CHANGES OF PERMEABILITY IN RESPONSE TO HISTAMINE AND TUMOR NECROSIS FACTOR-α AND THEIR POSSIBLE MECHANISM OF ACTION ON AN *IN VITRO* MODEL OF THE BLOOD-BRAIN BARRIER

MÁRIA ANNA DELI, M.D.

Ph.D. thesis

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1. PUBLICATIONS

I. Deli MA, Joó F, Krizbai I, Lengyel I, Nunzi GM, Wolff JR: Calcium/calmodulin stimulated protein kinase II is present in primary cultures of cerebral endothelial cells. *J. Neurochem.*, **60:** 1960-1963, 1993

II. Krizbai I, **Deli MA**, Lengyel I, Maderspach K, Pákáski M, Joó F, Wolff JR: In situ hybridization with digoxigenin labeled oligonucleotide probes: detection of CAMK-II gene expression in primary cultures of cerebral endothelial cells. *Neurobiology*, **1:** 235-240, 1993

III. Deli MA, Dung NTK, Joó F: Isolation and culture of endothelial cells from cortical microvessels of the rat and the piglet. *Drug transport across the blood-brain barrier (BBB): new experimental strategy. An overview of Endothelial Cell Culture and Microdialysis Techniques as Tools to Study BBB transport*, de Boer ABG and Sutanto W (eds), BIOMED-Concerted Action, 1994, Leiden, pp. 175-188

IV. Deli MA, Dehouck M-P, Ábrahám CS, Cecchelli R, Joó F: Penetration of small molecular weight substances through cultured bovine brain capillary endothelial cell monolayers: the early effects of 3',5'-cyclic adenosine monophosphate. *Exp. Physiol.*, **80:** 675-678, 1995

V. Huszti Z, **Deli MA**, Joó F: Carrier-mediated uptake and release of histamine by cultured rat cerebral endothelial cells. *Neurosci. Lett.*, **184:** 185-188, 1995

VI. Deli MA, Dehouck M-P, Cecchelli R, Ábrahám CS, Joó F: Histamine induces a selective albumin permeation through the blood-brain barrier *in vitro*. *Inflamm. Res.*, **44:** S56-S57, 1995

VII. Krizbai I, Szabó G, **Deli MA**, Maderspach K, Lehel Cs, Oláh Z, Wolff JR, Joó F: Expression of protein kinase C family members in the cerebral endothelial cells. *J. Neurochem.*, **65:** 459-462, 1995

VIII. Deli MA, Dehouck M-P, Descamps L, Cecchelli R, Joó F, Ábrahám CS, Torpier G: Exposure of tumor necrosis factor α to the luminal membrane of bovine brain capillary endothelial cells cocultured with astrocytes induces a delayed increase of permeability and cytoplasmic stress fiber formation of actin. *J. Neurosci. Res.*, 1995, **41:** 717-726.

2. RATIONALE

The most important function of the blood-brain barrier (BBB) is the maintenance of homeostasis of the central nervous system environment by separating the brain from the systemic blood circulation. Endothelial cells, connected by tight junctions, from brain microvessels form the structural and functional basis of this barrier, which limits the rate and amount of both paracellular and transcellular flux.

Under physiological conditions, the BBB not only regulates the entry of endogenous compounds, nutrients and drugs into the central nervous system (CNS), but also diminishes cellular infiltration compared to peripheral organs. The barrier function of the BBB can change dramatically during various diseases e.g. hypertension, hyperosmolarity, tumors of the CNS, cerebral ischemia, seizures, cerebral inflammation, and neurodegenerative diseases.

A new generation of the *in vitro* model systems to study the BBB have been developed during the last 15 years that seemed to be devoid of most of the problems experienced with the isolated cerebral microvessels. The tissue culture approach offered an easier, reproducible and mass-production method, where endothelial cells maintain both endothelial and BBB markers in long-term subcultures (Méresse *et al.*, 1989; Dehouck *et al.*, 1990).

Description of the presence of histamine H_2-receptors linked to adenylate cyclase in the cerebral endothelium (Joó *et al.*, 1975; Karnushina *et al.*, 1980) was the first notion in the literature, which suggested thet these cells may have receptors for vasoactive substances similar to those of peripheral vessels. In addition to mediating vasoregulatory responses, histamine H_2-receptors were shown to be involved in the induction of macromolecular transport leading to the formation of brain ooedema (Dux and Joó, 1982; Dux *et al.*, 1987).

The cytokine tumor necrosis factor-α (TNF-α) is a pleiotropic polypeptide that plays a significant role in brain immune and inflammatory activities. TNF-α is produced in the brain in response injuries induced by infectious, immune, toxic, traumatic and ischemic stimuli (Feuerstein *et al.*, 1994). TNF-α promotes inflammation by stimulation of capillary endothelial cell proinflammatory responses.

3. AIMS

The main aim of my studies was to gain a better insight into the changes in permeability of cerebral endothelial cells in response to histamine and TNF-α. I had to first (i) establish the *in vitro* reconstitution of the BBB under **tissue culture** conditions in our laboratory. (ii) **Histamine** has been shown in previous *in vivo* studies (Dux and Joó, 1982; Edvinsson *et al.*, 1993) to increase the permeation of circulating albumin through the BBB, supposedly by the induction of transcytosis in the cerebral endothelial cells rather than modifying the permeability properties of the tight junctions. The possible changes in permeability of cultured cerebral endothelial cells on the effect of histamine with three tracers of different molecular weight have been investigated. (iii) Experimental data, obtained from *in vivo* studies, indicated that **TNF-α** can increase the permeability of BBB, but it remained to be seen in *in vitro* studies if TNF-α exerts any effect on permeability when it reaches the endothelial cells from the luminal side. Therefore, a detailed study to clarify this point, which may be important in relation to the reactions of cerebral endothelial cells during immunological and inflammatory processes was done. (iv) There were controversial data reported in the literature on the role of **3',5'-cyclic adenosine monophosphate** (cAMP) in the regulation of permeability. So, we re-investigated the issue with direct measurements on the *in vitro* reconstituted BBB model. (v) It was not known in the literature if the **calcium/calmoduline-stimulated protein kinase II** (CaM PK-II), an enzyme being able to respond to elevations of intracellular calcium levels, was present in the cerebral endothelial cells. We decided to check on this apparently important aspect with immunohistochemical detection and with *in situ* hybridization techniques.

4. INTRODUCTION

It is now generally believed that apart from a few exceptions where the structural basis of restricted movement of certain substances is due to a glial barrier layer (Abbott, 1991), the cerebral endothelial cells regulate the entry of solutes and macromolecules from the blood circulation to the brain tissue in the majority of vertebrates. It was demonstrated in electron microscopic studies (Brightman and Reese, 1969) that the barrier, which prevented the passing of proteins from blood to brain, consisted of tight

junctions between cerebral endothelial cells. Another equally important feature of the cerebral endothelial cells, in contrast to those of most other tissues, is the relative lack of pinocytotic vesicles. These particular structural and functional characteristics of cerebral capillaries are determined by the surrounding central nervous tissue, especially by the influence deriving from astrocytes (Arthur *et al.*, 1987; DeBault and Cancilla, 1979; Stewart and Wiley, 1981; Tao-Cheng *et al.*, 1987; Tontsch and Bauer, 1991).

Since the advent of novel procedures, which have made possible not only the isolation of microvessels from the brain tissue (Joó and Karnushina, 1973) but also the culturing of cerebral endothelial cells (reviewed by Joó, 1992), a considerable amount of new information has been gained concerning the basic biochemical and physiological properties of the "blood-brain barrier", a term used to describe collectively the unique features of cerebral endothelial cells.

With the recognition that most of the endothelial cells resisted damage during isolation, remained viable, and could be maintained in tissue culture conditions (Panula *et al.*, 1978), a new generation of *in vitro* BBB model systems was developed. Several procedures and many modifications have been worked out for culturing rat cerebral endothelial cells (RCEC) in different laboratories since then (Phillips *et al.*, 1979; Spatz *et al.*, 1980; Bowman *et al.*, 1981; Diglio *et al.*, 1982; Rupnick *et al.*, 1988; Gordon *et al.*, 1991; Dux *et al.*, 1991; Abbott *et al.*, 1992). Except that of Phillips *et al.* (1979), all methods use gray matter as starting material, collagenase and/or collagenase-dispase for enzymic digestion. In most of the protocols cerebral endothelial cells are separated by Percoll gradient centrifugation step.

Monolayers of endothelial cells derived from cerebral capillaries, cultured and grown on inserts proved to be excellent objects for studying directly BBB permeability, as it was reviewed earlier (Joó, 1992), mainly because of the simplicity of the *in vitro* model system. Since astrocytes have been shown (Dehouck *et al.*, 1990) to be able of upregulating and maintaining certain BBB characteristics of cerebral endothelial cells, my studies on the effects of histamine, TNF-α and cAMP were carried out on bovine brain capillary endothelial cells (BBCECs) cocultured with rat astrocytes (Dehouck *et al.*, 1992).

Histamine is a known mediator of increased vascular permeability and oedema formation in peripheral tissues. It has been proposed as regulator of the cerebral blood flow and permeability of the BBB (Gross, 1982; Joó, 1986). It is also involved in the development of vasogenic brain oedema through the opening of the BBB (Joó, 1987; Mohanty, 1989). Three distinct cerebral histamine pools exist (Edvinsson *et al.*, 1993):

in neurons, in perivascular mast cells and in cerebral blood vessels, where the main source is presumably the endothelium (Karnushina *et al.*, 1979). Histamine receptors (mainly H_2-type) were supposed to be present on both sides of the BBB (Joó, 1986). Since the level of the histamine metabolizing enzyme, histamine-N-methyltransferase is very low in the cerebral capillaries (Karnushina *et al.*, 1979), it is crucial for the endothelium to operate an effective uptake and release system for histamine.

It has long been documented that different cytokines are important messenger molecules which mediate the communication between the immune system and brain. One of the proinflammatory cytokines, TNF-α has been shown to be particularly involved in the pathogenesis of CNS infections (Beutler and Grau, 1993; Tracey and Cerami, 1993), e.g. bacterial meningitis (Leist *et al.*, 1988; Waage *et al.*, 1989), cerebral malaria (Grau *et al.*, 1987), and human immunodeficiency virus type 1 encephalopathy (Grimaldi *et al.*, 1991). TNF-α was also reported to play a role in neurodegenerative diseases, such as multiple sclerosis (Hofman *et al.*, 1989; Tsukada *et al.*, 1991; Sharief and Thompson, 1992), Alzheimer's disease (Fillit *et al.*, 1991), Parkinson disease (Mogi *et al.*, 1994) and Guillain-Barré syndrome (Hofman *et al.*, 1989). Wherever TNF-α is released, either from circulating blood consituents or from intracerebrally located microglia, smooth muscle cells or astrocytes (Lieberman *et al.*, 1989; Sawada *et al.*, 1989; Warner *et al.*, 1989), it can reach with high probability the cerebral endothelial cells at the luminal or abluminal membrane, or at both.

According to the previous study of Megyeri *et al.* (1992), TNF-α, administered into the cerebrospinal fluid, induced the opening of the BBB for sodium fluorescein (m.w. 376 Da). On the other hand, Gutierrez *et al.* (1993) found no change in the permeability of the BBB for albumin (m.w. 67 kDa) after intravenous administration of TNF-α. It remained an open question what effects, if any, TNF-α could exert on the cerebral endothelial cells if the luminal surface of the BBB was exposed to the cytokine.

