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EXPRESSION OF CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE II AND PROTEIN KINASE C FAMILY MEMBERS IN CEREBRAL ENDOTHELIAL CELLS

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1. PUBLICATIONS RELATED TO THE THESIS

Full length papers

I. **I. Krizbai**, M. .Deli, I. Lengyel, K. Maderspach, M. Pákáski, F. Joó, J. R. Wolff: 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).

II. M. A. Deli, F. Joó, **I. Krizbai**, I. Lengyel, G. M. Nunzi, J. R. Wolff: Calcium/calmodulin Stimulated Protein Kinase II is Present in Primary Cultures of Cerebral Endothelial Cells. *J. Neurochem.* **60**, 1960-1963. (1993).

III. **I. Krizbai**, G. Szabó, M. Deli, K. Maderspach, Z. Oláh, CS. Lehel, J.R. Wolff and F. Joó: Expression of Protein Kinase C Family Members in the Cerebral Endothelial Cells. *J. Neurochem.* **65**, 459-462. (1995).

Abstracts

IV. F. Joó, M.A. Deli, **I. Krizbai**, I. Lengyel, G.M. Nunzi, J.R. Wolff: Second Messenger Systems in the Cerebral Endothelial Cells: Calcium/calmodulin Dependent Protein Kinase II. XVI. International Symposium on Cerebral Blood Flow and Metabolism, Sendai, Japan. *J. Cereb. Blood Flow Metab.* **13**, (Suppl.1) S23. (1993).

V. M.A. Deli, **I. Krizbai**, I. Lengyel, M.G. Nunzi, J.R. Wolff and F.Joó: Calcium/calmodulin Stimulated Protein Kinase II is Present in Primary Cultures of Cerebral Endothelial Cells. Molecular Mechanisms Regulating the permeability of the Blood-Brain Barrier: An International Workshop. *Neurobiology*, **1**, 274. (1993).

VI. M.A. Deli, **I. Krizbai**, I. Lengyel, F. Joó: Study on Calcium/calmodulin Stimulated Protein Kinase II in Primary Cultures of Cerebral Endothelial Cells. Fourteenth Meeting of the International Society for Neurochemistry, Montpellier, France. *J. Neurochem.* **61**, (Suppl.) S218D. (1993).

VII. **I. Krizbai**, G. Szabó, M. Deli, K. Maderspach, Z. Oláh, C. Lehel and F. Joó: PKC isoforms in cerebral endothelial cells. First Congress of the Hungarian Neuroscience Society, Pécs, January 27-29, 1994. *Neurobiology*, **2**, 78. (1994).

VIII. **I. Krizbai**, G. Szabó, M.A. Deli, K. Maderspach, Z. Oláh, C. Lehel and F. Joó: PKC isoforms in cerebral endotheliaL cells. Tenth Meeting of the European Society for Neurochemistry, Jerusalem, Israel. *J. Neurochem.* **63**, (Suppl1) S76C, (1994).

IX. F. Joó, M. Deli, **I. Krizbai**, A. Pestenácz and L. Siklós, Effects of glutamate on cultured cerebral endothelial cells. Tenth Meeting of the European Society for Neurochemistry, Jerusalem, Israel. *J. Neurochem.* **63**, (Suppl1) S49D, (1994).

X. **I. Krizbai**, G. Szabó, M. Deli, K. Maderspach, Z. Oláh, CS. Lehel, J.R. Wolff and F. Joó: Expression of PKC Isoforms in Cerebral Endothelial Cells. Second Congress of the Hungarian Neuroscience Society, Szeged 1995. *Neurobiology*, **3**, 89. (1995).

XI. **I. Krizbai**, G. Szabó, M. Deli, K. Maderspach, Z. Oláh, CS. Lehel, J.R. Wolff and F. Joó: Expression of PKC Isoforms in Cerebral Endothelial Cells. Fifteenth Meeting of the International Society for Neurochemistry, Kyoto, Japan. *J. Neurochem.* **65**, Suppl. S96C (1995).

2. RATIONALE

The cerebral endothelium is a single-cell layer lining the blood vessels of the brain and constitutes the principal component of the blood-brain barrier. By forming an active interface between blood and neuronal tissue it plays a key role in the maintenance of the homeostasis of CNS. Due to its important physiological function and involvement in pathological processes several attempts have been made to elucidate the molecular mechanisms, by means of which the permeability is regulated in the cerebral endothelium. The direct study of the endothelial cells has been rendered possible by the development of new methods like the isolation of cerebral microvessels, culturing of cerebral endothelial cells (CEC) or establishing immortalized cerebral endothelial cell lines. From these studies it turned out that the cerebral endothelial cells, like many other cell types, are equipped with a complex set of membrane receptors linked to different signal transduction pathways, which makes them reactive to different extracellular stimuli. However, due to its specialized function which differentiates it from other types of endothelia and epithelia, the analysis of components specific for the cerebral endothelial cells is crucial to understand the cell biology of the blood-brain barrier. Until recently only a few data were available about the expression of different key enzymes of the signal transduction in the CEC and the molecular characterization of their expression is almost completely lacking.

3. AIMS

At the beginning of our studies, the first goal was to introduce molecular techniques (in situ hybridization, PCR) into the study of signal transduction in the endothelial cells. Since protein phosphorylation plays a central role in the processing of the extracellular signal we turned our attention towards the expression of two key phosphorylation enzymes, calcium/calmodulin dependent protein kinase II (CAM-PK II) and protein kinase C (PKC).

The function of CAM-PK II has been well documented in the nervous tissue, but no data were available about its presence and possible role in cerebral endothelial cells. To get an insight into the mechanisms which may regulate the function of CAM-PK II under physiological and pathological conditions we decided to investigate its level of phosphorylation in response to glutamate, a substance which has been shown to play a key role in the initiation of ischemic brain injury.

The presence of the other key phosphorylation enzyme, the PKC, in the cerebral endothelium has already been demonstrated (Markovac and Goldstein, 1988). However, PKC consists of a family of isoenzymes with at least ten members with different distribution, sensitivity to calcium and phorbol esters. Our goal was to investigate which PKC isoforms are present in CEC and to compare the expression pattern in endothelial cells of different origin to determine the isoforms specifically expressed in the cerebral endothelium.

4. INTRODUCTION

Neuronal tissue due to its complex structure and function requires an activity dependent and finely tuned level of ions, nutrients and macromolecules and is very sensitive to changes in the extracellular environment. Paul Ehrlich's experimental observation that the CNS is not stained by intravascular water-soluble dyes provided the first demonstration that the entry of different substances to the CNS is restricted (Ehrlich, 1885). Later this discovery opened the era of the blood-brain barrier concept. According to this concept the walls of the cerebral microvessels represent a cellular interface between blood and brain with specialized physiological, biochemical and morphological properties. Now it is generally believed that apart from a few exceptions where the restricted movement of certain substances is due to an impermeable glial sheath (Cserr and Bundgaard, 1984; Abbott., 1991), the cerebral endothelial cells (CEC) make up the structural basis of the blood-brain barrier (BBB), a term first used by Stern and Peyrot (1927) and regulate the entry of solutes and macromolecules from the blood circulation to the brain tissue in the majority of vertebrates.

