EC morphology and resistance.

We found that increasing cAMP levels in ECs already treated with astrocyte CM caused a striking change in cell morphology that was particularly impressive when the cells were stained with rhodamine-phalloidin. Instead of the cytoplasmic pattern of staining described above, filamentous actin in cells treated with cAMP-elevating agents was highly enriched at cell borders (Fig. 2 b). In addition, treated cells showed enhanced staining with the anti-E-cadherin fusion protein antibody around their borders (Fig. 3 b). This enhanced staining was seen by 1 h after cAMP elevation. The distribution of ZO-1 also became more regular, but the effects were subtle (data not shown). PECAM and vascular cadherin staining did not change appreciably.

Effect of Elevated cAMP on Resistance of Brain ECs

We found that agents that increased cAMP levels produced a rapid increase in electrical resistance, again in cells already treated with astrocyte CM (Table I; Fig. 4). With time, the resistance increased, on average, to over 600 ohm-cm², with maximum resistances of ~1,500 ohm-cm² having been achieved.

For most experiments involving increases in cAMP, cells were treated with a combination of a cAMP analogue (CPT-cAMP) and a phosphodiesterase inhibitor (RO20-1724). However, other agents that elevate cAMP levels, including agonists whose receptors are coupled to G_s (such as isoproterenol and calcitonin gene-related peptide) cholera toxin, and forskolin were also effective. The most effective phosphodiesterase inhibitors in our studies were those that act on the class of cAMP-specific, cGMP noninhibitable phosphodiesterases (Beavo, 1988).

We also used an RIA to measure changes in cAMP levels

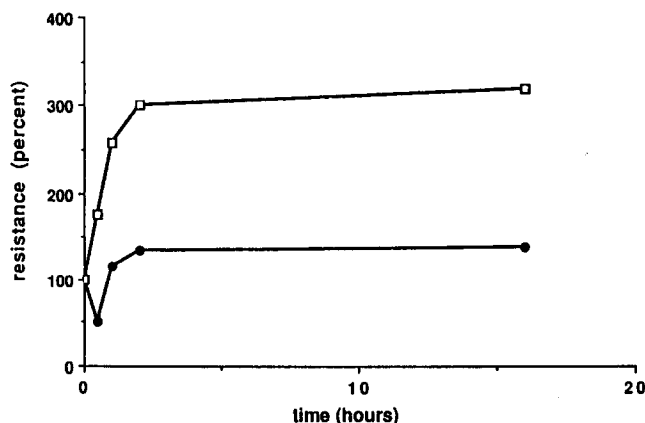


Figure 4. Effect of changes in cAMP on brain EC resistance. Bovine brain ECs were grown for 2 d on Transwells in medium containing astrocyte CM. At the beginning of the experiment, resistances were measured across cell monolayers. Resistance across individual filters was then measured at the specified times and expressed as percent of the initial value. In this, and all other figures, each point represents the average resistance of two to four individual monolayers. In this experiment, cells were either maintained in astrocyte CM alone (●) or treated with 17.5 μM RO20-1724 in astrocyte CM (□). The early transient fall in resistance is related to handling during resistance measurements. Resistance increases rapidly when cAMP levels increase, either in response to the RO20-1724 or after addition of CPT-cAMP or any of a variety of other agents that elevate cAMP.