The first results indicating that the lipid soluble derivative of cAMP, dibutyryl-cAMP, administered into the blood circulation *in vivo* can increase the albumin penetration in mature brain microvessels were published in 1972 by Joó. Later, the synthesizing and degrading enzymes of cAMP were detected in the cerebral endothelial cells (for review see Joó, 1985, 1992) indicating that these second messenger molecules are produced and metabolized locally. In addition, a correlation was reported (Ádám *et al.*, 1987) to exist between the activation of adenylate cyclase in cerebral endothelial cells and the induction of transcapillary albumin transport, suggesting that elevated cAMP may

trigger macromolecule permeation through the microvessels. In agreement with these findings, the data obtained also from *in vivo* studies of Sen and Campochiaro (1991) also pointed out that stimulation of intracellular cAMP accumulation may be a common feature of mediators that cause breakdown of the blood-retinal barrier. In contrast to the above-mentioned results, Kempski *et al.* (1987) found no increase of permebility to trypan blue-albumin complex in cultured cerebromicrovascular endothelial cells when the endothelial synthesis of cAMP was stimulated directly by forskolin. Later Rubin *et al.* (1991) reported that exposure of a cerebral endothelial monolayer to an elevated level of cAMP resulted in a rapid increase in transendothelial electrical resistance in the presence of astrocyte-conditioned medium (ACM) and a significant reduction in sucrose permeability for high resistance monolayers. Previous results of Oláh *et al.* (1988) also suggested that the effect of cAMP on BBB permeability is possibly mediated by protein phosphorylation in the cerebral endothelial cells and it may be exerted rapidly by interfering with certain cytoskeletal proteins. The influence of short term elevation of the intraendothelial cAMP level on the transport of small molecular weight substances have been studied in our experiments.

There has been little work on second messenger modulated kinases in the function of cerebral endothelial cells. One important candidate for attention is the CaM PK-II, also called multifunctional calcium/calmodulin-stimulated protein kinase because of its broad substrate specificity, that includes synapsin 1, tyrosine and tryptophan hydroxylases, the microtubule-associated proteins MAP-2 and tau, together with glycogen synthase, myosin light chain, intermediate filament proteins etc. (Schulman and Lou 1989). Transcripts for the α- and β-subunits are primarily found in brain, whereas transcripts for the γ- and δ-subunits are present in various tissues, among them in aorta as revealed by RNA blot analysis (Tobimatsu and Fujisawa 1989). The enzyme is involved in regulating the synthesis and release of neurotransmitters, motility and cell shape, amino acid, lipid and cyclic nucleotide metabolism.

5. MATERIALS AND METHODS

5.1 Preparation of primary RCEC cultures

Two-week-old CFY rats of either sex were anesthetized with ether. After thorough rinse with 70% ethanol, than with iodine in 70% ethanol heads were cut, and placed into

a sterile glass petri dish. In the laminar flow box forebrains were removed from the skulls with sterile microdissecting forceps and scissors, and collected in cold sterile phosphate buffered saline (PBS). Meninges were removed on sterilized filter paper (Whatman 3M) while at the same time white matter was "peeled off" with the aid of fine curved forceps. Grey matter was carefully collected from the filter paper (meninges sticked to it) and minced to approximately 1 mm^3 pieces by sterile disposable scalpels in the first incubation medium (3 mg/ml collagenase CLS2 from Worthington, 1 mg/ml bovine serum albumin (BSA) in Dulbecco's modified Eagle's medium (DMEM), containing antibiotics) in a sterile glass petri dish. The minced tissue was transferred into a centrifuge tube (35 ml, Oakridge-type with screw cap) with the rest of the collagenase solution (total: 15 ml/10 brains) and triturated with a pipet (10 up and down), than incubated at 37°C for 1.5 h in shaking waterbath. After this incubation, cold 25 ml of 25% BSA-DMEM was added to the homogenate, mixed well by trituration and centrifuged at 1000x *g* for 20 min. The myelin layer and the supernatant were aspirated, the pellet was washed once in DMEM (1000x *g* for 10 min) then further incubated in waterbath for an other, maximum 2 h in 10 ml of the second incubation medium containing 1 mg/ml collagenase-dispase in DMEM.

The cell suspension was centrifuged (700x *g* for 5 min). The pellet was suspended in 2 ml medium and carefully layered on a continuous 33% Percoll gradient and centrifuged at 1000x *g* for 10 min. For the gradient 10 ml Percoll (Pharmacia), 18 ml PBS, 1 ml fetal calf serum (FCS) and 1 ml 10x concentrated PBS were mixed, sterile-filtered and centrifuged at 4°C 30,000x *g* for 1 h. The band of the endothelial cell clusters (clearly visible as a white-grayish layer above the red blood cells) was aspirated, washed twice in medium (1000x *g*, 10 min). The cells were suspended in culture medium (DMEM/F-12 containing 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin, 2mM glutamine, 10mM Hepes, 20% heat inactivated FCS) and were seeded onto rat tail collagen-coated 35 mm plastic dishes or 25 mm cell culture inserts (seeding density 10 cm^2/brain). The medium was changed the next day, later on every third day. This protocol without any modification was used to obtain porcine cerebral endothelial cell (PCEC) cultures.

5.2 Preparation of BBCEC cocultures

BBCECs were cultured as described by Dehouck *et al.* (1990). Briefly: individual colonies of primary endothelial cells emerging from cerebral capillaries were subcloned

by microtrypsinization. Culture medium for BBCEC contained 10% FCS, 10% horse serum (Hyclone), 2 mM glutamine and 50 μg/ml gentamycine in DMEM. One ng/ml basic fibroblast growth factor was added every other day to the cultures.

Newborn rat astrocytes, prepared by the method of Booher and Sensenbrenner (1972) from 2-day-old rat cortex, were seeded on 6-well plates, kept in DMEM containing 10 % FCS, glutamine and gentamycine for 6-8 weeks before starting the coculture. Culture medium left for 48 h on 4-week-old cultures was collected, sterile-filtered and used as ACM. After 4-6 passages BBCEC were seeded on rat tail collagen coated cell culture inserts (Millicell-CM, pore-size: 0.4 μm, diameter: 30 mm, Millipore), which were placed into the astrocytes-containing wells. Thereafter, the medium was changed and added to both the apical and basolateral chambers. BBCEC and astrocytes were cocultured for at least 12 days.

Bovine aortic endothelial cell (BAoEC) cultures, prepared from aortic arch (Méresse *et al.*, 1989) were also used between passage 4-6 and grown in the same medium as BBCEC.

5.3 Characterization of primary RCEC cultures

Factor VIII (FVIII) immunohistochemistry: After a brief washing in PBS and fixing in ethanol at 4°C for 15 min, cells were treated with 1% H_2O_2 in PBS for 10 min, followed by washing in PBS. Non-specific binding sites were blocked by incubation in 3% normal goat serum in PBS at room temperature for 20 min. Anti-FVIII related antigen rabbit immunoglobulin (Dako) was used as primary antibody and biotin-labelled anti-rabbit IgG (Dako) as secondary antibody, both applied for 30 min. After a 30 min incubation with avidin-biotin-horseradish peroxidase (HRP) complex (ABC kit, Vector Lab). Diamino benzidine (DAB) was used as HRP substrate, followed by hematoxylin-eozin (HE) counterstaining.

Lectin-binding: RCECs were washed in PBS, fixed in 4% formalin and 70% ethanol in PBS for 15 min, treated with 1% H_2O_2 in PBS for 10 min, washed again in PBS, then incubated in 15 μg/ml HRP-conjugated *Bandeirea simlicifolia* lectin-I B4 (BSL-I B4) in 0.1% BSA-PBS for 90 min. DAB was used as HRP substrate. The preparations were counterstained by HE.

Alkaline phosphatase (AP) histochemistry: RCECs were washed in PBS, fixed in 2% paraformaldehyde-PBS for 2 min, washed again in PBS, then incubated in AP colour solution (0.41 mM nitro blue tetrazolium chloride, 0.40 mM 5-bromo-4-chloro-3-indolyl

phosphate in Tris-HCl buffered saline (TBS), pH 9.5) for 3 h.The reaction was stopped by washing in PBS, graded dehydration and mounting in Entellan.

Transmission electron microscopy: RCEC cultures were fixed for 30 min in long Karnovsky's fixative, then washed in 0.1 M phosphate buffer, pH 7.4. Cells were scraped off the surface, and centrifuged in buffer. The pellet was post-fixed in 1% OsO_4, dehydrated and embedded in Spurr resin. Thin sections were stained in lead citrate and finally examined in a Zeiss 902 electron microscope.

5.4 Transendothelial transport

Inserts were transferred into 6-well plates containing 2 ml prewarmed Ringer-Hepes buffer or serum-free DMEM (SF-DMEM) per well (abluminal or basolateral compartment) (**Fig. 1.**).

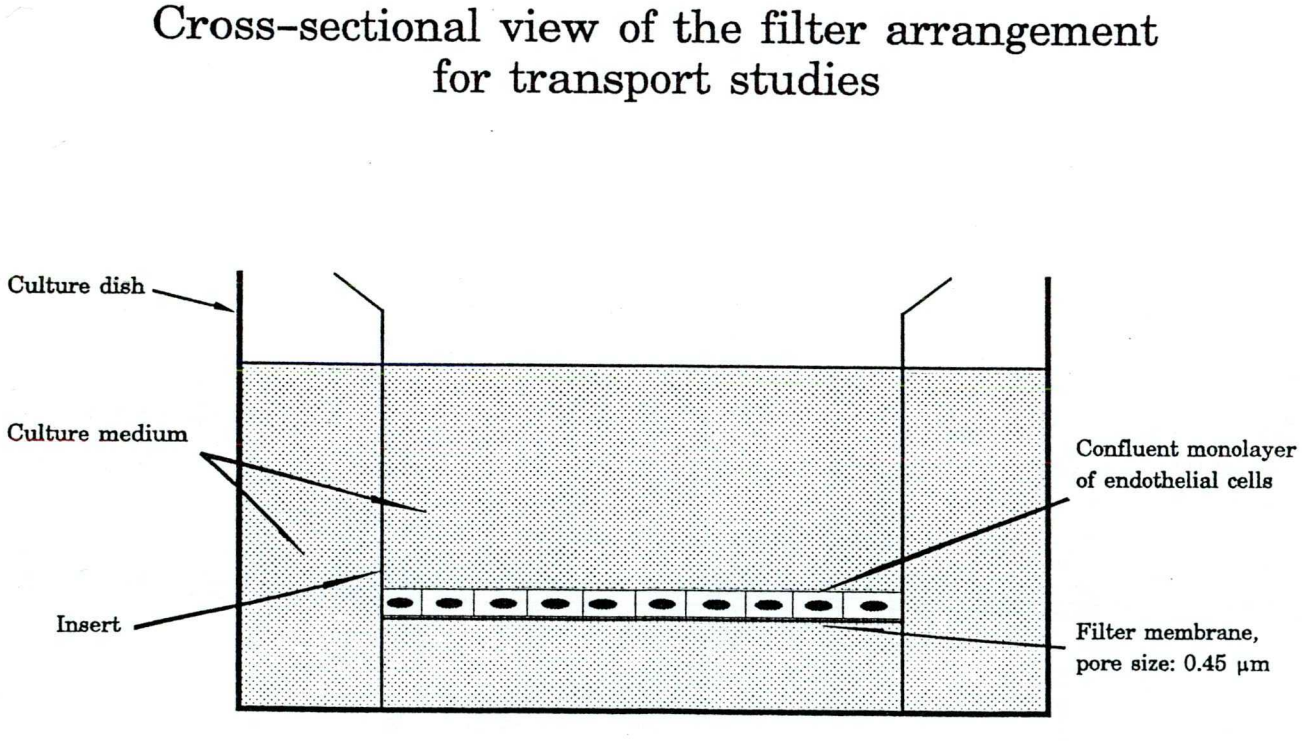


Fig. 1.

In each apical (luminal) chamber, culture medium was replaced by buffer or medium containing the labelled compounds: 5 μCi of[^{14}C]-sucrose (m.w. 342 Da) and 1.25 μCi

of [^{3}H]-inulin (m.w. 5 kDa), or 4% BSA with 0.67 mg/ml Evans blue dye (EBA, m.w. 67 kDa). Thereafter at regular intervals (e.g. 10, 20, 30, 60, 120, and 240 min) inserts were placed into the next well of the plate. Amounts of the labelled compounds were measured by scintillation counting (Wallac 14110 from Pharmacia) of 200 μl aliquots of the media from the the lower compartment. The concentration of EBA in the abluminal chambers was determined spectrophotometrically by its absorbance at 620 nm. For control, flux across cell-free, collagen-coated inserts was also measured. Fluid levels in the two compartments were equal (2 ml in both the luminal and abluminal chamber) and incubations were performed at 37 °C in a humidified atmosphere containing 5% CO_2 and 95 % air.