Since the advent of novel procedures, which made possible not only the isolation of microvessels from the brain tissue (Joó and Karnushina, 1973) but also the culturing of cerebral endothelial cells (Joó, 1992), a considerable amount of new information has been gained in respect to the basic chemical, biochemical and physiological properties of the blood-brain barrier. Subsequent studies (for reviews see Joó, 1985, 1986, 1992), carried out either on freshly isolated brain microvessels and cultured CEC, or more recently on immortalized cerebral endothelial cell lines revealed that the microvascular endothelium is equipped with specific receptors for vasoactive substances and neurotransmitters making the blood vessels responsive to specific changes of pericapillary microenvironment. Similarly to many other cell types, CEC have been shown to contain certain receptors (Karnushina *et al.*, 1980, Spatz, 1986) linked to different signal transduction pathways

and second messenger molecules, which in turn may induce changes in the permeability of the CEC like cAMP (Joó, 1972; Rubin *et al.*, 1991) and/or cGMP (Joó *et al.*, 1983).

Changes in intracellular Ca^{2+} concentration, which can be induced by different stimuli (Hess *et al.*, 1989, Hess *et al.*, 1991, Revest *et al.*, 1991) -similarily to many other cell types- regulate a large number of endothelial cell functions. One of the key molecules mediating the effect of Ca^{2+} is CAM-PK II also called multifunctional CaM kinase because of its broad substrate specificity. Isoenzymes of CAM-PK II are widely distributed in nature: in mammalian tissues, like brain, liver, skeletal muscle, heart, pancreas, retina, lung, intestinal brush border and testis as well as in neural tissues of Aplysia, electric eel, squid and Drosophyla (reviewed by Colbran *et al.*, 1989, Walaas and Greengard, 1991). 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). CAM-PK II constitutes up to 2% of total brain protein and has been established to play an important role in neurotransmitter release (Nicoll *et al.*, 1990), organization of postsynaptic receptors, long term potentiation and memory formation (Lisman *et al.*, 1988). In the nervous system the enzyme is composed of α and β subunits which forms an active holoenzyme with different subunit ratios. Inspite of its importance in neurons and astrocytes no data were available about its presence and function in cerebral endothelial cells. One of the most important biochemical properties of CAM-PK II is that when the intracellular Ca^{2+} concentration raises above a threshold level the enzyme undergoes autophosphorylation which in turn leads to the loss of its Ca^{2+} dependency (Hashimoto *et al.*, 1987).

In the CNS glutamate, the most widespread neurotransmitter, has been shown to play an important role in regulating the intracellular Ca^{2+} level by acting through different glutamate receptors (Westbrook and Jahr, 1989). Activation of NMDA receptors can lead to a thousandfold increase of the intracellular Ca^{2+} concentration which in turn may trigger the autophosphorylation of CAM-PK II in neurons. Since cerebral endothelial

cells have been shown to possess glutamate receptors (Koenig *et al.*, 1992) the question arises, if present in the CEC, how can be CAM-PK II regulated by glutamate.

One of the other candidate molecules mediating the effect of Ca^{2+} is protein kinase C (PKC), a key regulatory enzyme involved in both signal transduction and cellular proliferation (Tanaka and Nishizuka, 1994). The presence and translocation of PKC in microvessels isolated from rat brain were observed in response to phorbol esters as well as to transforming growth factor β (Markovac and Goldstein, 1988), a widely distributed regulatory peptide that promotes endothelial cell differentiation. In addition, stimulation of PKC translocation was found by Catalán *et al.* (1989) in isolated brain microvessels on the effect of Substance P. It has been shown that under basal conditions 85% of the PKC activity is in the cytoplasmic fraction and substance P cause a significant translocation to the particulate (membrane) fraction. The finding suggested that Substance P may be involved in the regulation of processes underlying protein phosphorylation in the BBB. Molecular cloning and biochemical studies have revealed, that PKC consists of a family of isoenzymes with at least ten members with different distribution, sensitivity to calcium and phorbol esters. PKC-α, -βI, -βII as well as PKC-γ are conventional isoforms, whereas PKC-δ, -ε, -η and -θ are novel PKC related protein kinases (Hata *et. al.*, 1993). In addition, two atypical isoforms -ζ and -λ were more recently characterized. The conventional isoforms are calcium and phospholipid dependent, whereas the novel isoforms do not require calcium for their activation. PKC-ζ is both Ca^{2+} and phorbol ester independent, and can be activated by phosphatidyl serine (for review see Tanaka and Nishizuka, 1994).

The distribution of the different isoforms has been characterized in the central nervous system and several other tissues, but the PKC expression pattern in endothelial cells has been unknown so far.

Since certain family members of PKC may mediate different cellular functions, we decided to study with PCR technology the expression pattern of 7 PKC isoforms in brain microvessels and cultured cerebral endothelial cells comparing the results to those

obtained with aortic endothelium and brain homogenate. The studied probes were designed to represent all the three known PKC subclasses: (i) PKC-α, -β and -γ for conventional, (ii) PKC-δ, -ε, and-η for novel, and (iii) -ζ for atypical PKC isoforms.

5. MATERIAL AND METHODS

5.1 Isolation of microvessels from brain tissue

A fraction of isolated capillary vessels was purified from the grey matter of the rat cerebral cortex by the micromethod described by Joó and Karnushina, (1973). Prior to decapitation, the anaesthetized animals were perfused through the heart with 100 ml of Krebs-Ringer solution. Then, the cortex tissue was homogenized manually through nylon sieves (three times through a sieve of 250 m pore size, followed by three times through a sieve of 110 mm pore size), suspended in 10 vol. of 0.25 M-sucrose, 50 mM-Tris-HCl, pH 7.2, 5 mM EDTA, and centrifuged at 1000 g for 10 min; the pellet was thoroughly suspended in 0.25 M-sucrose buffer and centrifuged repeatedly. The final pellet was suspended in 3 vol. of 0.25 M-sucrose and placed on a stepwise sucrose gradient, 1.0 M, 1.3 M, and 1.5 M (6 ml each) and centrifuged in a Beckman SW 25.I rotor at 20,000 r.p.m. at 4°C for 30 min. The capillaries were recovered from the bottom of the tube and checked for purity by light and electron microscopy.