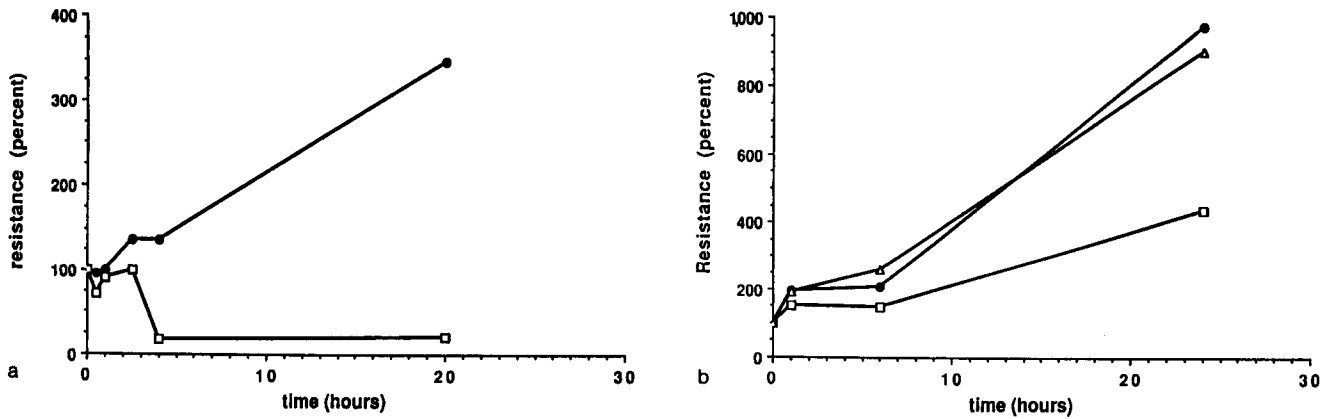


Figure 5. Effect of astrocyte CM on the response to cAMP elevation. (a) Primary cultures of bovine brain ECs were grown in either 50% astrocyte CM (●) or 50% MEM with 10% FCS (□) from time of initial plating. Resistances across sets of filters were measured (average starting resistance was ~ 100 ohm-cm² for CM-treated cells and 25 ohm-cm² for untreated cells), and the cells were then treated to elevate their cAMP levels. In this particular example, primary cells grown in astrocyte CM responded rapidly to increased cAMP, whereas those not pretreated with CM did not respond over a 24-h period. Note that in the experiments presented in Table I, measurements were made 48–72 h after elevation of cAMP. Results were similar if cells were treated with either CPT-cAMP or CPT-cAMP plus RO20-1724. (b) Cloned bovine brain ECs were maintained for 10 passages in astrocyte CM and passaged into tissue culture flasks in the presence (●) or absence (□, Δ) of CM. After 7 d, the cells were passaged onto Transwells, and some of the cells were re-fed with CM (Δ). After two additional days, all cells were treated with CPT-cAMP plus RO20-1724. Before treatment, cells grown in the presence of astrocyte CM had resistances of ~ 35 ohm-cm²; those grown without CM were ~ 25 ohm-cm². As seen in this example, cloned brain ECs responded less well to cAMP increases if kept without CM. However, switching them back to CM restored their response. All resistances are given as percentages of initial values.

in response to the various treatments. The level of cAMP in untreated cultures varied from 60 to 100 pmol/mg total protein, similar to that reported by Stelzner et al. (1989) for peripheral ECs. Both RO20-1724 and forskolin treatment produced a two to threefold increase in cAMP levels within 1 h of addition.

Importance of Astrocyte CM in cAMP Effects

Although astrocyte CM itself did not have a large effect on resistance or on cell morphology, it was an important determinant of the response to elevation of cAMP. For instance, in $\sim 25\%$ of the experiments, the resistance of cells grown without astrocyte CM did not increase significantly within 24–48 h of cAMP elevation (Fig. 5 a; such experiments are included when averages are presented). In fact, even when cells that were maintained in the absence of astrocyte CM did show increased resistance, they generally took much longer to respond than did the astrocyte CM-treated cells (16 h, rather than 2 h or less). This was also reflected in cell morphology since the type of rhodamine phalloidin staining illustrated in Fig. 2 b was seen only when cells were subjected to the combination of CM and elevated cAMP.

Other types of cell-derived CM were not as effective as that from type 1 astrocytes (Table II). Cells grown in rat fibroblast CM, for example, remained in a much lower resistance state even when treated with agents that elevate cAMP. Also, compared to cells maintained in astrocyte CM, cells switched from astrocyte CM to other types of CM, including those derived from either U2-51 astrocytoma or C6-glioma cells, showed a smaller increase in resistance upon elevation of cAMP (Table II).