Transport, i.e. clearance was expressed as microliters of tracer diffusing from the luminal to the abluminal compartments and was calculated from the initial concentration of tracer in the luminal and the final concentration of tracer in the abluminal chamber (Dehouck *et al.*, 1992):

$$Clearance(\mu l) = \frac{[C]_A x V_A}{[C]_L}$$

where $[C]_L$ is the initial luminal tracer concentration, $[C]_A$ is the abluminal tracer concentration, and V_A is the volume of the abluminal chamber (Siflinger-Birnboim *et al.*, 1987). During the experiments the clearance volume increased in a linear fashion with time. The average volume cleared was plotted *versus* time, and the slope was estimated by linear regression analysis to give the mean and the SE to the estimate. The slope of the clearance curves for the BBCEC culture on insert was denoted PS_t, where PS is the permeability x surface area product (in μl/min). The slope of the clearance curve for the control filter was denoted as PS_f. The PS value for the endothelial monolayer (PS_e) was calculated by the following formula:

$$\frac{1}{PS_e} = \frac{1}{PS_t} - \frac{1}{PS_f}$$

The PS_e values were divided by the surface area of the Millicell inserts (4.2 cm^2) to generate the endothelial permeability coefficient P_e (in cm/min).

Mannitol treatment: For osmotic opening of the endothelial monolayers the cells were preincubated with 1.4 M mannitol in SF-DMEM for 5 min.

Histamine treatment: In the luminal chambers culture medium was replaced by Ringer-Hepes solution containing the labelled compounds, or BSA-EBA and 10^{-4} or 10^{-5} M dilutions of histamine dihydrochloride (Sigma). At 15, 30, 45, 60, 90 and 120 min thereafter inserts were placed into the next well of the plate.

TNF-α treatment: BBCEC monolayers were incubated in wells without astrocytes with 3 different concentrations of the cytokine in SF-DMEM for 1 to 4 h. In the first group of treatments, recombinant human TNF-α (Genzyme) was added in concentrations of 50, 250, and 500 U/ml concomitantly with the permeability tracer compounds to the luminal chamber, thereby it could exert its effect until the end of the transport study, for a maximum of 4 h in total. In the second type of experiments, after the treatment with the same concentrations of TNF-α, inserts were replaced to their original, astrocytes-containing wells in medium for BBCEC overnight. Delayed effects were studied on the subsequent day, without any re-exposure to TNF-α.

cAMP treatment: Either chlorophenylthio-cAMP (CPT-cAMP, 250 μM), which elevates intraendothelial cAMP, or the lipid-soluble dibutyryl-cAMP (DB-cAMP, 500 μM), which mimicks the intracellular action of cAMP, were used in the presence of 4-(3-butoxy-4-methoxy-benzyl)imidazolidin-2-one (RO 20-1724, 17.5 μM), a phosphodiesterase inhibitor. Six experimental groups of BBCEC monolayers were set up. In three of them monolayers were cocultured with astrocytes and left in the astrocyte-containing well during 1 h of different treatments: A/ culture medium (control); B/ CPT-cAMP and RO 20-1724; C/ DB-cAMP and RO 20-1724. Two groups of monolayers were cocultured with astrocytes, then removed from the astrocyte-containing well and kept in 50% ACM during 1 h of the different treatments: D/ CPT-cAMP and RO 20-1724; E/ DB-cAMP and RO 20-1724. In the sixth group, F/ monolayers were cultured without any astrocytic influence and then treated with DB-cAMP and RO 20-1724 for 1 h. After 1 h of treatment the culture medium was changed, and transendothelial transport was measured at 10, 20, 30, 60 and 120 min thereafter.

5.5 F-actin localization with immunofluorescence

After finishing the transport study for TNF-α, the same inserts were gently washed in prewarmed (37°C) SF-DMEM and two of the triplicate filters were replaced to the original coculture system for an additional 18 and 42 h, respectively. One set of

filters (representing each TNF-α concentration) was immediately immersed into a modified paraformaldehyde-based fixative developed by Schliwa *et al.* (1981) for structural preservation of cell fibrous components of the cytoskeleton. The other two sets of inserts were treated in the same way at the end of the above mentioned additional coculture period. Fixation conditions were 4% paraformaldehyde in PHEMS buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$ and 140 mM NaCl, pH 6.9) for 15 min at 4°C. After washing in PHEMS, the cells on filter fragments were permeabilized with cold (-20°C) acetone for 10 min and were held in PHEMS until being processed for fluorescent microscopy. For F-actin localization, cells were stained by incubation with 165 nM bodipy-phallacidin (Molecular Probes, Inc.) in PHEMS for 30 min at room temperature. For localization of vimentin, free aldehyde groups were quenched with 0.1 M L-lysine for 4 h at 4°C and incubated for 30 min in TBS pH 7.0 containing 5% (w/v) ovalbumin (OVA) and 1% heat-inactivated normal goat serum. This was followed by a 1 h incubation with a mouse monoclonal antibody to swine vimentin (Dako, clone V9, diluted 1:10 in TBS-OVA). Fluorescein isothiocyanate (FITC) conjugated affinity purified goat anti-mouse IgG (Cappel Laboratories) secondary antibody was used at 1:100 in TBS-OVA. After several washes filter-grown cells were mounted on a microscope slide in Mowiol. All specimens were viewed for optical sectioning with a Leica confocal laser scanning microscope, equipped with 63x, 1.4N.A. oil immersion lens and an argon/krypton ion laser. Optical sections were recorded at 0.5 μm intervals and confocal images were photographed on Kodak Tmax film with a Polaroid Freeze frame directly from the monitor.

5.6 Methods used for the detection of CaM-PK II in RBECs

In situ hybridization: Oligonucleotides complementary to the nucleotides 1439-1470 of the α-subunit mRNA and 1090-1128 of the β-subunit mRNA of the CaM-PK II were 3-end labelled with DIG 11-dUTP (Boehringer) by terminal deoxynucleotidyl transferase (Boehringer). After removal of the medium and washing with PBS (pH=7.4) the cells were fixed in 4% paraformaldehyde in PBS for 5 min, then washed with PBS for 10 min, treated with HCl (0.1 N) for 20 min, and washed again with PBS (10 min) followed by a proteinase K treatment (20 μg/ml in TrisHCl 100 mmol/l, EDTA 50 mmol/l pH=8) for 10 min. After a washing step each coverslip was prehybridized with hybridization solution for 1 h and incubated with 200 μl of hybridization solution (50% formamide, 4× saline-sodium citrate (SSC), 1×Denhardt solution, 5% dextran sulfate, 0.5

mg/ml salmon sperm DNA and 0.25 mg/ml yeast tRNA) containing the labelled probe in a concentration of 100 ng/ml. Hybridizations were performed for 12-16 h at 37°C in a humidified chamber. Following hybridization the coverslips were washed with 2×SSC (1 h), 1×SSC (1 h), 0.5×SSC (30 min) and 0.2×SSC (30 min). The immunological detection was carried out with anti-digoxigenin polyclonal antibody conjugated with AP using a DIG-DNA-Detection kit (Boehringer).

Immunocytochemistry: Cultures grown on collagen-coated glass coverslips were briefly washed in physiological saline, cells were fixed for 10 min in 2% paraformaldehyde and 0.2% glutaraldehyde in PBS, then permeabilized for another 10 min in 0.1% Triton X-100 in PBS. Nonspecific binding of the antibodies was reduced by a 20 min preincubation in blocking buffer containing 3% normal goat serum and 0.2% BSA in PBS. Coverslips were first incubated for 2 h with monoclonal antibodies (in form of ascitic fluids) specific for α and β subunits of the kinase (Scholz *et al.*, 1988) in dilutions of 1:2000 and 1:500, respectively, then for 1 h with biotinylated goat anti-mouse IgG [1:1000] (Amersham), finally for another hour with streptavidin conjugated AP [1:1000] (Boehringer), all reagents being diluted in blocking buffer. Coverslips used as controls were identically treated except that primary antibodies were omitted. Extensive washing in PBS, 3 x 5 min each, was repeated between and after these steps. For the detection of the immunostaining we used AP colour solution (as described in section 5.3).

Western blot analysis: Protein samples (0.5μg purified kinase/lane, 100μg endothelial cell homogenate/lane) were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore) at 70 V constant voltage for 90 min. The lanes were cut into strips, which were soaked in PBS for 10 min, then preincubated in blocking buffer (2% nonfat dry milk powder in PBS, containing 0.1% sodium azide) for 2.5 h. All the steps of the immunostaining were done on a rocker platform and were followed by exhaustive washing (once with 0.25% Tween 20 in PBS for 5 min, twice with 0.05% Tween 20 in PBS for 5 min, finally once with PBS). Strips were first incubated overnight at 4°C with α- and β-subunit specific monoclonal antibodies [1:4000 and 1:2000], respectively. This was followed by 1 h incubation with biotinylated goat anti-mouse IgG 1:1000 (Amersham), finally for 30 min with streptavidin conjugated AP [1:1000] (Boehringer) at room temperature, all reagents being diluted in blocking buffer. The immunoreactive bands were visualized by AP colour solution.

Protein phosphorylation: Samples containing 50 μg protein each, prepared from primary RCEC cultures, were preincubated for 5 min in 50 μl assay medium containing

25 mM Tris-MES buffer and 2 mM EGTA to eliminate contaminating Ca^{2+} from the control samples. Calmodulin-stimulated protein kinases were assayed in the presence of Ca^{2+} (100 μM) and bovine brain calmodulin (5 μM) and 10 μCi [γ-^{32}P]ATP for 2 min. The incubations were stopped by addition of SDS sample buffer, and the proteins were separated by 10% SDS-PAGE. For autoradiography the gels were dried and exposed to X-ray films (Kodak XAR-5) for 3 days using intensifying screens.

5.7 Statistical analysis

Statistical analysis was performed using the Student's *t*-tests and Friedman's repeated measure analysis of variance (ANOVA) on ranks followed by the Student-Newman-Keuls test as appropriate.