5.2 Primary cultures of cerebral and aortic endothelial cells

Cultures were prepared, as described in detail earlier (Deli *et al.*, 1993), from 2-week old rats, maintained in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum, 2.0 mM glutamine, 10 mM Hepes and antibiotics at 37°C in a humidified atmosphere of 5% CO and 95% air. To obtain pure cultures of brain

endothelial cells we performed complement killing (Risau *et al.*, 1990). After 8-10 days, endothelial cells developed a monolayer, which were used for the experiments.

5.3 Immortalized RBE4 cell cultures

An immortalized cell line was produced (Durieu-Trautmann *et al.*, 1993) by transfection of primary CEC with the plasmid pE1A-*neo*, containing the adenovirus E1A encoding sequence followed by the neomycin resistance gene. One clone, called RBE4, has been characterized in detail (Roux *et al.*, 1994) and found to retain the main features of the CEC kept in primary cultures, so this cell line seems to provide an ideal model to study the BBB *in vitro*.

5.4 In situ hybridization

Synthetic oligonucleotide probes complementary to the nucleotides 1439 - 1470 of the α-subunit mRNA and nucleotides 1090 - 1128 of the β-subunit mRNA of the calcium/calmodulin dependent protein kinase II (CAM-PK II) were synthesized and purified. The sequence is not present in the mRNA of other known subunits of CAM PK II (Burgin *et al.*,1990). A sense probe was also synthesized and used for control experiments. Oligonucleotides were 3-end labeled with DIG 11-dUTP (Boehringer) by terminal deoxynucleotidyl transferase (Boehringer) according to the following protocol.

About 100 pmol oligonucleotides were incubated in 50 ml solution containing 1 x tailing buffer (5 x tailing buffer contains potassium cacodylate 1 mol/l, TrisHCl 125 mmol/l, BSA 1.25 mg/ml, pH=6.6), $CoCl_2$ 2 mmol/l, dATP 0.2 mmol/l, DIG 11-dUTP 20 mmol/l and terminal transferase 50 units for 1 hour at 37^0 C. The labeled probe can be used without further purification.

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 the cultures were 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 mg/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 hour and incubated with 200 µl of hybridization solution (50% formamide, 4'SSC, 1'Denhardt solution, 5% dextrane sulfate, 0.5 mg/ml salmon sperm DNA and 0.25 mg/ml yeast tRNA) containing the labeled probe in a concentration of 100 ng/ml. Hybridizations were performed for 12 - 16 hours at 37°C in a humidified chamber. Following hybridization the coverslips were washed with 2XSSC (1 hour), 1XSSC (1 hour), 0.5XSSC (30 min) and 0.2XSSC (30 min). The immunological detection was carried out with anti-digoxigenin polyclonal antibody conjugated with alkaline phosphatase using a DIG-DNA-Detection kit (Boehringer) and following the manufacturer's recommendations.

5.5 Immunocytochemistry

Cultures grown on collagen-coated glass coverslips from the same separation than those used for in-situ mRNA hybridization were processed in parallel for immuno-histochemistry. All the steps of the procedure were undertaken at room temperature. Following a brief wash 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% bovine serum albumin (BSA) in PBS. Coverslips were first incubated for 2 hours 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 hour with biotinylated goat anti-mouse IgG [1:1000] (Amersham), finally for another hour with streptavidin conjugated alkaline phosphatase [1:1000] (Boehringer), all reagents were diluted in blocking buffer. Coverslips used as controls were identically treated except that primary antibodies were omitted. Extensive washing in PBS, 3 times 5 min each, was repeated between and after these

steps. For the detection of immunostaining, we used alkaline phosphatase color solution (0.41 mM nitro blue tetrazolium chloride and 0.40 mM 5-bromo-4-chloro-3-indolyl phosphate in buffer containing 100 mM Tris-HCl, 100 mM NaCl and 10mM $MgCl_2$, pH 9.5)

5.6 Western blot analysis

Protein samples (0.5 μg of purified kinase and 100 μg of endothelial cell homogenate per lane) were separated by sodium dodecyl sulfate-polyacrylamidegel electrophoresis 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 and then preincubated in blocking buffer (2% nonfat dry milk powder in PBS, containing 0.1% sodium azide) for 2.5 hours. 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 a- and b-subunit specific monoclonal antibodies [1:4000 and 1:2000], respectively. This was followed by 1 hour incubation with biotinylated goat anti-mouse IgG 1:1000 (Amersham), finally for 30 min with streptavidin conjugated alkaline phosphatase [1:1000] (Boehringer) at room temperature, with all reagents being diluted in blocking buffer. The immunoreactive bands were visualized by alkaline phosphatase colour solution as described above. The reaction was stopped by placing strips in 50 mM EDTA solution. Membrane strips were rinsed in distilled water and air-dried.

5.7 Protein phosphorylation

The experiments were carried out at day in vitro 7 on confluent rat brain endothelial cultures grown on petri dishes coated with collagen. The cells were washed with PBS and homogenized in 50mM Tris-MES buffer (pH 7.4) with a glass teflon homogenizer. Protein concentration was measured according to Bradford (1976). Protein extracts from each sample

were incubated in 50 µl assay medium containing 25 mM Tris-MES buffer, 2 mCi [γ-^{32}P]ATP and 2 mM EGTA or Ca^{2+} (100 mM) and bovine brain calmodulin (2 mM) for 2 min. The incubations were stopped by addition of SDS sample buffer, and the proteins were separated by 10% SDS-PAGE as described by Laemmli (1970). For autoradiography the gels were dried and exposed to X-ray films (Kodak XAR-5) for 3 days using intensifying screens. Autoradiograms were quantified using a laser densitometer (Pharmacia LKB). To study the effect of glutamate cerebral endothelial cells cultures were exposed to 2 mM glutamate for 30 min., then possible changes in phosphorylation pattern were checked at 0, 10 and 60 minutes recovery time after exposure as described above.

5.8 Preparation of RNA and PCR analysis

Total RNA from rat brain, freshly isolated brain microvessel fraction, primary cultures of rat brain endothelial cells, cultured aortic endothelial cells and an immortalized rat brain endothelial cell line was extracted according to Chomzynsky and Sacchi (1987). Briefly, samples were homogenized in 0.5 ml denaturing solution containing 4M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sodium lauroylsarcosine, 20mM DTT. After homogenization 0.05 ml sodium acetate (2M, pH 4.5), 0.5 ml phenol (water saturated) and 0.1 ml chloroform-isoamyl alcohol mixture (49:1) were added to the homogenate, vortexed and kept on ice for 15 min. Samples were centrifuged at 10.000 g for 20min. The aqueous supernatant was transferred to a fresh tube and the RNA was precipitated with equal amount of isopropanol at -20^{0}C. Following a second precipitation step the RNA pellet was washed with ethanol and dissolved in sterile water.