Influence of Astrocyte CM on Cloned Bovine Brain ECs

Primary EC cultures were not entirely free of contaminating

cell types, and it was possible that either astrocyte CM or cAMP elevation affected other cells, such as pericytes, that in turn influenced the ECs. To address this issue, we isolated several individual clones of bovine brain ECs from colonies of cells maintained from their time of isolation in astrocyte CM. These cells also were capable of being induced to form high resistance tight junctions when cAMP levels were elevated. When cloned cells were removed from astrocyte CM for several days, they did not respond to elevated cAMP as well (Fig. 5 b). When astrocyte CM was added back to those cells for 24–48 h, they again showed an enhanced ability to respond to cAMP elevation (Fig. 5 b).

Table II. Effects of Various Conditioned Media on Brain EC Resistance

	Treatment		Relative resistance	
	Initial CM	Final CM	pre-cAMP	post-cAMP
			%	%
Expt. 1	ACM	ACM	100	945
	FCM	FCM	98	219
Expt. 2	ACM	ACM	100	921
	ACM	FCM	78	113
	ACM	U2-51 CM	89	593
Expt. 3	ACM	ACM	100	1,181
	ACM	C6 CM	85	427

Bovine brain ECs were isolated and maintained in flasks in either astrocyte CM (ACM) or neonatal rat fibroblast CM (FCM). This is referred to as the initial CM. They were then passaged onto Transwells and maintained in ACM, FCM, U2-51 astrocytoma CM, or C6 glioma CM (C6 CM). This is referred to as the final CM. Each type of CM was collected from confluent cultures maintained in MEM with 10% FCS and was diluted 1:1 with fresh medium before use. After two days, resistances were measured (pre-cAMP), and the cells were treated with CPT-cAMP plus RO20-1724. Resistances were measured again after an additional two days (post-cAMP). Resistances are given as percentages, with the resistances of pre-cAMP cells maintained in ACM defined as 100%; resistances of these cells ranged from 35 to 45 ohm-cm².

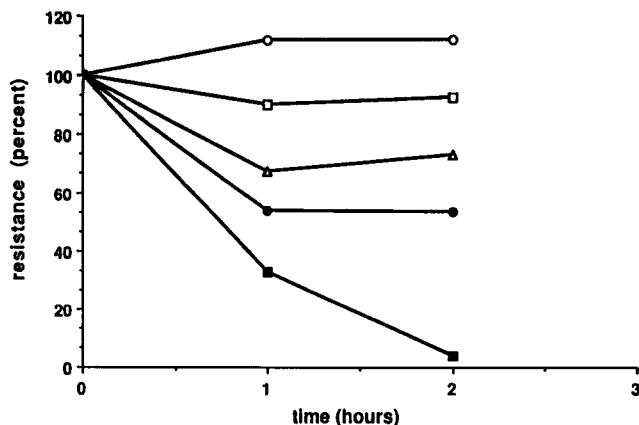


Figure 6. Effect of elevated cGMP on resistance of brain ECs. Cells were treated for 48 h with CPT-cAMP plus RO20-1724, but then received various concentrations of sodium nitroprusside to increase cGMP levels (O, control; □, 0.1 μ M; Δ , 1 μ M; ●, 10 μ M; ■, 100 μ M). Resistances decreased rapidly when cGMP levels were increased even in cells that had elevated cAMP. Again, resistances are expressed as percent of initial values.

Preliminary Characterization of Astrocyte CM Factor

To begin to study the active factor(s) in astrocyte CM, we carried out a preliminary characterization. Astrocyte CM was collected in either defined medium or in MEM without additional additives over a 24–48-h period. Both types of CM were effective compared to the appropriate control media. The active factor was also heat-sensitive. The activity of astrocyte CM could not be accounted for fully by PDGF (Pettmann et al., 1985; Richardson et al., 1988), CNTF (Lilien et al., 1988), IGF-1, or FGF (data not shown).

Response of Human Brain ECs to Astrocyte CM and Elevated cAMP

To test the generality of these observations, we isolated ECs from a limited number of human brain biopsies and treated them with a combination of astrocyte CM and agents that elevate cAMP (Table I). Again, we were able to obtain cells with relatively high electrical resistance and a similar belt-like distribution of filamentous actin. Interestingly, on average, the resistance was somewhat lower than we were able to obtain with bovine brain ECs, and, correspondingly, the filamentous actin staining was not restricted exclusively to cell borders.