6. RESULTS

6.1 Tissue culture of RCECs

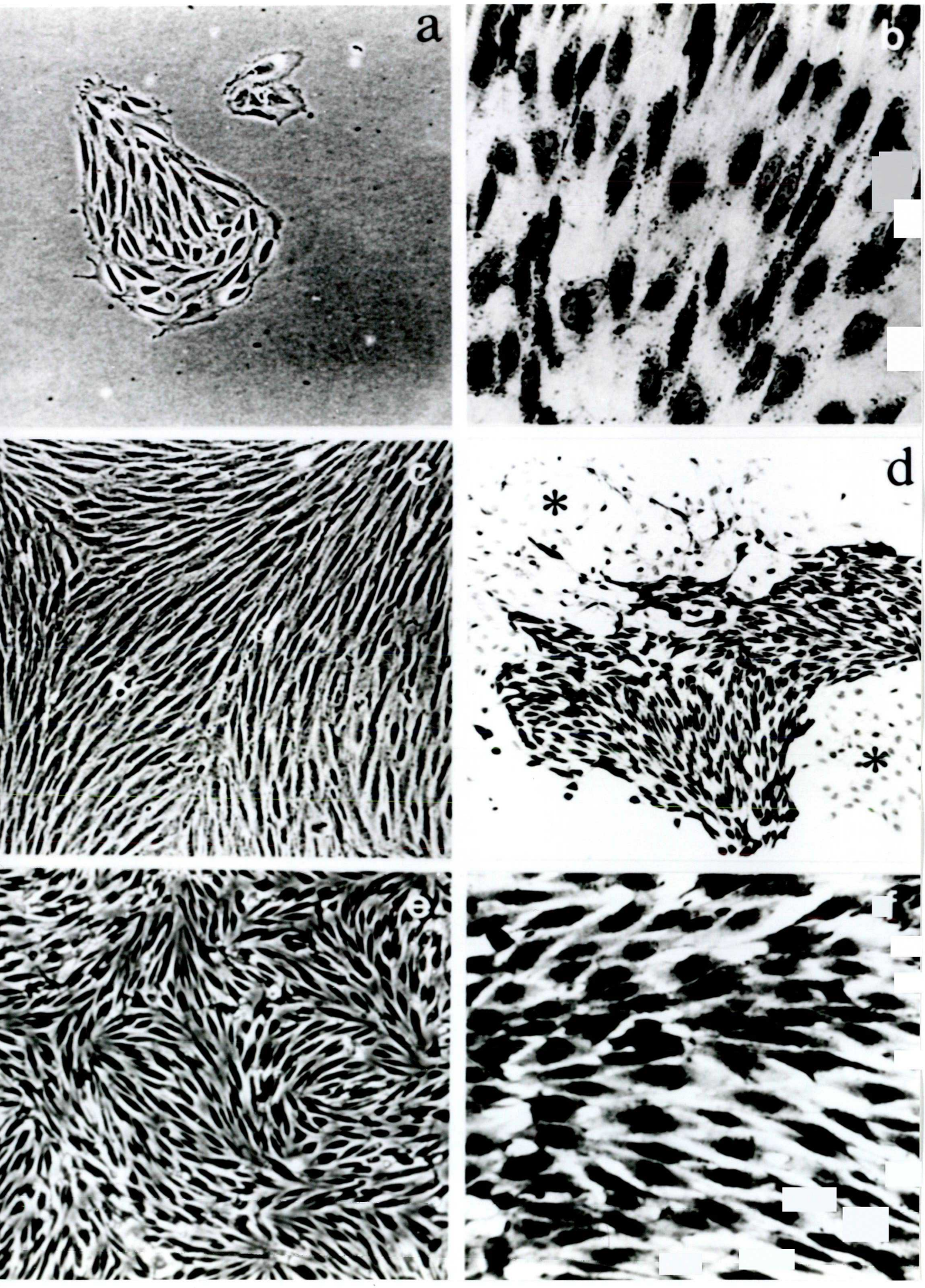
First the microvessels were separated from rat brain tissue, then basement membrane and perivascular cells were removed by the second enzymic treatment. Endothelial cell clusters were further purified by Percoll gradient centrifugation. The small vessel fragments attached rapidly to the rat tail collagen coated surfaces, and by the second or third day *in vitro* colonies of CECs emerged (**Plate I.a.**), and formed a non-overlapping continuous monolayer at the end of the first week (**Plate I.c. and e.**) with some swirling patterns. Both RCEC and PCEC displayed a so called "fibroblast-like" morphology: cell-shape was fusiform with an oval nuclei in the center, neighbouring cells tightly apposed to each-other. Cells gave specific immunohistochemical staining with anti-FVIII antibody, bound the galactose-specific BSL-I B4 isolectin and showed positive histochemical staining for AP. Cells were abundant in mitochondria and endoplasmic reticulum. Some attachment sites were without specialization, while others were gap junction-like (**Publ. II., Fig. 2.**). The passage of 70 kDa FITC-dextran was restricted through RCEC: 99.12 ± 8.79 μg/cm^2/h *vs.* 655.80 ± 12.37 μg/cm^2/h (n=6; $p<0.001$) in the case of cell-free filters.

In confluent primary cultures older than 7-10 day *in vitro* angiogenesis, occasionally overgrowth by non-endothelial cells could be observed (**Plate I.d**).

Plate I. Light microscopy

a, **c**, and **e**: phase contrast pictures from cerebral endothelial cells
a: a colony of primary RCEC 3 days after seeding; magnification x200
c: confluent primary culture of RCEC, 7 days after seeding; x200
e: confluent primary culture of PCEC, 7 days after seeding; x200

b and **d**: FVIII immunohistochemistry, HE counterstaining
b: specific, perinuclear cytoplasmic dot-like staining of RCEC; x400
d: an islet of RCEC is stained for FVIII, while contaminating cells are not (asterisks); x80
f: *Bandeirea simplicifolia* isolectin B4 binding to RCEC; x400


a
b
c
d
e
f

6.2 Barrier function

As it is shown in **Figure 2.**, the clearance of both tracers through BBCEC cocultured with astrocytes was only 8.5-12.5 % of the clearance of cell-free filters, indicating the maintenance of permeability barrier (P_e of sucrose = $0.49 \cdot 10^{-3}$ cm/min, P_e of inulin = $0.28 \cdot 10^{-3}$ cm/min). Exposure of the luminal membrane of BBCEC to 1.4 M mannitol for 5 min resulted in significant increases in the permeability of reconstituted BBB for both [^{14}C]-sucrose (31.81 ± 5.89 μl vs. 12.02 ± 1.02 μl, n=3, $p<0.05$) and [^{3}H]-inulin (10.26 ± 1.65 μl vs. 4.75 ± 0.61 μl, n=3, $p<0.05$) at 10 min. However, this effect disappeared at later stages (20, 30, and 60 min) after mannitol exposure, showing the reversible nature of the osmotic BBB opening. In contrast to BBCEC, confluent monolayers of BAoEC restricted 4-fold less the flux of both tracers. The clearance values were 44-61 % for [^{14}C]-sucrose and 35-48 % for [^{3}H]-inulin of clearances of the collagen-coated inserts (P_e of sucrose = $3.46 \cdot 10^{-3}$ cm/min, P_e of inulin = $1.43 \cdot 10^{-3}$ cm/min). The permeability enhancing effect of 1.4 M mannitol on BAoEC proved to be irreversible up to 60 min ($p<0.001$); the clearances for both tracers through mannitol-treated BAoEC monolayers did not differ from values obtained on cell-free filters.

6.3 Effects of histamine on BBCEC monolayer permeability

Luminal application of histamine (10^{-5} and 10^{-4} M) resulted in no significant changes in the clearance of monolayers for [^{14}C]-sucrose or [^{3}H]-inulin within 2 hours. Evans blue labelled BSA permeation through BBCEC was, however, significantly ($p<0.05$) increased after the administration of histamine in both concentrations (**Fig. 3.**).

6.4 Histamine uptake and release by RCECs

[^{3}H]-histamine was taken up into cultured rat cerebral endothelial cells; the uptake of 0.04 μM of [^{3}H]-histamine was linear up to 6 min and inhibited by Na^+-deprivation (when 60 mM of NaCl was substituted with choline chloride) and by pre-incubating the cells with 0.1 mM of ouabain for 15 min (**Publ. V., Fig. 1.**).

Varying the concentration of [^{3}H]-histamine in the incubation medium, the initial (5 min) velocity of the uptake was shown to be concentration dependent and saturable, following the Michaelis-Menten kinetics. The linearized form of the kinetics, the Lineweaver-Burk and the Hofstee plots resulted in an apparent K_m = 0.3 ± 0.02 μM and a V_{max} = 4.6 ± 0.04 pmol/mg protein per min for the uptake (**Publ. V., Fig. 2.**).

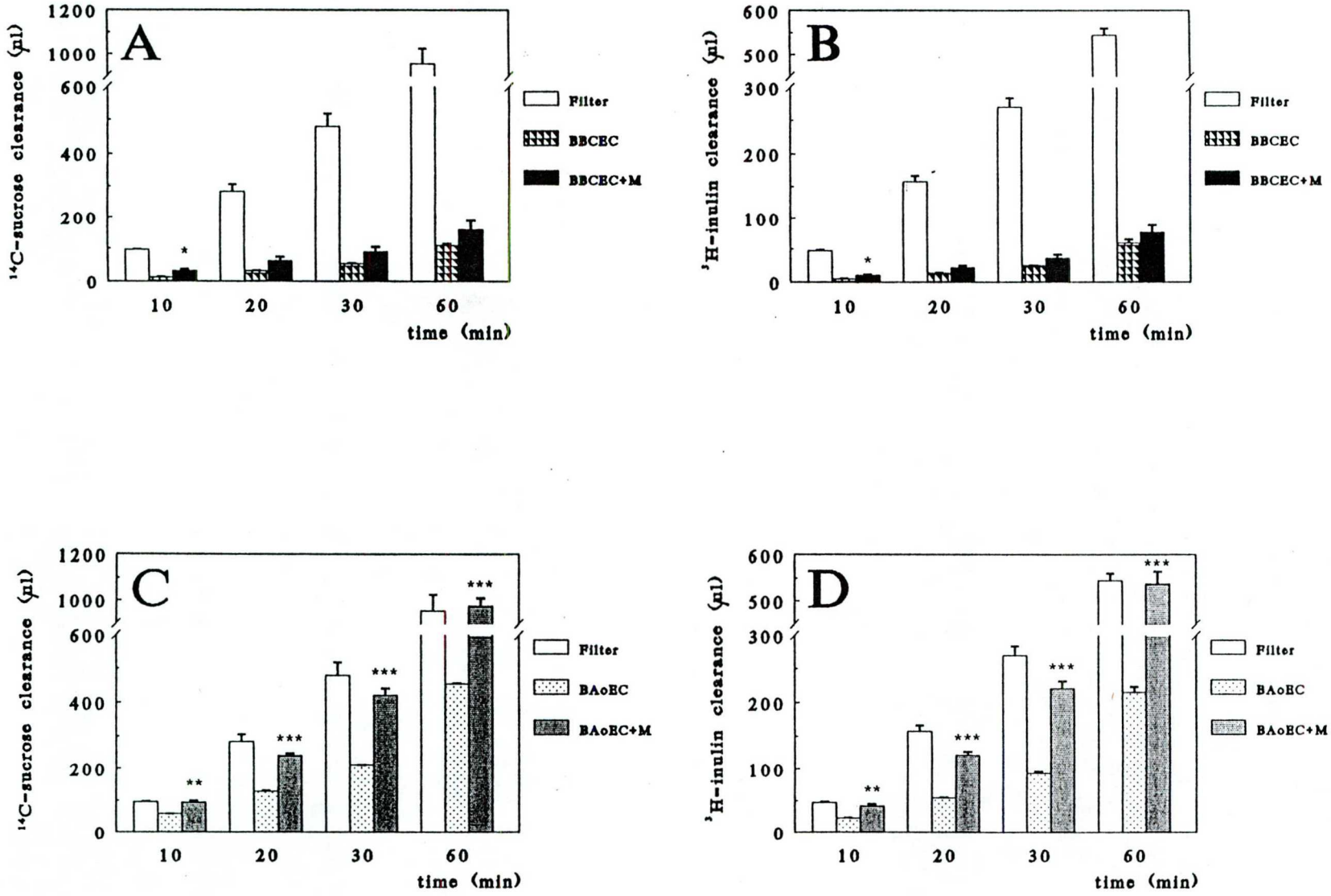


Fig. 2. Clearance of [^{3}H]-sucrose (**A,C**) and [^{14}C]-inulin (**B,D**) across BBCEC (A,B) and BAoEC (C,D) monolayers vs time. Values are expressed as mean ± SEM (n=3). *p<0.05, **p<0.01, ***p<0.001.