Five µg RNA from each sample was transcribed into first strand cDNA with a Superscript reverse transcription kit (GIBCO-BRL). Oligonucleotide primers specific for each PKC isoform were synthesized and used for amplification. The primers, specific for different isoforms, were designed on the basis of previous data (Freire-Moar *et al.*, 1991) and had the following sequence:

for **PKC-α**: 5'-TGAACCCTCAGTGGAATGAGT

and 5'-GGCTGCTTCCTGTCTTCTGAA;

for **PKC-β**: 5'-TTCAGATTTCAGCTGAAGGAA

and 5'-TGTCAAATTTGGATATAGTGTTCG;

for **PKC-γ**: 5'-CGGGCTCCCACATCAGATGAG

and 5'-CGTCCTGGGCTGGCAC;

for **PKC-δ**: 5'-CACCATCTTCCAGAAAGAACG

and 5'-CTTGCCATAGGTCCCGTTGTTG;

for PKC-ε: 5'-CATCGATCTCTCGGGATCATCG

and 5'-CGGTTGTCAAATGACAAGGCC;

for **PKC-η**: 5'-GGATAATGCGACAAGGACTTC

and 5'-GAAGAGTTAACACCGATCCCA;

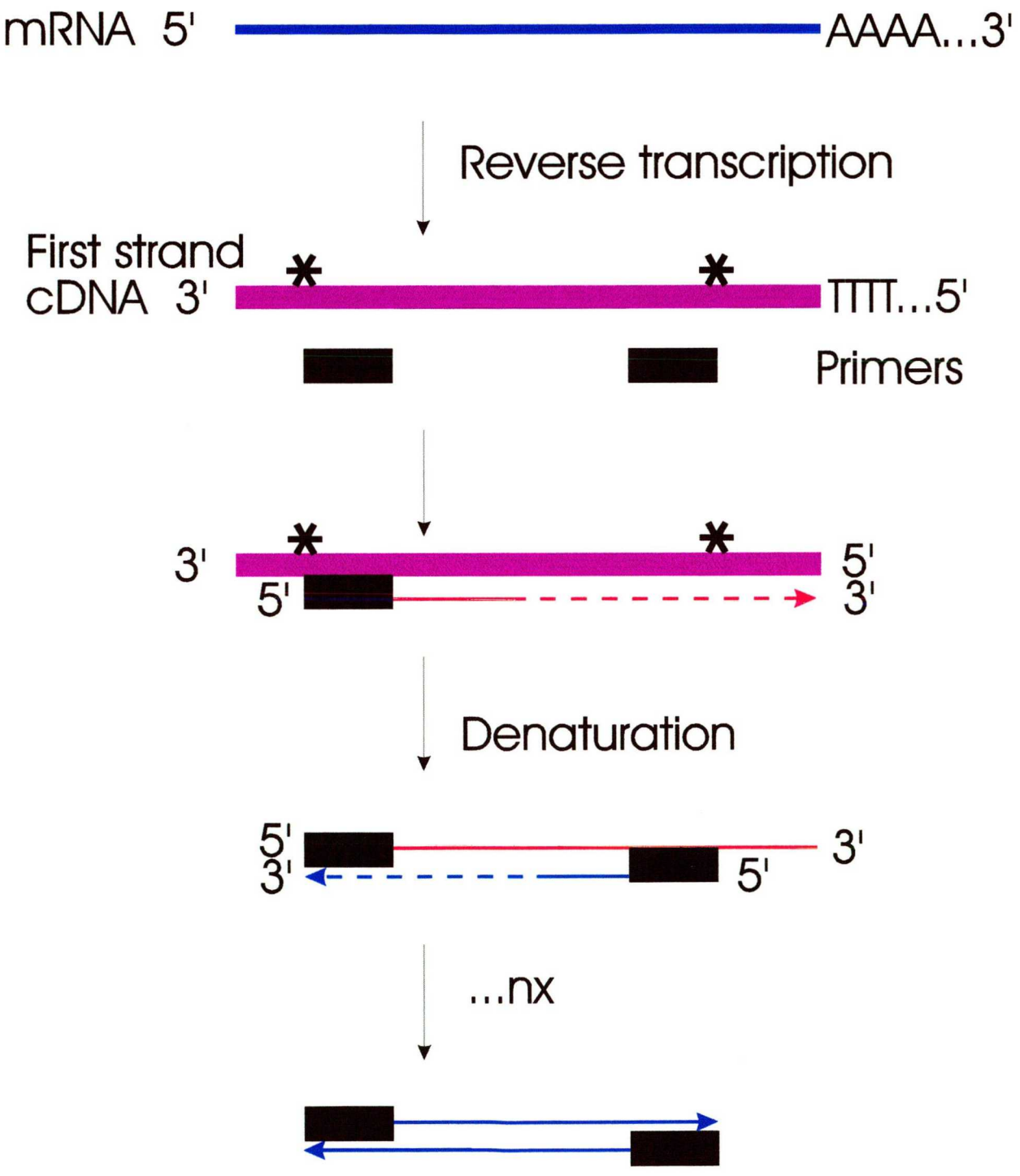
for **PKC-ζ**: 5'-CGATGGGGTGGATGGGATCAAAA

and 5'-GTATTCAT GTCAGGGTTGTCTG.

The amplification conditions were as follows: 94°C 1 min 20 s denaturation, 55-65°C (depending on primers) 1 min 30 s annealing and 72°C 2 min synthesis. The PCR products were separated on 1.6% agarose gel containing ethidium bromide and examined in ultraviolet light. The amplification products obtained with all the seven primer pairs were of the expected size: 325 bp for PKC-α, 309 bp for PKC-β, 544 bp for PKC-γ, 352 bp for PKC-δ, 732 bp for PKC-ε, 681 bp for PKC-η, and 238 bp for PKC-ζ. In addition the specificity of the products was controlled by digestion with appropriate restriction enzymes. In each case fragments with expected size were obtained. The principles of the polymerase chain reaction are shown in Fig. 1.

Fig. 1. Principle of polymerase chain reaction. Purified total RNA or mRNA (1 - 5 μg) is transcribed into first strand cDNA in the presence of reverse transcriptase. The amplification of the DNA is achieved by using two oligonucleotide primers specific for the target DNA and thermostable DNA polymerase. Following 30 - 35 cycles (each cycle consists of denaturation, annealing and synthesis) the DNA sequence between the two primer pairs will be amplified by several orders of magnitude.