Effects of Elevated cGMP on Brain EC Resistance

cAMP was not the only second messenger capable of altering resistance of brain ECs. Cells whose resistance had been increased by treatment with CPT-cAMP were treated in addition with sodium nitroprusside to elevate cGMP levels (Waldman and Murad, 1987). Fig. 6 shows the dose-dependent decrease in resistance produced by this treatment. Thus, increased cGMP appears to oppose the effects of increased cAMP. Atrial natriuretic factor, another agent that elevates cGMP levels in ECs (Waldman and Murad, 1987), had similar effects (data not shown).

Transport of Compounds across High Resistance Monolayers of Brain ECs

The high electrical resistance of induced bovine brain EC

cultures suggests that they should be rather impermeable to hydrophilic compounds. This was tested with fluorescent dextrans of different molecular weights and with radioactively labeled compounds that are not transported by specific membrane transporters. We found that the high resistance cells offered a significant permeability barrier to even small hydrophilic compounds such as sucrose (Fig. 7, top) whereas lower resistance monolayers were more permeable (Fig. 7, bottom). However, as expected, there was marked flux of hydrophobic compounds across even high resistance monolayers (Fig. 7, top). Hydrophobic compounds are capable of crossing the BBB in vivo as well.

Fluid-Phase Endocytosis in Brain and Peripheral ECs

Another hallmark of ECs in the brain is their low rate of fluid-phase endocytosis (Reese and Karnovsky, 1967). This is a nonsaturable, temperature-dependent process that is assayed by incubating cells with labeled compounds for which there are no specific transport mechanisms (Steinman et al.,

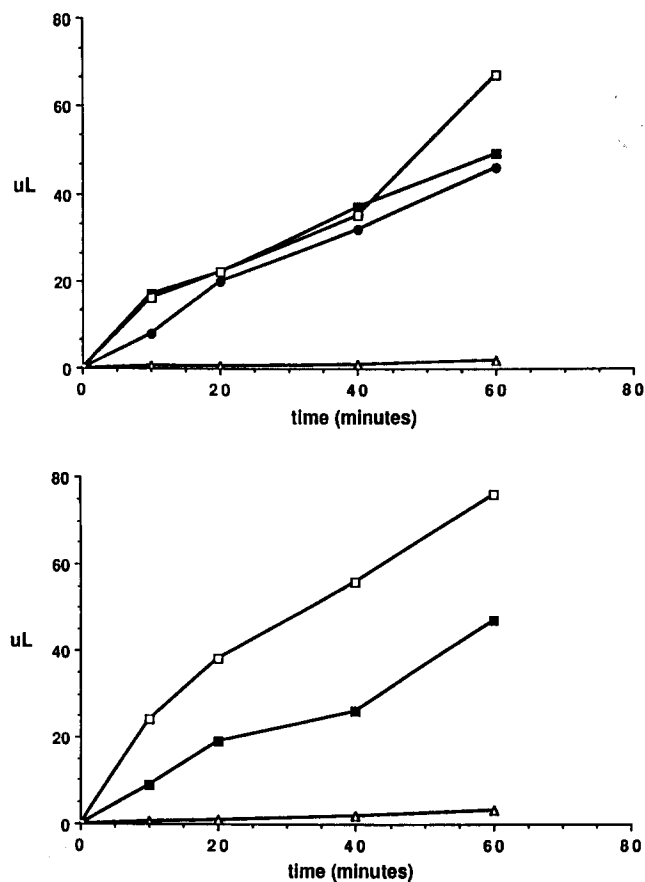


Figure 7. (Top) Flux of compounds across high and low resistance monolayers of brain ECs. Flux was measured using radioactively labeled compounds added to one side of filters with or without cells. Leakage of small, hydrophilic compounds, such as sucrose across high resistance monolayers (Δ) was limited compared to that occurring across filters without cells (\square). However, hydrophobic compounds, such as propranolol, moved rapidly across high resistance monolayers (\bullet) and across cell-free filters (\blacksquare). (Bottom) Sucrose flux across low resistance monolayers (\blacksquare) is less than that occurring across cell-free filters (\square), but much greater than that across high resistance monolayers (Δ).