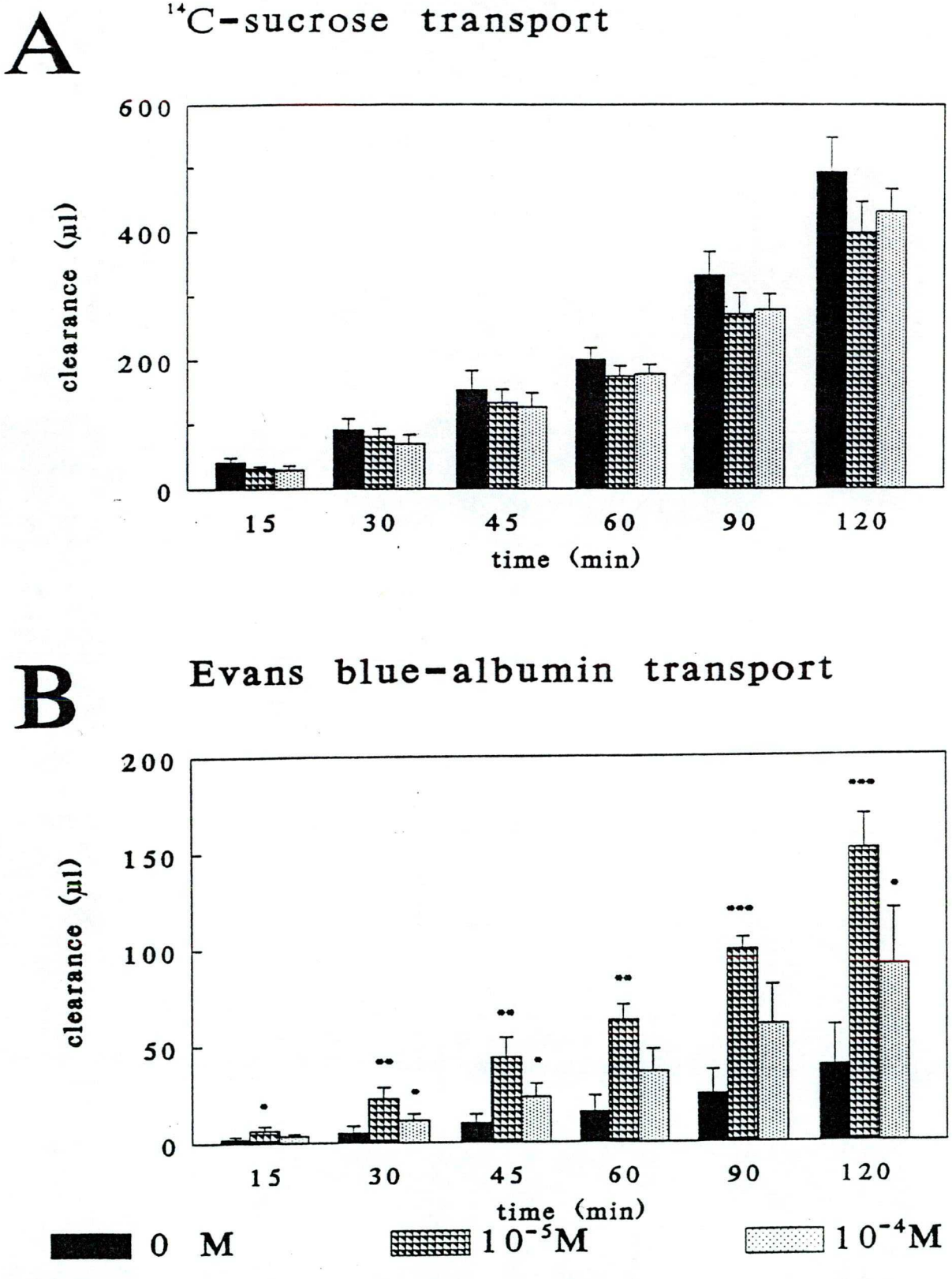


Figure 3. Effect of histamine on the permeability of BBCEC monolayers. Clearance of ^{14}C-sucrose (A), and Evans blue-albumin (B) is plotted *vs* time; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Efflux of [^{3}H]-histamine, previously accumulated by the cells (0.04μM for 10 min incubation) was observed when the cultures were incubated in a histamine free Krebs buffered medium (**Publ. V., Fig. 3.**). The time course showed an exponential curve reaching an equilibrum in 12 min. Plotting the logarithm of the % radioactivity remained in the cells against the duration of the incubation period yielded a straight line in 12 min. The slope of this line varied with the constituents of the incubation medium; Na^+-deprivation (either with choline or with K^+-substitution) was considered to produce a remarkable enhancement in the efflux of [^{3}H]-histamine.

In a series of experiments, uptake studies were performed by using an *in vitro* reconstructed model of the BBB. Incubating the RCEC monolayers grown on culture inserts with 0.04μM [^{3}H]-histamine, the amine was taken up in closely equal rates accross the luminal and abluminal membranes: 0.120 ± 0.010 and 0.100 ± 0.008 pmol/mg protein per min, respectively, suggesting that the uptake system is present both luminally and abluminally (**Publ. V., Fig. 4.**). Histamine, taken up either luminally or abluminally, was released from the cells into the medium in the upper compartment of the inserts representing the "blood side" of the system. This suggests a preferential luminal release of histamine from the monolayers. Ouabain, like Na^+-deprivation, produced a remarkable increase in the efflux of [^{3}H]-histamine from both the luminally or abluminally preloaded cells.

6.5 Effects of TNF-α on BBB permeability

Exposure of TNF-α to the luminal membrane of BBCEC monolayers caused no significant change in either [^{14}C]-sucrose or [^{3}H]-inulin clearance up to 4 h, *early phase*, except for a decrease ($p<0.05$) in [^{14}C]-sucrose transport 2 and 4 h after the beginning of the treatment with 500 U/ml TNF-α (**Fig. 4.**).

On the other hand, 16 h after the 1-h challenge, *delayed phase*, TNF-α induced a significant increase ($p<0.05$) of permeability for both markers (**Fig. 5.**).

6.6 TNF-α stimulates delayed stress fiber formation

In normal condition, immunofluorescence staining with bodipy-phallacidin, which specifically binds to F-actin microfilaments, reveals the preferentially cortical membrane associated actin and the fine actin fibers running linearly across the cell (**Plate II.**, left panel). Within 1-4 h of treatment with TNF-α no perturbation of F-actin characteristics was observed. In the TNF-α treated cells, which were replaced to the original coculture

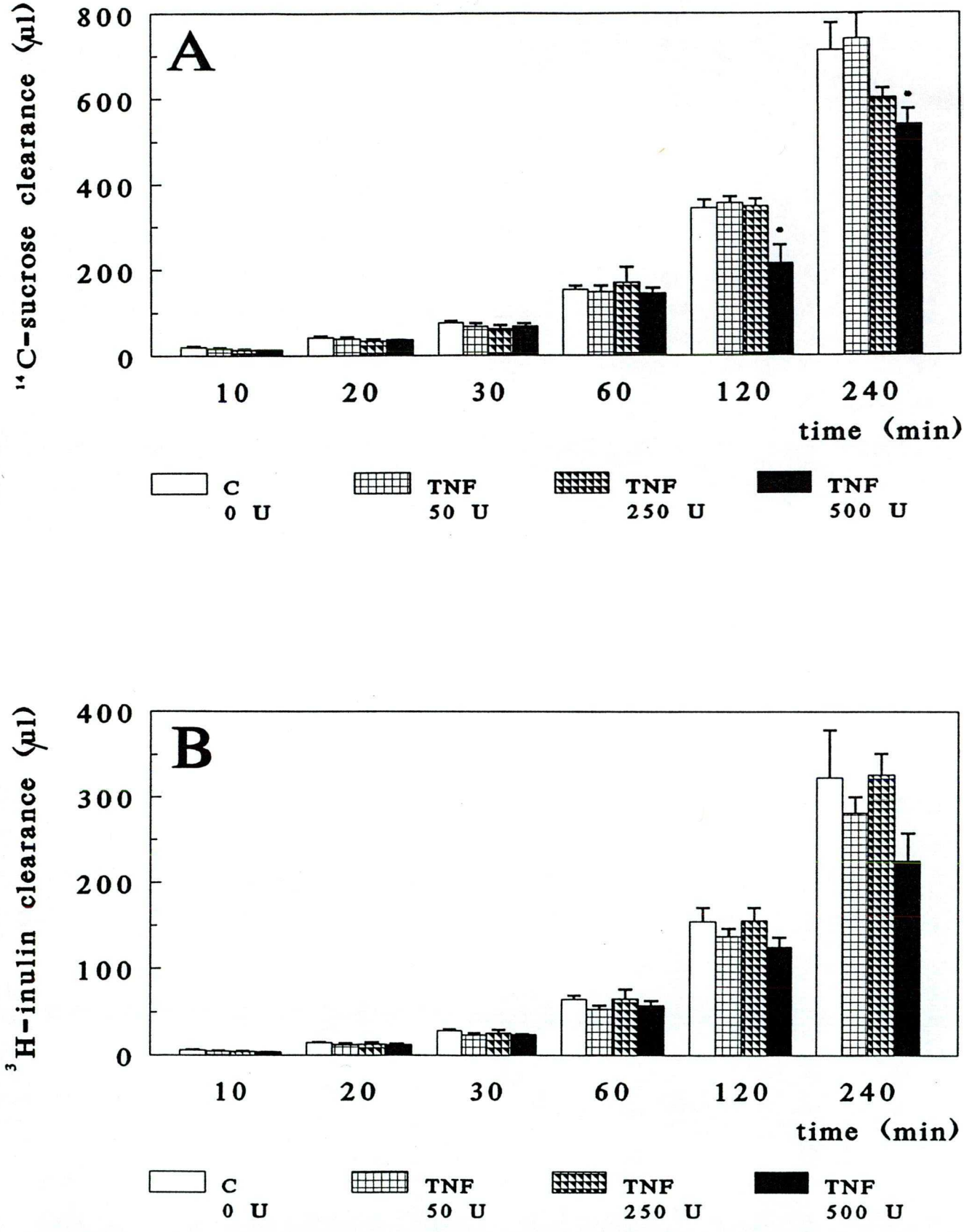


Fig. 4. Effect of 50, 250 and 500 U/ml TNF-α treatment on the permeability of BBCEC monolayers up to 4 h, *early phase*. Clearance of [^{3}H]-sucrose (**A**) and [^{14}C]-inulin (**B**) across BBCEC in µl is plotted vs time. Values are expressed as mean ± SEM (n=12). Differences between control and TNF-treated groups were determined by Student's *t*-test; *p<0.05, **p<0.01.

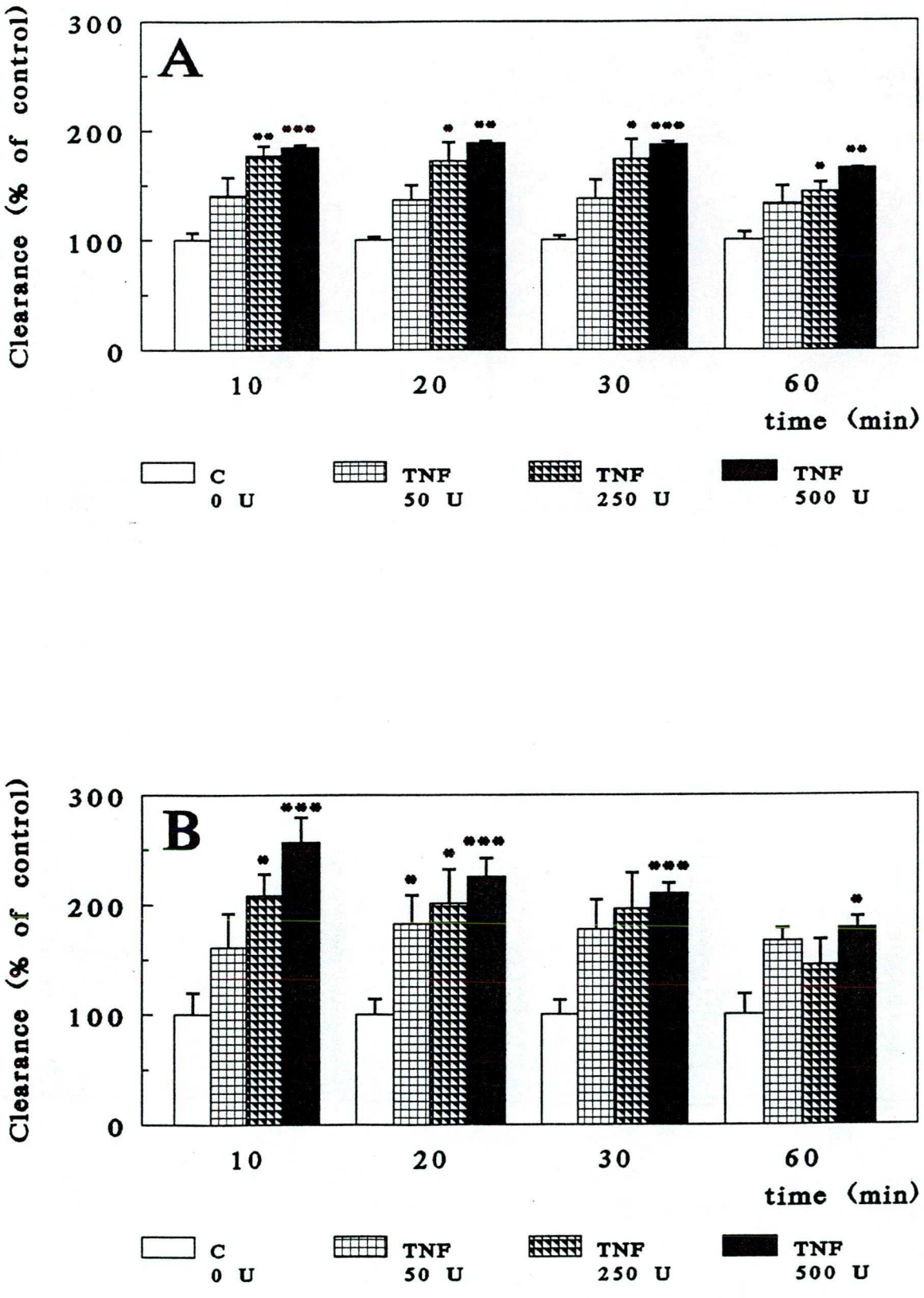


Fig. 5. Effect of 50, 250 and 500 U/ml TNF-α pretreatment on the permeability of BBCEC monolayers 16 h after the 1-hour challenge, *delayed phase*. Clearance of [^{3}H]-sucrose (A) and [^{14}C]-inulin (B) across BBCEC is expressed as % of the control. Differences between control and TNF-treated groups were determined by Student's *t*-test; *p<0.05, **p<0.01, ***p<0.001.