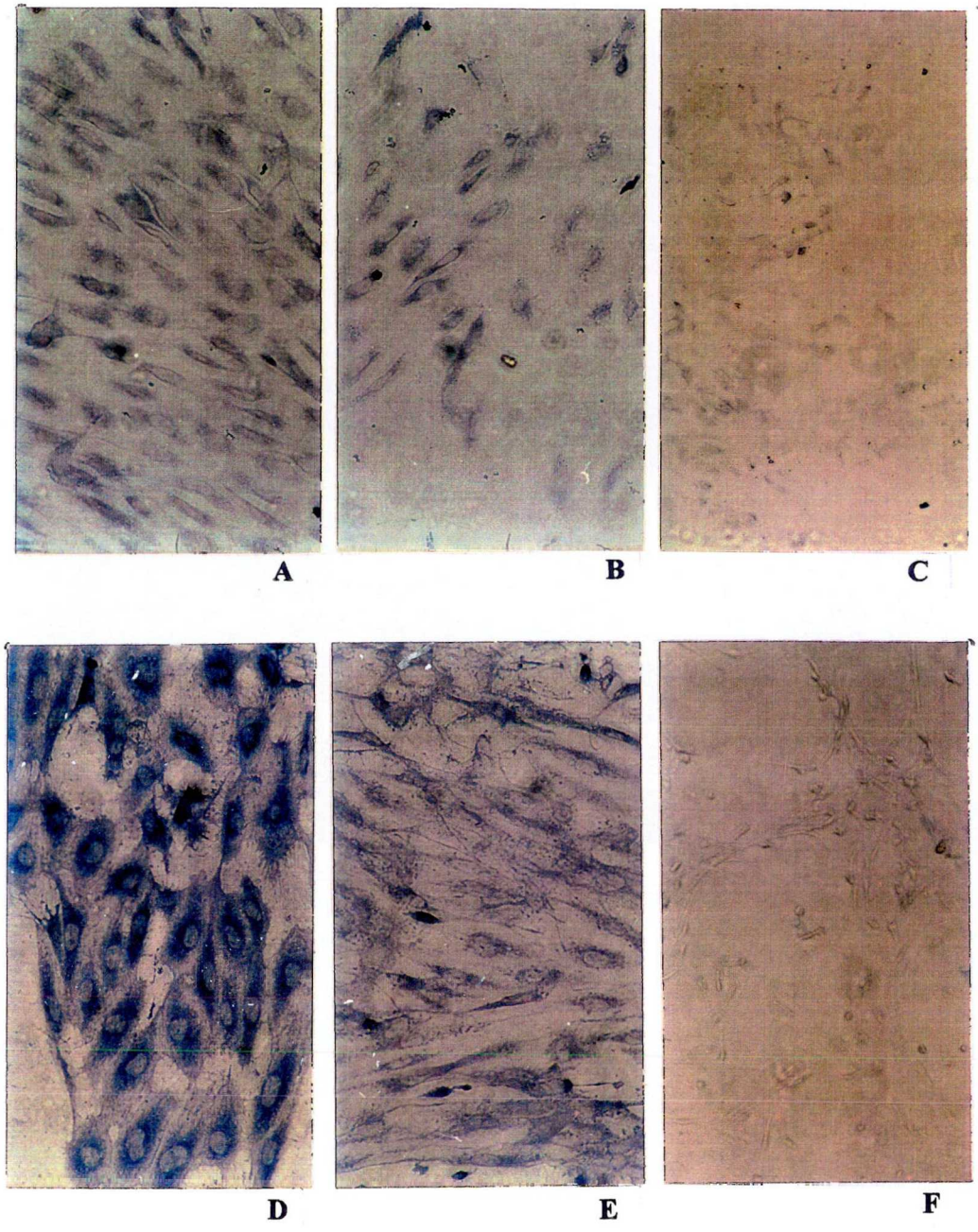
PRINCIPLE OF POLYMERASE CHAIN REACTION



RESULTS

6.1 CAM-PK II in the CEC

To determine if the cerebral endothelial cells were capable of syntesizing CAM-PK II, we examined the expression of its mRNA by in situ hybridization in primary cultures of cerebral endothelial cells. In order to obtain a high cellular resolution we decided to use a non-radioactive method based on digoxigenin labeled oligonucleotide probes and with several modifications we made the technique applicable on endothelial cell culture. The synthetic antisense oligoprobes were 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 corresponding to the same region were also synthesized and used for control experiments. Strong staining indicating the expression of α subunit of CaM-PK II was observed (Fig. 2A) in the perinuclear region of cytoplasm of almost all cerebral endothelial cells, whereas no staining was seen after the use of sense probe (Fig. 2C). On the other hand, weak labeling was found with the use of oligonucleotide probe for the β-subunit of CaM-PK II (Fig. 2B), which did not differ essentially from the low labeling obtained with the sense probe (not shown). Similar results were obtained with immunohistochemical methods using monoclonal antibodies raised against the α- and β- subunits of the CAMK-II. Fig. 2D shows the strong immunoreactivity obtained with the α-subunit antibody, whereas weak staining could be obtained with the β-subunit antibody (Fig. 2E). No staining was seen in the controls (Fig. 2F). The Western blot analysis using the same antibodies confirmed our finding that the α-subunit of the CAMK-II is present in the cerebral endothelial cells in considerably higher amount than the β- subunit (Fig. 3).



Figs. 2A-C : Detection of mRNA by in-situ hybridization for the α- (Fig. 2A) and β-subunits (Fig 2B) of CaM-PK II in cerebral endothelial cells. Fig. 2C shows no labelling after the use of sense probe for the α-subunit.

Figs. 2D-F: Immunohistochemical detection of CaM-PK II in primary cultures of cerebral endothelial cells. Strong staining was found with the use of a specific antibody raised against the α-subunit of CaM-PK II (Fig. 2D); whereas almost negligible staining was seen with the use of antibody raised against the β-subunit (Fig. 2E). In control experiments, where no primary antibody was used lack of staining was observed (Fig. 2F).