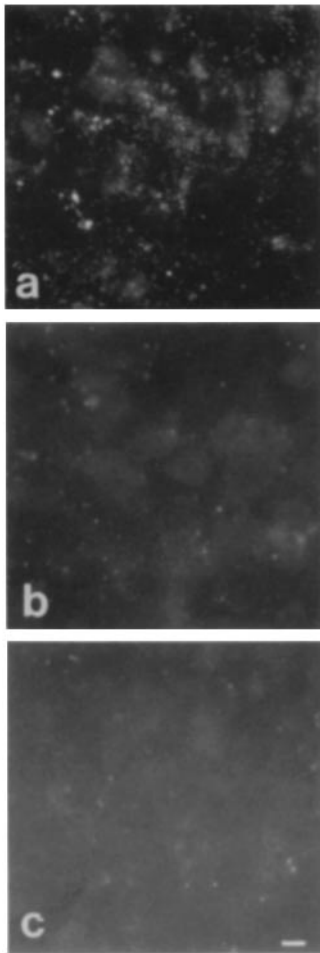


Figure 8. Fluid-phase endocytosis in bovine brain and aortic ECs. Aortic cells showed relatively pronounced uptake of fluorescein-labeled dextran at 37°C (a), but not at 0°C (b). Brain ECs, on the other hand, pinocytosed significantly smaller amounts of the tracer at 37°C (c). Quantitative studies, using either fluorescein-dextran or HRP, indicated that the rate of uptake of aortic or bovine microvasculature ECs was approximately 1.0 nl/filter/h at 37°C while that of brain ECs (either primary or cloned cells) was 0.25 nl/filter/h. Bar, 10 μm.

1983). For measurements of endocytosis, brain ECs (treated with astrocyte CM and CPT-cAMP) and bovine aortic and peripheral microvasculature ECs were incubated with fluorescent dextran. Uptake into the peripheral ECs was readily observed at 37° (Fig. 8 a), but barely detectable at 0° (Fig. 8 b). Uptake into brain ECs was low at 37° (Fig. 8 c).

The rate of fluid-phase endocytosis by brain ECs remained low under all growth conditions. Removal of astrocyte CM did not affect the rate of pinocytosis, nor did elevation of cAMP. Similarly, the rate was not altered specifically in aortic ECs by either elevation of cAMP or by the addition of astrocyte CM (data not shown).

Effect of Decreasing cAMP Levels on EC Resistance

Regardless of the agent used to elevate levels of cAMP in brain ECs, upon its removal, transmonolayer resistances dropped relatively rapidly (within 15–30 min; Fig. 9). The filamentous actin distribution also changed, but more slowly (see Fig. 11).

The most straightforward explanation for the ability of cAMP to increase resistance is that protein kinase A mediates the phosphorylation of one or more proteins important in regulating the resistance of tight junctions. The relatively rapid decrease in resistance following removal of cAMP-elevating agents suggests the presence of an active phosphoprotein phosphatase. This further suggests that while cAMP levels remain elevated, kinase A must act to keep

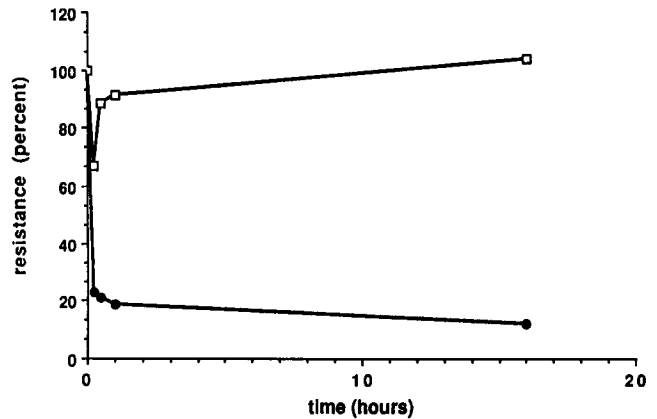


Figure 9. Reversibility of cAMP effects on brain EC resistance. Cells were maintained in astrocyte CM and were treated with CPT-cAMP plus RO20-1724 for 48 h before the start of experiment. At the start of the experiment, resistances were measured, and filters were rinsed and replaced in either the same medium (□) or in astrocyte CM alone (●). The resistance is seen to decrease rapidly after removal of cAMP elevating reagents.

regulatory proteins in a phosphorylated state in spite of the presence of active phosphatases. Thus, inhibitors of protein kinase A should act to decrease resistance even in the presence of elevated cAMP.