system for an additional 18 and 42 h, the association between the underlying actin cytoskeleton and the cell membrane was always observed, but became indistinct. The diffuse and filamentous actin condensed in most of the cells and became more prominent (**Plate II.**, right panel). Pre-exposure to high concentration (500 U/ml) of TNF-α led to the maximal TNF-induced formation of stress fibers without detectable cell retraction and was fully observed by 42 h. X-Z views of the fluorescent planes confirm the dramatic intracytoplasmic reorganization of actin filaments following incubation of BBCEC with TNF-α. In contrast, in the same samples the distribution of intermediate filaments of vimentin, localized by indirect immunofluorescence, did not change significantly between TNF-treated and control groups throughout the study period (**Publ.VIII., Fig. 5.**).

6.7 Effects of cAMP on BBCEC monolayer permeability

One hour of treatment with either of the cAMP concentration-elevating drugs, plus the phosphodiesterase inhibitor, decreased significantly ($p<0.05$) the permeability of both sucrose and inulin in all groups, and at all timepoints except for the DB-cAMP +RO 20-1724 group at 120 min (see **Fig. 6.**). There were no significant differences between the two groups treated with CPT-cAMP + RO 20-1724 (B and D). The transport for both tracers was significantly ($p<0.05$) lower in BBCEC monolayers grown above astrocytes (group C), but not in monolayers treated with 50% ACM (group E), compared with that measured in monolayers without any astrocytic influence (group F) after DB-cAMP + RO 20-1724 treatment.

6.8 Detection of CaM-PK II in RBECs

The expression of CaM-PK II mRNA in primary cultures of cerebral endothelial cells was examined with the use of synthetic oligonucleotide probes complementary to the nucleotides 1439-1470 of the α-subunit mRNA and 1090-1128 of the β-subunit mRNA of the CaM-PK II. Neither of these sequences could be found in the mRNA of other known subunits of CaM-PK II (Burgin *et al.*, 1990). Sense probes were also synthesized and used for control experiments. Strong staining indicating the expression of α-subunit of CaM-PK II (**Publ. I., Fig. 1a.**) was observed with *in situ* hybridization in the perinuclear region of the cytoplasm of cerebral endothelial cells, whereas no staining was seen after the use of sense probe (**Publ. I., Fig. 1c.**). On the other hand, weak labelling was found with the use of the oligonucleotide probe for the β-subunit of CaM-PK II (**Publ. I., Fig. 1b.**), which did not differ essentially from the low labelling obtained

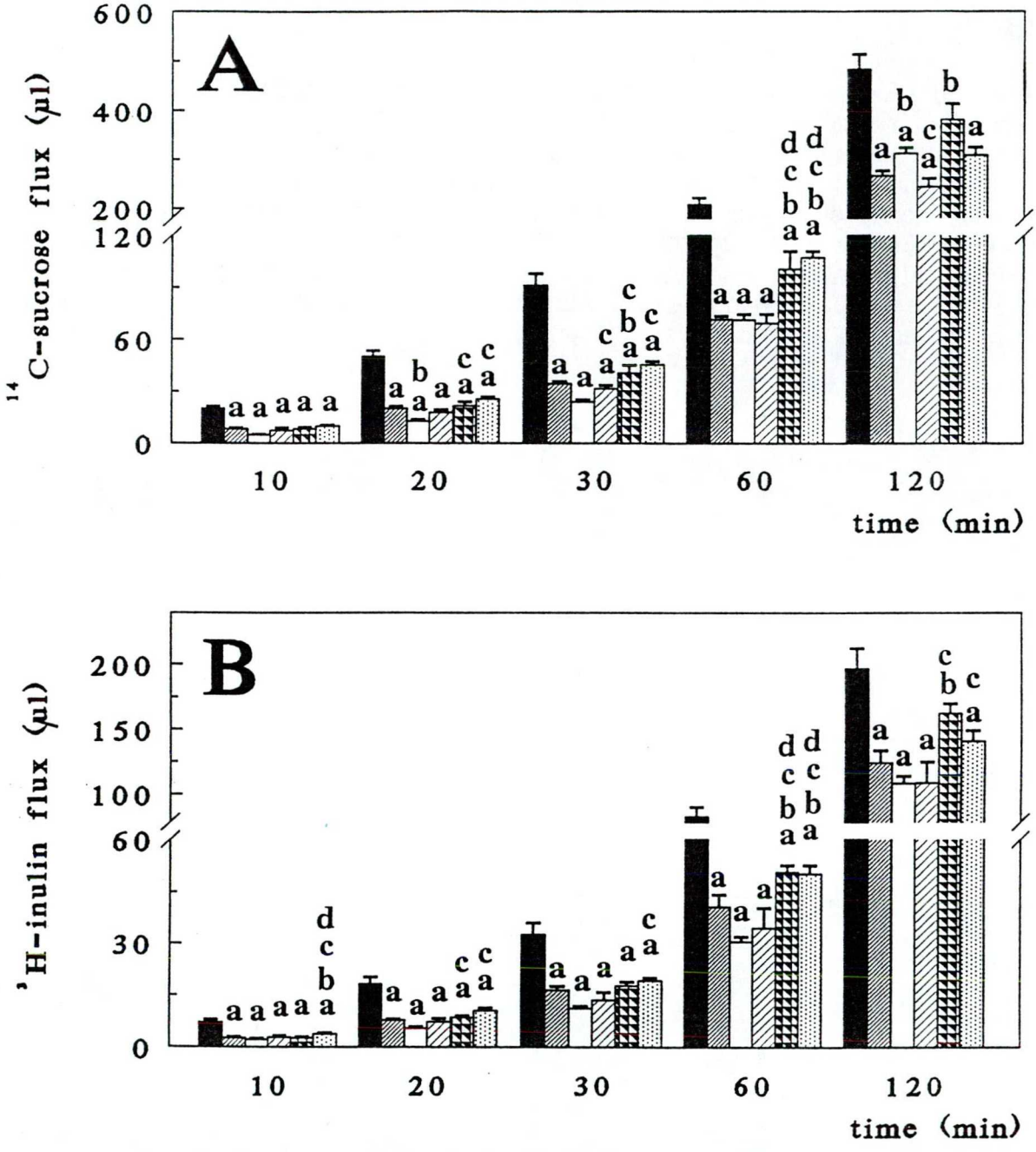
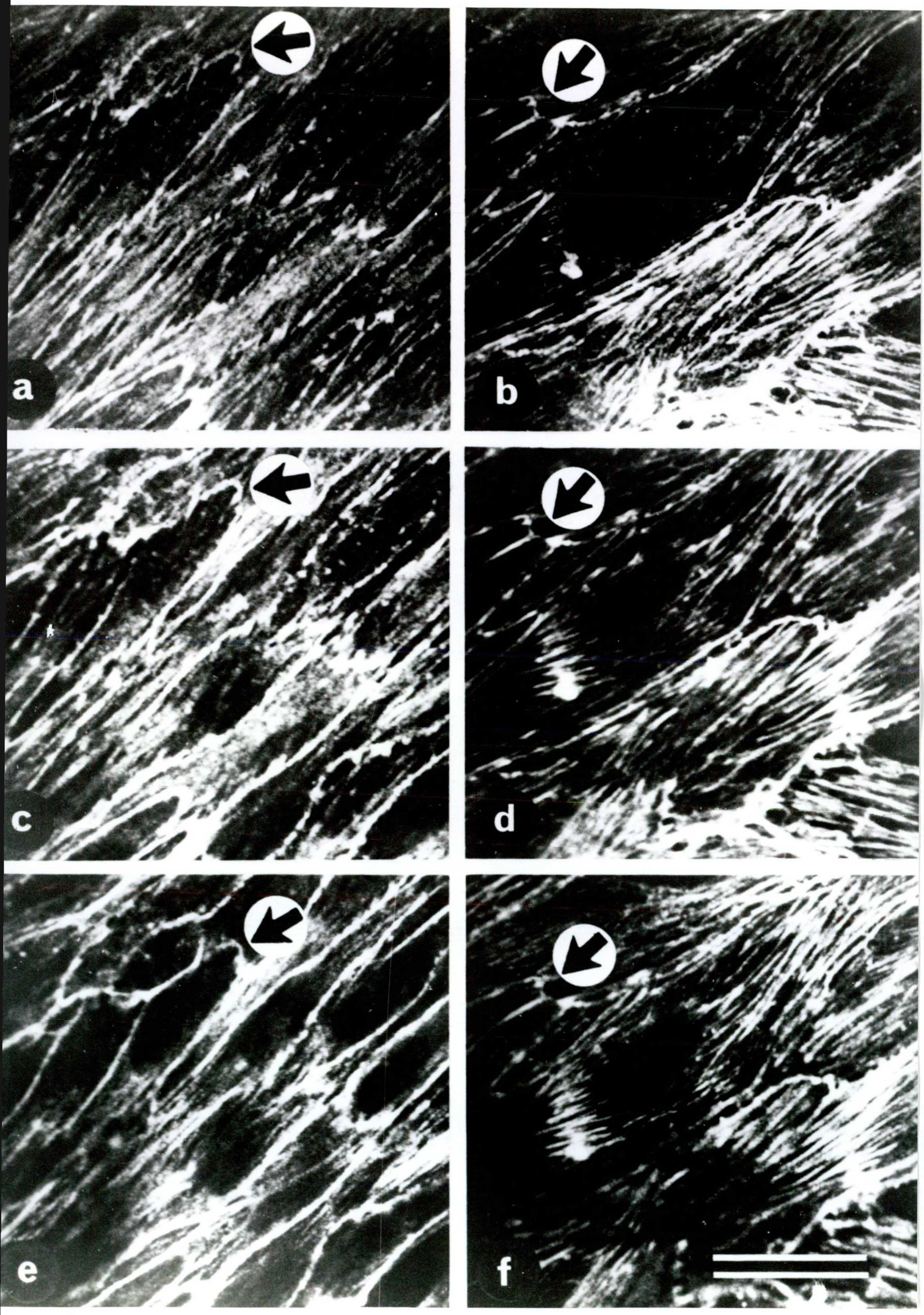


Fig. 6. The effect of intraendothelial cAMP-elevating drugs on flux of ^{14}C-sucrose (**A**) and ^{3}H-inulin (**B**) across monolayers of bovine brain capillary endothelial cells. Experimental groups: A, monolayers cocultured with astrocytes, no further treatment (■); B, monolayers cocultured with astrocytes then treated with CPT-cAMP and RO 20-1724 in the presence of astrocytes (▨); C, monolayers cocultured with astrocytes then treated with DB-cAMP and RO 20-17243 in the presence of astrocytes (□); D, monolayers cocultured with astrocytes then treated with CPT-cAMP and RO 20-1724 in astrocyte-conditioned medium (▨); E, monolayers cocultured with astrocytes then treated with DB-cAMP and RO 20-1724 in astrocyte-conditioned medium (▩); F: monolayers cultured without astrocytes then treated with DB-cAMP and RO 20-1724 (▨). Means ± S.E.M.; n=5. a, b, c, d, e, significant different ($p < 0.05$; ANOVA) from values for A, B, C, D and E, respectively.