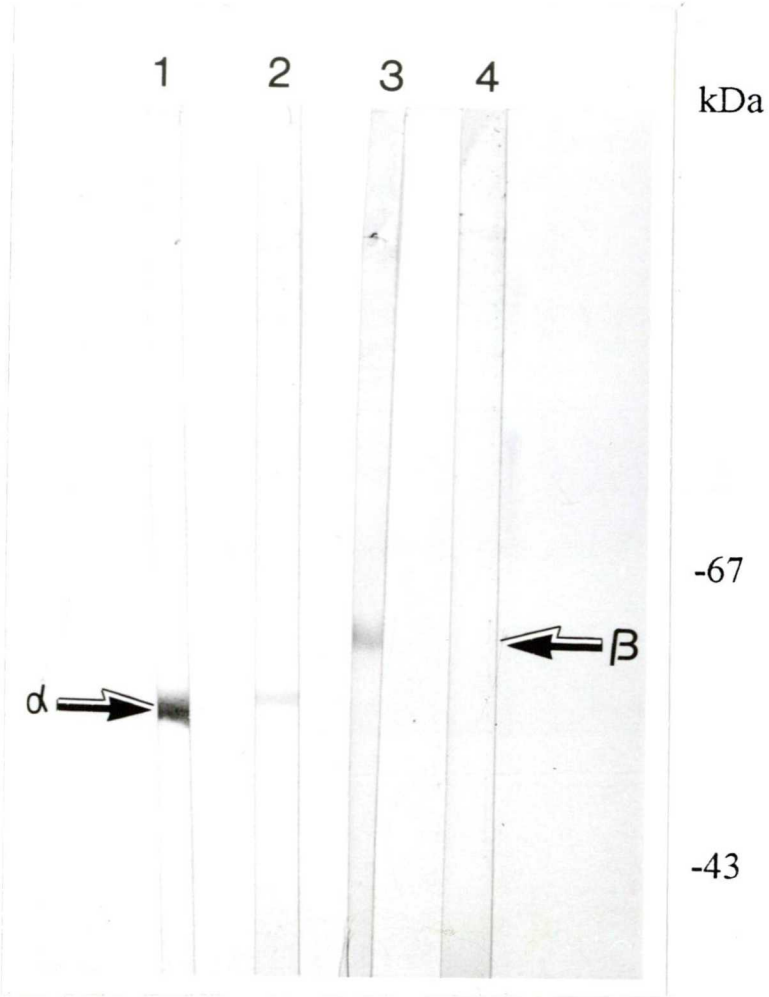


Fig. 3. shows immunoblot analysis of CAM-PK II. The α-subunit specific momoclonal antibody recognized a 50 kDa protein band in the case of both purified kinase (lane1) and cerebral endothelial cell homogenate (lane2). The β-subunit specific antibody reacted with a 58 kDa protein band, as expected, on the strip containing the purified kinase (lane 3), altough the intensity was weaker, whereas it did not give detectable reaction with cerebral endothelial cell proteins (lane 4).

In order to gain some information of the functional reactivity of CaM-PK II to calcium and/or calmodulin in cultured cerebral endothelial cells, we carried out protein phosphorylation studies. The most prominent substrate of calmodulin-dependent phosphorylation was the 50-54 kDa polypeptide (Fig. 4, lane 2), 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 immuno-histochemistry. Phosphorylation of β-subunit, if it occurs in cultured cerebral endothelial cells, was under the sensitivity of our assay.

6.2 Effect of glutamate on CAM-PK II

In order to get an insight into the molecular mechanisms which may regulate the function of CAM-PK II, we have investigated the effect of glutamate on its phosphorylation. Exposure to 2 mM glutamate for 30 min increased two to threefold the Ca^{2+} /calmodulin stimulated phosphorylation of CAM-PK II which persisted even at 10 and 60 min recovery time after glutamate exposure. (Fig. 4A). The effect of glutamate could be blocked by the NMDA receptor blocker MK-801 (Fig. 4B). The increase in phosphorylation was parallelled by an increase in intracellular Ca^{2+} concentration as revealed by electron microscopical localization of intracellular Ca^{2+} (Joó *et al*, 1994). Fig. 4 shows one representative of two independent experiments. The densitometric evaluation of the results is shown in Fig. 5.

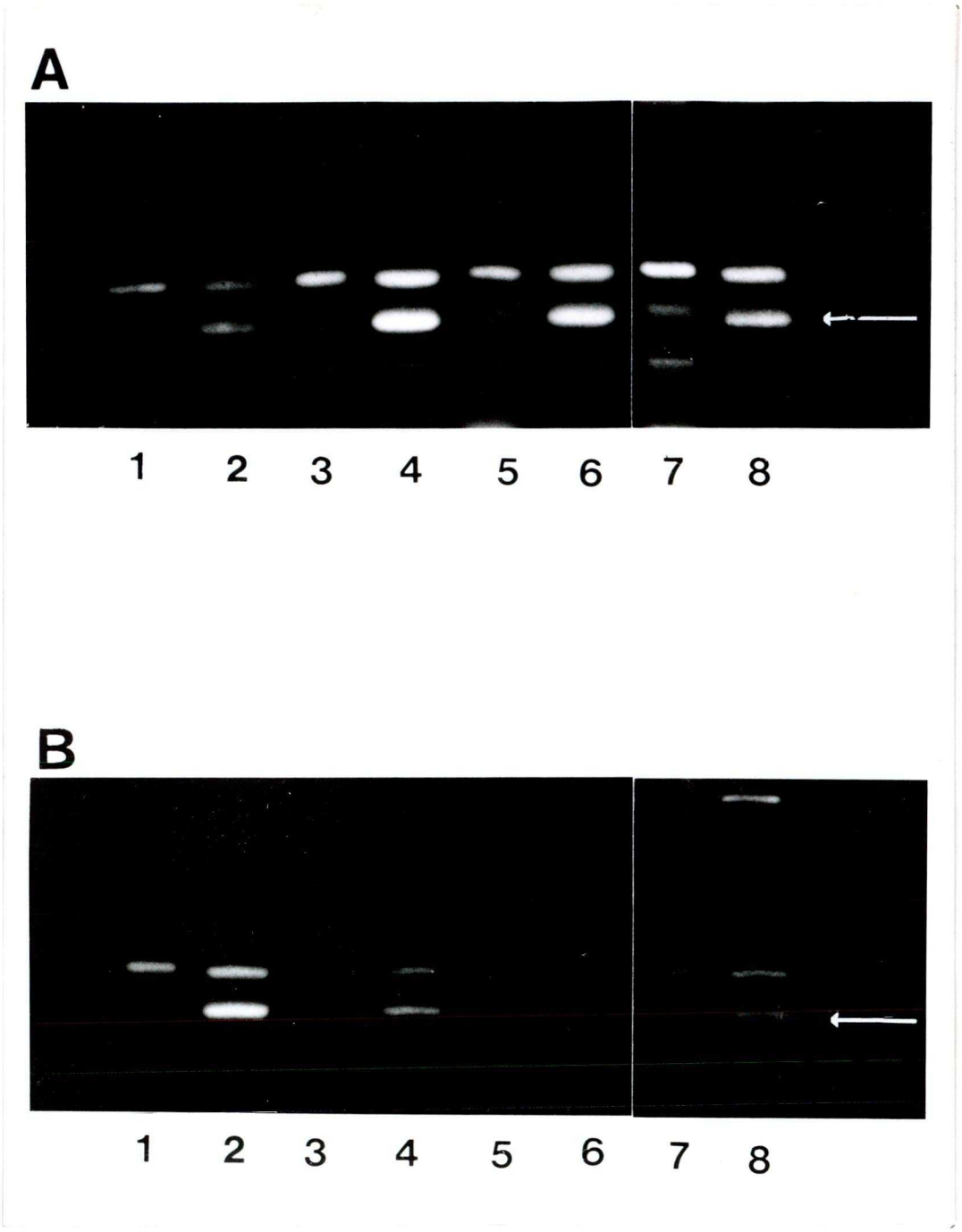


Fig. 4. shows the phosphorylation of CAM PK II α in response to glutamate (Fig. 4A) and glutamate and MK-801 (Fig. 4B). Lane 1 control, lanes 3, 5 and 7 basal phosphorylation after 0, 10 and 60 min of the glutamate exposure. Lanes 2, 4, 6, 8 Ca^{++}/calmodulin stimulated phosphorylation under control conditions and after 0, 10 and 60 min of the glutamate exposure. Significant increase in the Ca^{2+}/calmodulin stimulated phosphorylation of CaM-PK II was observed 0 and 10 minutes after 30 minutes exposure with glutamate, which could be prevented by pretreatment with MK-801 (100 mM).