We tested the effects of a variety of protein kinase inhibitors on resistance of brain ECs continuously treated with CPT-cAMP. We did find that two potent, but somewhat non-specific, protein kinase inhibitors, staurosporine and K-252a (Koizumi et al., 1988; Hashimoto and Hagino, 1989), were quite effective in decreasing resistance across EC monolayers (Fig. 10). We also found that H-89 (Chijiwa et al., 1990), a newly described kinase inhibitor, that is less potent,

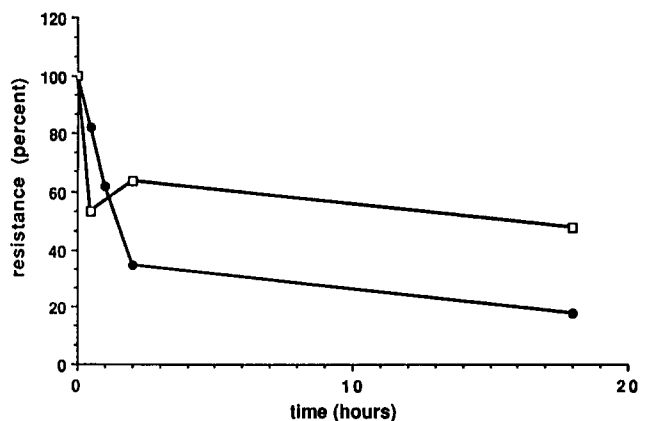


Figure 10. Effects of protein kinase inhibitors on resistance of brain ECs. Cells were grown for 48 h in astrocyte CM containing CPT-cAMP plus RO20-1724. Resistances were measured and cells were incubated with either 100 nM staurosporine (●) or 25 μM H-89 (□) in the continued presence of CPT-cAMP plus RO20-1724. Resistances were measured at various intervals and expressed as percentages of their initial values. Kinase inhibitors were dissolved in DMSO, which, at the concentrations used (<0.25%), had no effects on resistance. Both kinase inhibitors caused the resistance to decrease even in the continued presence of elevated cAMP, but staurosporine was more effective.

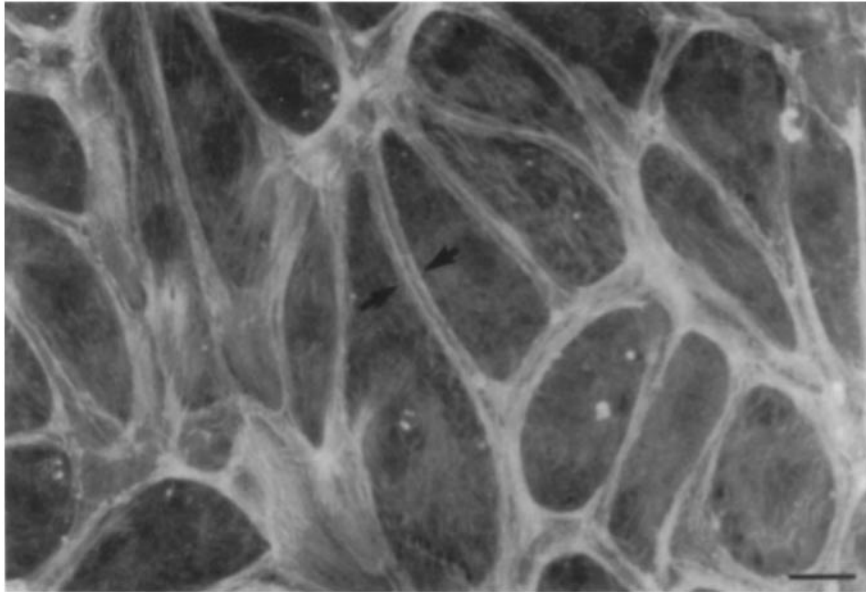


Figure 11. Effect of staurosporine on distribution of filamentous actin. Brain ECs were grown as in the legend to Fig. 10 and treated with 100 nM staurosporine. Cultures were fixed after 4 h and labeled with rhodamine-phalloidin. Concomitant with the decrease in electrical resistance, staurosporine caused a significant change in the distribution of filamentous actin, similar to that seen when CPT-cAMP was removed from treated cultures. Arrows point to gaps between bands of filamentous actin in adjacent cells. Bar, 20 μ m.

but more selective for protein kinase A, also decreased resistance in the presence of elevated cyclic AMP (Fig. 10).