Plate II.

Confocal laser beam immunofluorescence localization of actin in BBCECs incubated without (**a,c,e**) or with (**b,d,f**) 250 U/ml of TNF-α. Each panel of pictures consists of serial confocal slices (acquired at 0.5 μm intervals) of a X-Y view of the same field (arrows point to the same position). F-actin thin filaments were diffusely distributed and appear linearly distributed in the cytoplasm of cells incubated in control medium (a,c). However, TNF-α treated endothelial cells displayed thick stress fibers (b,d,f). In contrast, the cortical membrane-associated actin, particularly well observed in (e), was not altered by TNF-α treatment. Bodipy-phallacidin was used to stain actin filaments, as described in Materials and Methods. Bar = 30 μm.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with the sense probe.

The presence of CaM-PK II in the cerebral endothelial cells was confirmed by immunohistochemical visualization and Western blot techniques. Strong immunoreactivity in primary cultures of RCECs was obtained with the use of a monoclonal antibody raised against the 50 kDa α-subunit of CaM-PK II (**Publ. I., Fig. 1d.**). In good agreement with the result of *in situ* hybridization, almost negligible staining was seen in the cerebral endothelial cells when we used a monoclonal antibody specific for the 58/60 kDa β-subunit of CaM-PK II (**Publ. I., Fig. 1e.**). No staining was seen in the controls (**Publ. I., Fig. 1f.**). Western blot analysis confirmed the immunohistochemical observations suggesting that the α-subunit of CaM-PK II is present in the cerebral endothelial cells in considerably higher amounts than the β-subunit (**Publ. I., Fig. 2.; lanes 2 and 4**). The most prominent substrate of calmodulin-dependent phosphorylation was the 50-54 kDa polypeptide (**Publ. I., Fig. 2.; lanes 5 and 7**), which is supposedly identical to the α-subunit of the CaM-PK II (Mackie *et al.*, 1986). This finding is in good agreement with the results of *in situ* hybridization and immunohistochemistry. Phosphorylation of the β-subunit, if it occurs in cultured cerebral endothelial cells, was below the sensitivity of our assay. It is important to mention that in cerebral microvessels, freshly isolated from adult rats, phosphorylation of the β-subunit of CaM-PK II was observed (Oláh *et al.*, 1988) suggesting that β subunit expression may be a maturation phenomenon dependent on the brain tissue environment.

7. DISCUSSION

Model system

The *in vitro* BBB model systems are applicable not only to biochemistry and physiology but also to the drug research, and may contribute to the improvement of the transport of substances through the BBB. The *in vitro* approach has been and should remain an excellent model to help unravel the complex molecular interactions underlying and regulating the permeability of the cerebral endothelium.

In the interpretation of the data, obtained from permeability studies with small molecular weight substances (**Fig. 2.**), it should be recalled that the impermeability of tight junctions and absence of transendothelial pathway represent the cellular basis of the low P_e values for both tracers in the cerebral endothelial cells. Consequently, our results

deriving from the use of hyperosmolar mannitol treatment may suggest that, in contrast to the intercellular junctions of the BAoEC, the tight junctions connecting the BBCEC were able to regain their impermeability with time after a short-lasting opening.

Histamine

Microvessels isolated by centrifugation from brain tissue have been shown to contain histamine, which can be liberated on environmental influences to modulate endothelial permeability (Joó *et al.*, 1981; Joó, 1985; Karnushina *et al.*, 1979). Cerebral microvessels comprise primarily histamine receptors of H_2-type that are linked to the endothelial adenylate cyclase (Karnushina *et al.*, 1980), but the involvement of H_1-receptors has also been noted (Dux *et al.*, 1987). Recent results of Butt and Jones (1992) showed that histamine resulted in a 75% decrease in transendothelial electrical resistance in brain-surface microvessels of rats and caused an increase in BBB permeability which was mediated *via* endothelial H_2-receptors. It has been previously found in a model of neonatal asphyxia (Temesvári *et al.*, 1984), that antihistamines could prevent hypoxic brain ødema in newborn pigs (Dux *et al.*, 1987). Histamine receptor antagonists, not only H_2-type but also H_1-type, have a protective effect on increased BBB permeability and brain ødema formation in animal models (Butt and Jones, 1992; Dux and Joó, 1982; Joó and Klatzo, 1989; Schilling and Wahl, 1994; Tósaki *et al.*, 1994). Protective effect of antihistamines suggested that histamine plays a pathogenetic role in cerebral ødema formation (Edvinsson *et al.*, 1993).

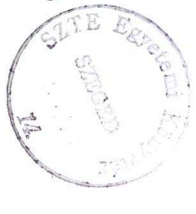
Histamine increased the permeability of the BBB for albumin, but not for markers with smaller molecular weights, known as markers of paracellular route. Our *in vitro* data may suggest a selective induction of transcellular passage of albumin through the BBB. These observations are in accordance with *in vivo* data indicating increased pinocytotic activity after *intracarotid* injection of histamine (Dux and Joó, 1982). As regards the possible molecular mechanisms, it should be mentioned that the effects of histamine were shown to be mediated by both H_2-receptors linked to the microvascular adenylate cyclase (Joó *et al.*, 1975) and H_1-receptors that are coupled with the inositol triphosphate pathway (Revest *et al.*, 1991). In other studies (Rubin *et al.*, 1991; **Publ. I.**), elevated intraendothelial levels of cAMP have been shown to enhance the barrier for the penetration of both sucrose and inulin. However, it awaits further studies to see how elevated cAMP levels can influence the synthesis and/or release of other second messengers, like cyclic GMP, and vasoactive substances, such as nitric oxide.

The finding of a rapid and high affinity uptake of histamine into cerebral endothelium and a rapid efflux of the amine from these cells suggests that endothelium may regulate brain extracellular histamine concentration besides other cell types. Earlier studies of Huszti *et al.* (1990) showed that histamine uptake into astroglial cells is also a carrier-mediated, high affinity, Na^+-dependent and reversible system, which takes part in histamine inactivation. Present results imply similarities between the astroglial and the endothelial uptake of histamine by a carrier operating bidirectionally. Moreover, our preliminary data indicate the presence of a histamine-specific carrier in both the luminal and the abluminal membranes of cerebral endothelial cells with an asymmetrical function on the abluminal (brain) side. In the abluminal membrane of the monolayers, the reverse uptake of histamine might be inhibited, however other explanations of this observation cannot be excluded. A rapid uptake and efflux of histamine by RCECs with a preferential release of histamine to the luminal side indicating an active role for the BBB in the regulation of cerebral histamine level has been observed.

Kovács *et al.*, (1995) found on a model of asphyxiated newborn pigs a rise of histamine content in the venous blood, that may originate from blood corpuscules and endothelium; an early increase in histamine content of CSF, that may be released from intracerebral sources (neuronal elements and/or perivascular mast cells), triggered by, at least in part, from the by-products of severely disturbed brain purine metabolism, (i.e. free radicals) (Temesvári *et al.*, 1990) and a late increase in the histamine content of brain microvessels, that could be derived mainly from the brain intercellular space. We have recently demonstrated (Kondo *et al.*, 1994) the direct effect of oxygen free radicals on BBB permeability. Cerebral endothelial cell monolayers from Cu-Zn superoxide dismutase transgenic mice, which overexpress the enzyme, showed a significant decrease of transendothelial electrical resistance earlier than the control ones in response to free radical challenge elicited by menadione. The elevated microvascular histamine may be connected with triggering of selective albumin transport through the brain microvessels *in vivo* (Ádám *et al.*, 1987). Taken together *in vitro* and *in vivo* data, it may be concluded that in cases of ischemic challenges, cerebral endothelial cells can not only accumulate but also release histamine towards the blood circulation and thereby take part actively in the elimination of histamine from the extracellular space.

TNF-α

To our best knowledge our finding is the first, which document the possible



effects TNF-α exerted directly on the permeability of cerebral endothelial cells after exposure to the luminal membrane in an *in vitro* reconstituted BBB model. During the *early phase* (up to 4 h), TNF-α exposure in all concentrations, varying from 50 to 500 U/ml, did not result in significant change in the [^{3}H]-inulin clearance. On the other hand, [^{14}C]-sucrose clearance was significantly ($p<0.05$) decreased 120 and 240 min after the beginning of challenge. However, 16 h after 1-h exposure to TNF-α (*delayed phase*), an enhancement in transendothelial flux for both tracers was observed. Concomitantly, cytoskeletal changes, such as reorganization of F-actin, but not of vimentin filaments, were observed 24 and 48, but not 4 h after the TNF-α challenge.

TNF-α exposure did not change significantly [^{14}C]-sucrose or [^{3}H]-inulin clearance up to 4 h, i.e. during the *early phase*. This result indicates that TNF-α has no influence on the tight junction permeability and the transendothelial passage of these small molecular weight substances in either concentrations examined. The decrease of [^{14}C]-sucrose permeation observed at 2 and 4 h after 1-h exposure to 500 U/ml TNF-α indicates also the tightening of intercellular junctions. The molecular basis of this interesting effect should be elucidated in further studies; at present it can only be assumed on the basis of data of literature (Stelzner *et al.*, 1989; Rubin *et al.*, 1991, **Publ. IV.**) that it may be related to the activation of microvascular adenylate cyclase. On the other hand, [^{3}H]-inulin clearance did not change significantly during the *early phase*, which is in agreement with the result of Gutierrez *et al.* (1993), showing no change for albumin (m.w. 67 kDa) after TNF-α exposure. However, it should be emphasized that the molecular weight of inulin is one order of magnitude higher (5 kDa) than that of sucrose (m.w. 342 Da), and therefore, it is not considered to be a selective tight junction permeability tracer substance, but may reflect the functional state of a cell barrier integrity.

Although certain endothelium-damaging effects of TNF-α have been reported in endothelial cells derived from the periphery (Shimada *et al.*, 1990) and the brain tissue (Terada *et al.*, 1992), transport studies, however, have not yet been carried out in BBCEC cocultured with astrocytes. As we have documented, TNF-α did not influence permeability to small molecular weight tracers in the *early phase*, but 18 h after 1-h cytokine exposure to the luminal membrane of BBCEC (*delayed phase*) it significantly increased the permeability for both tracers. As regards what second messengers could mediate this delayed effect of TNF-α, it may be possible that the recently discovered sphingomyelin pathway is involved (Kolesnick and Golde, 1994), since brain microvessels

have been shown to contain high amount of neutral sphingomyelinase (Carré *et al.*, 1989). In renal epithelial cells, in contrast to our findings, TNF-α has been shown to induce a rapid and reversible paracellular leakage, which was followed by a long-term increase in transepithelial resistance (Mullin and Snock, 1990; Mullin *et al.*, 1992; Marano *et al.*, 1993).

There is a growing interest in understanding the regulated changes in the actin cytoskeleton that occur in response to extracellular factors. The observations resulting from our studies indicate that TNF-α induces a delayed reorganization of F-actin to form stress fibers in highly differentiated cerebral endothelial cells. It seems important to underline that all staining studies were made with the same filter-grown endothelial cells used in the permeability experiments. It is of particular interest that the stabilization buffer employed for actin staining results in good preservation of all cytoskeletal fibers and permits to obtain reproducible results following paraformaldehyde fixation.

BBCEC used in the study form a pertinent physiological endothelial monolayer (Dehouck *et al.*, 1992), the principal actin structures observed include the cortical actin network and intracytoplasmic actin fibers. Using bodipy-phallacidin, together with antibodies to vimentin, we have documented that TNF-α exposure, even in 500 U/ml concentration, had no influence on the localization pattern of cell fibrous components of the cytoskeleton during the *early phase*, i.e. up to 4 h. These results could not be accounted for inactivation of TNF-α, which is routinely tested on L929 cells in the presence of actinomycin D, as described (Robbins *et al.*, 1987). Profound changes in permeability and actin rearrangements after exposure to TNF-α were observed with a variety of endothelial cells isolated from vessels of peripheral origin (Brett *et al.*, 1989; Kohno *et al.*, 1993).