GLUTAMATE INDUCED PHOSPHORYLATION OF CAMK-II α IN CEC

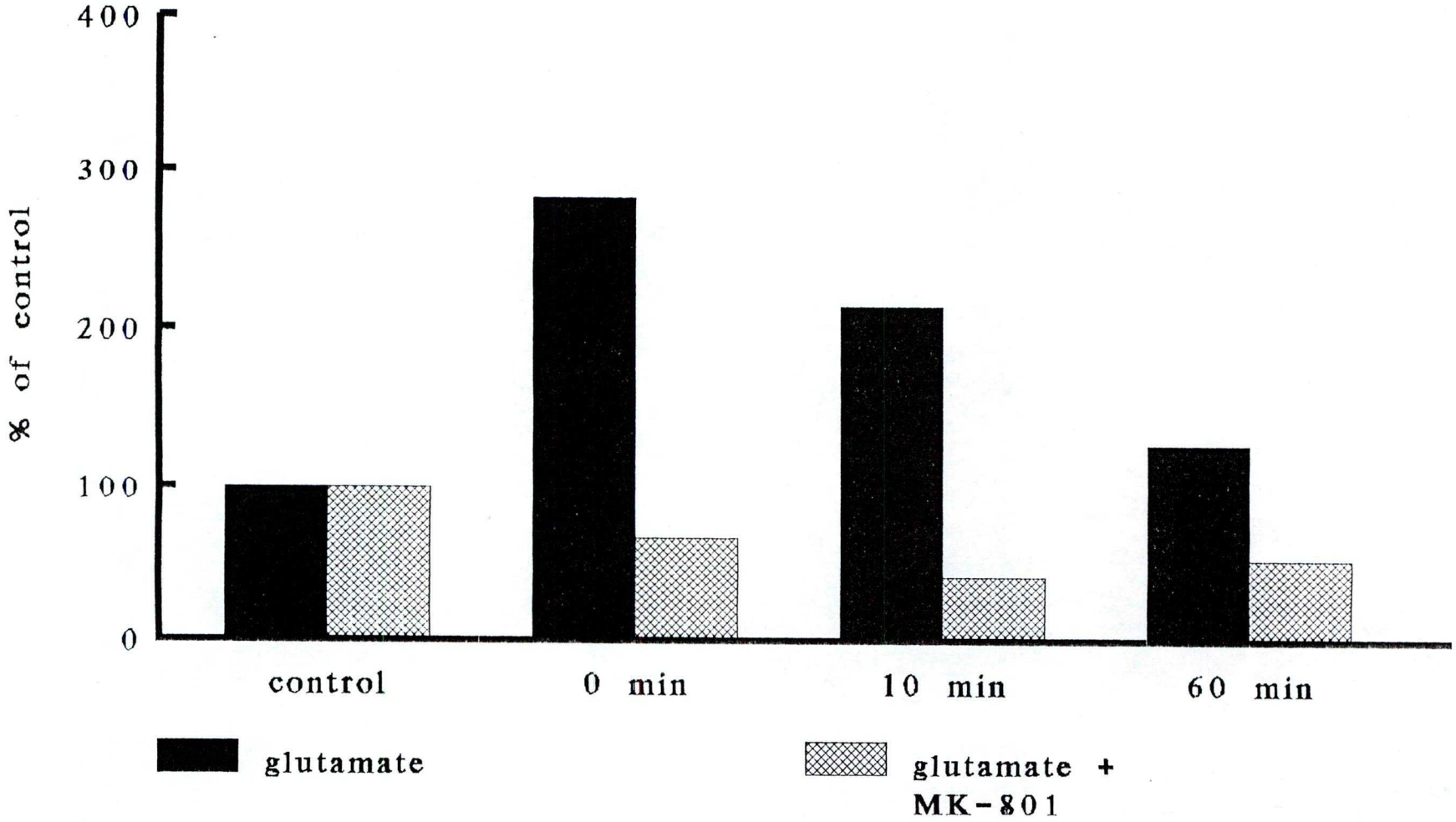


Fig. 5

6.3 PKC family members in CEC

For the characterization of the expression of PKC family members in isolated microvessels and endothelial cells of different origin we have used reverse transcription polymerase chain reaction (RT-PCR). This method makes possible the detection with high sensitivity and specificity of gene expression. Since the results and interpretation of PCR analysis is extremely dependent on the purity of the starting material we have paid special attention to the purity of the brain microvessel fraction and primary cultures of endothelial cells. The purity of the microvessel fraction was checked by light and electron microscopy and contaminating cells neither of glial nor of neuronal origin were detected (not shown). In case of the primary cultures, the Thy-1. 1. and complement treatment successfully eliminated the eventual pericytic contamination. No contaminants of neural, glial or other origin were detected by using antibodies against specific markers such as GFAP or neurofilament protein (not shown).

Fig. 6 demonstrates the distribution of PKC-α (Fig. 6A), PKC-β (Fig. 6B), PKC-γ (Fig. 6C), PKC-δ (Fig. 6D), PKC-ε (Fig. 6E), PKC-η (Fig. 6F) and PKC-ζ (Fig. 6G) in rat brain homogenate (lanes 1), cultured aortic endothelial cells (lanes 2), freshly isolated brain microvessel fraction (lanes 3), primary cultures of rat brain endothelial cells (lanes 4) and immortalized rat brain endothelial cell line (lanes 5). One representative result of two independent experiments is shown for each case. Brain contained all the seven investigated isoforms. The same expression pattern was found in freshly purified microvessels but the PKC-γ isoform was absent. Primary cultures of endothelial cells expressed PKC-α,-β,-δ,-ε and -η isoenzymes, whereas the immortalized cell line expressed PKC-α,-δ,-ε and η. Rat aortic endothelium contained PKC-α and δ, only. The results are summarized in Table 1.