Staurosporine also produced morphological changes in the cells, visualized by staining with rhodamine-phalloidin, that took place several hours after the decrease in resistance (Fig. 11). These changes, which were similar to those seen after removing CPT-cAMP, did not represent a simple reversion to a state of diffuse staining. Rather, the cortical-type staining still was predominant, but became broader in the regions of cell-cell contact, and there were visible gaps of actin stain between adjacent cells.

Discussion

Establishment of a Cell Culture BBB Model System

Understanding how the permeability of the BBB is regulated is important for several reasons. First, initial therapeutic treatments for a variety of neurodegenerative disorders will likely be based upon the use of compounds, neurotrophic factors, for example, that do not enter freely into the CNS, and it will be important to have methods for enhancing their CNS penetration. Second, there are a set of neurological disorders, such as vasogenic brain edema and multiple sclerosis, that involve a compromised BBB. In fact, because of the possible presence of peripheral circulating amyloid, it has even been speculated that Alzheimer's disease involves an alteration in BBB permeability (Joachim et al., 1989). Finally, there are instances of undesired cell trafficking into the brain, such as that occurring with HIV-infected monocytes or metastatic cells. These considerations make an understanding of the factors that control BBB permeability and means for manipulating them *in vivo* of great importance.

In this paper, we describe methods for producing monolayer cultures of brain ECs whose properties are similar to those expressed by these cells *in vivo*. These properties include high resistance tight junctions, low rates of fluid-phase endocytosis, and restricted paracellular flux. The major modification that we made of previous procedures was to in-

clude astrocyte CM from the time of cell isolation and subsequently treat the cells with agents that elevate EC cAMP levels. Most of our results were obtained with ECs derived from bovine brain. However, in a limited number of experiments similar treatments seemed to be effective with ECs derived from adult human brain. These experiments thus potentially provide an increased understanding of factors that regulate the BBB.

Until recently, there had been some difficulty in establishing EC monolayers with properties appropriate to the BBB, particularly high resistance tight junctions and minimal leakage of small hydrophilic molecules. Generally, in these cases, primary cultures of brain ECs were isolated and maintained using standard cell culture conditions without further inductive factors (Audus and Borchardt, 1986; van Bree et al., 1988). In addition, based on the observation that astrocytes are important in EC differentiation, attempts had been made to influence the differentiation of brain ECs with astrocytes or astrocyte-derived factors. Dehouck et al. (1990) found that coculturing astrocytes with ECs increases their resistance even more although no effect was seen with astrocyte CM. Others found that astrocytes or astrocyte-derived factors produced an increase in the complexity or frequency of tight junctions, as seen with freeze-fracture microscopy (Arthur et al., 1987; Tao-Cheng et al., 1987; Shivers et al., 1988; Tao-Cheng and Brightman, 1988). We found that CM derived from neonatal type 1 astrocytes alone caused only a small increase in resistance and, at best, subtle changes in cell morphology. As pointed out above, it has never been demonstrated that astrocytes exert a major influence on tight junction resistance *in vivo*, and we feel that this question remains to be resolved.

To overcome some of the inconsistencies associated with primary cultures, Rutten et al. (1987) and Dehouck et al. (1990) isolated clones of brain ECs that formed high resistance monolayers in the absence of added factors. We also isolated clones of brain ECs; however, they still required astrocyte CM and elevated cAMP to form high resistance tight junctions. At this time, we cannot account for the differences

between our results and those obtained by these other groups.