In the present study, the TNF-induced formation of stress fibers was evidenced in a time- and dose-dependent manner, but no physical disruption of the actin filaments in the cell cortex was observed. The cellular distribution of the cortical actin network is consistent with the idea that membrane-associated actin confers rigidity on the cell membrane, and thus account for the absence of morphological changes in BBCEC. Although we observed a dramatic formation of stress fibers in TNF-treated BBCEC at the delayed phase, there appeared to be no comparable rearrangement of intermediate filaments of vimentin. Growth factors or readdition of serum have been shown to regulate the formation of actin stress fibers in Swiss 3T3 cells (Ridley and Hall, 1992). These studies provide possible role for lysophosphatidic acid, bound to serum albumin, in

reorganization of actin. Because of this property, we have shown that reincubation of non-treated BBCEC monolayers replaced to their original, astrocytes-containing wells, in medium for additional 18 and 42 h, did not induce any detectable alteration to actin filaments. Surprisingly, we have demonstrated that bovine recombinant γ-interferon (IFNγ) leads to a pronounced reduction of cytoplasmic F-actin staining, whereas it has no effect on the staining of vimentin in the same monolayers exposed for 18 h (data not shown). In this context, it is interesting to note, that cortical membrane associated actin remains prominent. Thus, the present findings suggest that IFNγ may therefore act through a different signal transducer to elicit a pronounced reduction of cytosolic F-actin staining. It is tempting to speculate, that TNF-α might activate the rearrangement of stress fibers by regulating intracellular Ca^{2+} levels (Aderem, 1992), or other actin-binding proteins (Finkel *et al.*, 1994; Gumbiner, 1993). In this context, the presence of CaM-PK II, as one of the possible targets of calcium actions, has been detected (**Publ. I.**) in the brain endothelial cells. Although we cannot exclude a role of some other factors in inducing finer or transitory changes within the cell membrane underlying cortical actin network. Obviously, additional experiments are required to determine the precise mechanism by which TNF-α participates in the endothelial activation and the changes in microfilamentous system.

The effects of TNF-α on endothelium of peripheral origin has been extensively studied, since this cell type is one of the main target cells of the cytokines. It has been shown (Brett *et al.*, 1989; Camussi *et al.*, 1991; Kohno *et al.*, 1993) that TNF-α increase or modulate the permeability, and influence cytoskeletal organization in endothelial cells isolated from vessels of peripheral origin. In another study, Goldblum *et al.* (1990) reported on the augmentation by TNF-α of transendothelial albumin flux through bovine pulmonary endothelial cell monolayers. Similar results were obtained on vascular permeability of BAoECs (Royall *et al.*, 1989; Brett *et al.*, 1989). Langeler *et al.* (1991) published an interesting observation on endothelial cells derived from human umbilical artery: during short-term incubation (< 4 h), TNF-α induced a reduction in the passage rate of macromolecules, whereas during prolonged incubation (up to 24 h) the permeability of monolayers increased significantly at a concentration of 500 U/ml. In contrast to these data, endothelial cells from human aorta and vena cava did not respond to TNF-α challenge (Langeler *et al.*, 1991).

As regards to the effects of TNF-α on cerebral endothelial cells, however, much less information is available. At first, adhesion of lymphocytes to the cerebral endothelium

was described (Hughes *et al.*, 1988; Male *et al.*, 1990, 1992) and the validity of results were confirmed by Fábry *et al.* (1992). In another experiment, Yoshida *et al.* (1989) observed that high doses of TNF-α inhibited cell proliferation and caused morphologic alterations in cultured microvascular endothelial cells derived from gerbil brain. Later, Terada *et al.* (1992) described that TNF-α (50 U/ml) caused a free oxygen radical-mediated lactate dehydrogenase release in cultured BBCECs. As it turns out from the comparison of endothelial cells of CNS to that of peripheral origin, the reactivity to TNF-α-exposure of cells from various origin is different. BBCEC seemed to be more resistant to TNF-α challenge during the *early phase* of exposure than the endothelial cells of peripheral origin.

Whether or not exposure to TNF-α of abluminal ("brain") side of cerebral endothelial cells evokes similar changes in the permeability and intracytoplasmic localization of F-actin filaments, further studies are required.

cAMP

Our data confirm the validity of previous findings obtained from *in vitro* culture systems (Kempski *et al.* 1987; Rubin *et al.* 1991) and indicate that cAMP can rapidly reduce sucrose and inulin flux through the monolayers, i.e. after a 1-h exposure. It was previously shown that a 1 h addition of CPT-cAMP in the presence of phosphodiesterase inhibitor resulted in a two-to-threefold increase in cAMP levels in cerebral endothelial cells (Rubin *et al.* 1991). In our study, intraendothelial elevation of cAMP was able to reduce the transport of small molecular weight substances even through monolayers not cocultured *in vitro* with astrocytes. This observation indicates that BBCEC monolayers alone can provide a certain permeability barrier to the tracers studied, but astrocytic influences can further strengthen the tightness of the intercellular junctions.

To explain the difference between the results of *in vivo* and *in vitro* experiments reported on the effect of cAMP on the permeability of cerebral endothelial cells, the following points should be taken into consideration. Recent results of Warren *et al.* (1993) draw the attention to the fact, that agents that increase concentration of cAMP can either promote or inhibit the development of inflammatory ødema despite acting *via* this common second messenger. According these findings, increasing intracellular cAMP concentrations in vascular smooth muscle cells promotes ødema *via* increased blood flow, whereas increasing cAMP concentrations in endothelium may supress ødema by enhancing the permeability barrier. Contribution of cAMP to the regulation of cerebral

vascular tone has been recently documented in pial arterioles by Parfenova *et al.* (1993). This opposing role of cAMP may be responsible, at least in part, for the dissimilarity obtained on cAMP effects in *in vivo* and *in vitro* studies.

Dibutyryl cGMP (DB-cGMP) increases the permeability of brain microvessels to albumin, possibly by increasing pinocytotic activity (Joó *et al.*, 1983), suggesting its involvement in the regulation of transcellular transport mechanisms. Vigne *et al.* (1994) has described a cross talk between cAMP and cGMP formation mechanisms in cloned RCECs. They found that high intracellular cAMP levels elevated cGMP level supposedly *via* the competitive prevention of cGMP degradation by nonspecific phosphodiesterases, thereby cAMP enhanced the actions of agonists of guanylate cyclases. Another explanation for the observation that DB-cAMP increases pinocytotic activity in brain capillaries (Joó, 1972) could be that the effects of DB-cAMP on albumin transport were mediated by cGMP.

CaM-PK II

Activation of cell surface receptors by several hormones, neurotransmitters (e.g. excitatory amino acids) and other external stimuli evoke changes in Ca^{2+} fluxes leading to an increase in cytosolic Ca^{2+}, an acknowledged mediator of numerous cell responses. High intracellular Ca^{2+} concentration has been reported to be responsible for eliciting an increase in the permeability of the endothelial barrier in microvessels of peripheral origin (Curry, 1992). Increases in cytosolic free Ca^{2+} concentration were observed in response to histamine, bradykinin and ATP, substances known to increase the permeability of BBB, in cultured RCECs (Revest *et al.*, 1991). In addition, brain endothelial cells were shown with the use of sensitive oxalate-pyroantimonate electron microscopic detection to accumulate calcium under pathological conditions like brain ooedema evoked by kainic acid (Sztriha and Joó, 1986). However the detailed mechanism of the Ca^{2+} action remains largely unknown. Our finding that the Ca^{2+}/calmodulin-stimulated enzyme, CaM-PK II is present in cerebral endothelial cells raises the possibility that this enzyme, besides its possible involvement in the physiological function of these endothelial cells such as facilitated transport of nutrients, may play a role in BBB opening during the pathogenesis of brain ooedema.

General considerations

Tight junction resistance and vascular permeability are regulated by cyclic

nucleotides, and second messengers originating from the hydrolysis of phosphatidylinositol by phospholipase C. Cyclic AMP decreases the BBB permeability by narrowing tight junctions, as evidenced by large increases of electrical resistance across cell monolayers (Rubin *et al.*, 1991; **Publ. IV.**). Cyclic GMP has the opposite effect: it increases vascular permeability. Sodium nitroprusside, a nitric oxide donor molecule that activates soluble guanylate cyclase and atrial natriuretic peptide decreases the resistance across BBCEC monolayers (Rubin *et al.*, 1991), and DB-cGMP enhances macromolecular transport (Joó *et al.*, 1983). The potentiating action of cAMP on cGMP formation in RCECs (Vigne *et al.*, 1994) may link together the controls of paracellular and transcellular diffusion pathways. When paracellular diffusion is reduced by cAMP, transcellular diffusion could be increased by cGMP, thus amplifying the cell control of blood-to-brain exchanges.

The rapid increase in transendothelial resistance and decrease in BBB permeability could be mediated by protein kinase A (PK A), *via* the phosphorylation of one or more proteins important in regulating the resistance of tight junctions. While cAMP levels remain elevated, PK A must act to keep regulatory proteins in a phosphorylated state in spite of the presence of active phosphatases. Accordingly, protein kinase inhibitors decreased the transendothelial resistance in brain endothelial cells (Rubin *et al.*, 1991) in the presence of elevated cAMP.

Primary cultures of RCEC expresses several isoforms of protein kinase C (PK C) (**Publ. VII.**), a key regulatory enzyme involved in both signal transduction and cellular proliferation. Beside PK C-α and -δ, thought to be universally occurring enzymes, PKC-β, PK C-epsilon and -eta are also present in the cells. As regards the possible function of PK C in cerebral endothelium, phorbol esters were shown to increase hexose uptake (Drewes *et al.*, 1988). Phorbol myristate acetate significantly stimulated fluid phase endocytosis in BBCEC (Guillot *et al.*, 1990). Another important function of PK C may be the regulation of cell growth, since tumor-promoter phorbol esters stimulated BBCEC growth through high-affinity receptors in a dose-dependent manner (Daviet *et al.*, 1989). In the cerebral microvessel fraction several proteins were phosphorylated with a different time course by the addition of purified PKC (Oláh *et al.*, 1988). Since PK C isoenzymes show differences in subcellular localization, Ca^{2+}-dependence, and responsiveness to phospholipid metabolites, the presence of different isoforms may confer a sophisticated intracellular regulatory mechanism to the brain endothelial cells. Further experiments may explore its role in the regulation of BBB permeability.

8. PERSPECTIVES

During the last 15 years the tissue culture approach has become a suitable model system for studying the BBB *in vitro* (Joó, 1992), and has led to a better understanding of the exact molecular basis of permeability regulation in the cerebral endothelium. It can be predicted that, beside basic research application, this new generation of the BBB model systems will be used more frequently in drug research. Results obtained on cultured brain endothelial cells of human origin can be valuable (Kása *et al.*, 1991; Joó, 1992).

Looking at clinical areas of possible application, it is crucial to enhance the penetration of nutrients (glucose, amino acids, choline etc.) and therapeutic substances through the cerebral endothelium for treatment of several diseases in the CNS. Several drug delivery strategies are under investigation and seem to be promising. The synthesis of more lipophilic analogues is one of them, since increased lipid solubility provides enhanced transport. Other approaches are the use of specific carrier systems, encapsulation of drugs into liposomes, coupling of drugs to brain vectors as anti-transferrin receptor antibody OX-26, cationized or glycosylated albumin, cationized IgG. An efficient delivery system designed to enhance the penetration of compounds into the CNS might be based on agents that selectively decrease cAMP activity in brain endothelial cells.

Prevention and treatment of brain oedema, inflammatory and immunological diseases of the brain are also important tasks from clinical point of view. Histamine receptor blockers, cyclooxigenase inhibitors, calcium channel blockers and free radical scavangers proved to be effective in the prevention of cerebral oedemas (Joó and Klatzo, 1989). The use of specific antibodies directed against adhesion molecules on the cerebral endothelial cell surface (ICAM-1, VCAM-1) or their leucocytic counterpart (Mac-1, VLA-4) may suppress the onset and progression of several cerebral inflammatory diseases. The administration of interleukin-1 and TNF-α receptor antagonists, cytokine-specific antibodies or drugs, which down regulate the release of cytokines, may also protect the CNS.

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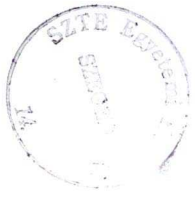

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