In our studies, we used some assays that had been employed routinely by other investigators studying brain ECs, namely, resistance measurements and molecular flux. However, we also carried out a fairly extensive immunocytochemical analysis of these cells that allowed us to achieve a monolayer characterized by the uniform localization around cell borders of several proteins known to be associated with epithelial cells that form high resistance tight junctions. Of the proteins that we examined, the distributions of filamentous actin and a protein immunologically related to E-cadherin were substantially different in treated than in untreated cells. ZO-1 staining was present between peripheral ECs and did not change as dramatically following cAMP elevation. Thus, consistent with other observations (Schnabel et al., 1990), it appears to be a less sensitive indicator of the degree of tight junction formation. The distributions of PECAM and vascular cadherin also were not altered by our treatments, and, hence, do not seem to be strongly associated with the formation of tight junctions of high resistance. On the other hand, the distribution of E-cadherin immunoreactivity altered more drastically during the induction of high resistance tight junctions, suggesting that it might somehow be involved in the formation of these junctions. We have not yet completely characterized the E-cadherin-like protein.

Regulation of Tight Junction Resistance in Brain ECs

One of the major observations in this paper is that tight junction resistance in brain ECs is regulated by second messengers, including cAMP and cGMP. Similar results were achieved with bovine and human brain ECs. Further, we and others (Duffey et al., 1981; Balda et al., 1990; Rubin, L., unpublished observations) have accumulated evidence that tight junction resistance in epithelial cells is also subject to second messenger modulation. A likely explanation, for at least the cAMP effect, is that the state of phosphorylation of one or more proteins associated with the tight junction is important in determining the permeability of the tight junction. In this way, the tight junction can be viewed like an ion channel or like the gap junction channel, with its permeability being modified by the physiological state of the cell. This is consistent with suggestions made previously (Madara, 1988).

The effects of elevated cAMP on brain ECs were particularly pronounced only when they were pretreated with astrocyte CM. This was seen both in primary cultures of brain ECs and also in clonal brain EC cultures. This makes it likely that the effects of astrocyte CM (and of elevated cAMP) were directly on ECs. There are other instances in which growth or serum factors have been shown to potentiate the effects of second messengers (Barres et al., 1989). The identity and the manner in which the astrocyte factor primes ECs to respond to cAMP elevation are currently under investigation. Our model system should facilitate greatly the identification of this factor.

CM derived from other cell types was not nearly as effective as that obtained from astrocytes. In particular, that derived from an astrocytoma cell line and a commonly used glioma cell line were not as effective even if the cells were grown initially in astrocyte CM. They were much less effective still if ECs were maintained from time of isolation in

these other types of CM. These results are potentially interesting in that CNS tumors are associated with a leaky BBB (Greig, 1989). It may be that CNS tumors either secrete a factor that increases the permeability of brain ECs (like the vascular permeability factor produced by peripheral tumor cells; Connolly et al., 1989) or that these cells simply fail to produce the normal astrocyte factor.

Regulation of BBB Permeability In Vivo

The real question that arises from the cAMP experiments is the degree to which they are relevant to understanding the BBB in vivo. Recently, we have attempted to address this in a preliminary fashion by giving rats intracarotid infusions of staurosporine, which, as shown in this paper, increases tight junction permeability substantially in vitro. This inhibitor increased three to fivefold the ability of peripherally administered sucrose and BSA (both radioactively labeled) to enter the brain, supporting the possibility that cAMP may be an important regulator in vivo. However, more work needs to be done to validate this theory.

This hypothesis also suggests that there might be physiological factors that regulate cAMP levels in brain ECs in vivo. Such factors might include ones derived from astrocytes or a variety of neurotransmitters and peptides released from nerve terminals on cerebral capillaries (Harik et al., 1981; Goldstein and Betz, 1986).

Conclusions

In summary, we have derived an in vitro model of the BBB in which brain ECs are induced to maintain a differentiated state with a combination of astrocyte CM and treatment with agents that elevate cAMP. Our results also suggest that agents that elevate cAMP levels increase tight junction resistance and those that decrease cAMP levels or block its physiological activity decrease tight junction resistance. Thus, we would suggest that a delivery system designed to enhance the penetration of compounds into the brain might be based on agents that selectively decrease cAMP activity in brain ECs.

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