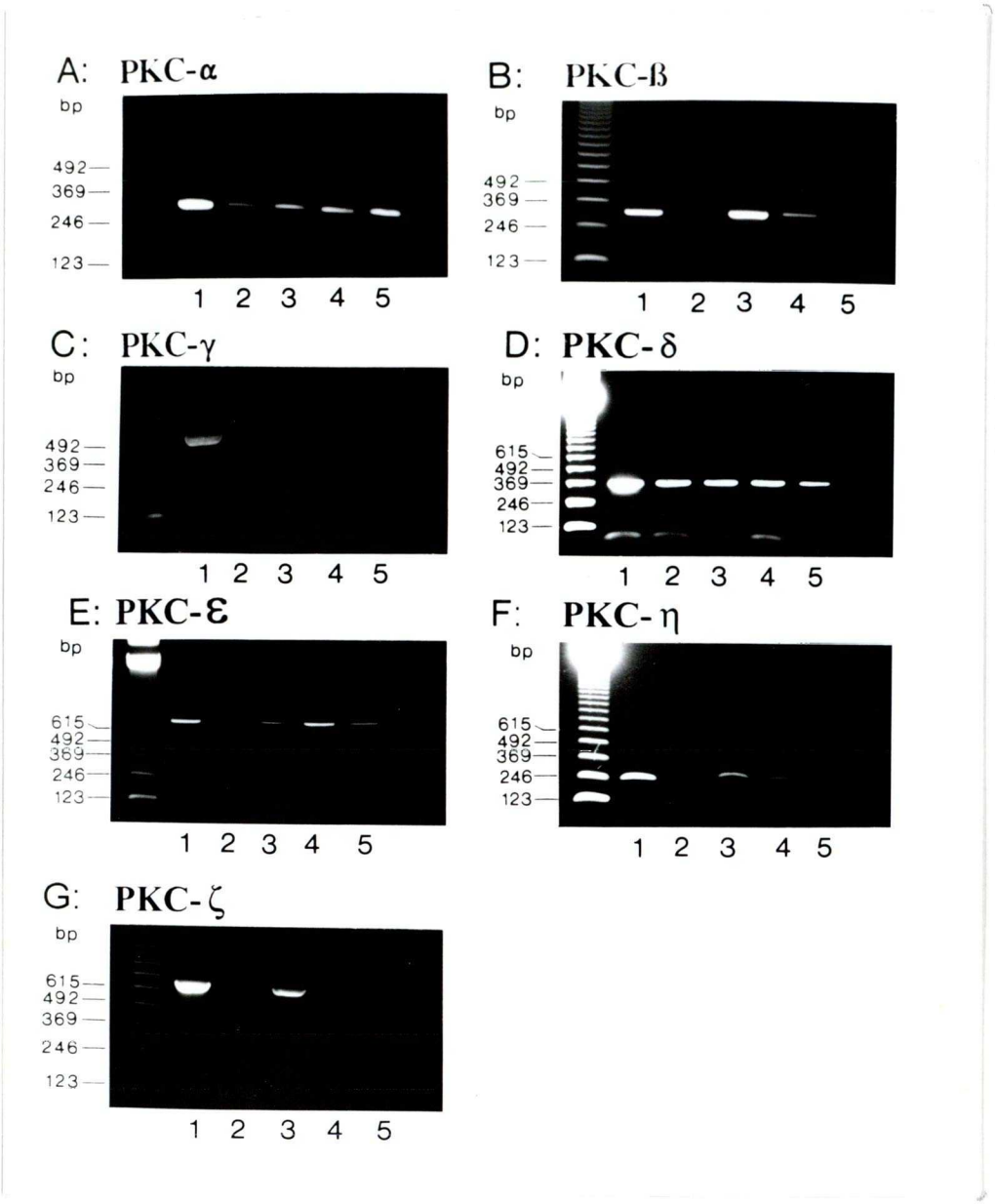


Fig. 6: Detection by RT PCR method of different PKC family members. Seven isoforms are shown, representing the three main subclasses of PKC isoforms. Lanes 1 = brain homogenate; lanes 2 = aortic endothelial cells; lanes 3 = brain microvessel fraction; lanes 4 = primary cultures from brain capillaries; lanes 5 = RBE4, an immortalized line from CEC. One representative result experiment of two independent experiments are shown.

	BRAIN HOMOGENATE	AORTIC ENDOTHELIAL CELLS	BRAIN MICROVESSEL FRACTION	PRIMARY CULTURES OF BRAIN CEC	RBE4 CELL LINE
PKC-α	+	+	+	+	+
PKC-β	+	-	+	+	-
PKC-γ	+	-	-	-	-
PKC-δ	+	+	+	+	+
PKC-ϵ	+	-	+	+	+
PKC-η	+	-	+	+	+
PKC-ζ	+	-	+	-	-

Tab. 1

7. DISCUSSION

Activation of cell surface receptors by hormones, neurotransmitters (like excitatory amino acids) or other external stimuli evoke changes in Ca^{2+} fluxes, which apparently lead 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 blood-brain barrier, in cultured rat brain capillary endothelial cells (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 oedema evoked by kainic acid (Sztriha and Joó, 1986). However the detailed mechanism of the Ca^{2+} action remains largely unknown. Possible candidates to mediate the effect of Ca^{2+} are protein kinases which may participate in the coupling of signal transduction to endothelial metabolism. The fact that protein phosphorylation occurs in brain microvessels was first shown by Pardridge *et al.* (1987) and even several proteins involved in it have been characterized (Weber *et al.*, 1987; Dechert *et al.*, 1991)

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 oedema.

It is well known that in its phosphorylated form, CAM-PK II looses its Ca^{2+} dependency and retains its activity even after the removal of Ca^{2+} (Bronstein *et. al.*, 1993) This phenomenon has been shown to mediate important cellular processes, such as LTP and memory formation in neurons (Lisman *et al.*, 1988). The persistence of CAM-PK II phosphorylation even after removal of glutamate may cause a prolonged change in the

responsiveness of cerebral endothelial cells after exposure to increased glutamate concentrations in ischemic conditions. Since the phenomenon can be blocked by MK-801, the effect of glutamate seems to be mediated by NMDA receptors. The assumption that glutamate may exert a direct effect on the cerebral vasculature is further supported by the finding that glutamate induced cerebral arteriolar dilatation is mediated by NO through NMDA receptors (Meng *et al.*, 1995). These results suggest that NMDA receptors may take part in physiological function and pathological reactions of cerebral endothelial cells.

Beside CAM-PK II, the other major protein kinase whose activity can be regulated by Ca^{2+} is protein kinase C. To reveal some specific features in the cerebral endothelial cell signal transduction we have compared the expression of PKC family members in endothelial cells of different origin, isolated cerebral microvessel fraction and brain. PKC-α and -δ, thought to be universally occurring enzymes, were indeed detected in all the tissues and cell types studied . PKC-β, beside being present in brain, could be observed only in the microvessel fraction and in endothelial cells which have the best capability to form tight junctions. On the other hand PKC-γ was found to be present only in brain homogenate. The lack of this isoform in all endothelial preparations may prove the absence of contaminating neurons in our preparations, and can possibly be interpreted on the basis of a recent finding (Moriya and Tanaka, 1994) revealing a correlation between the prominent expression of PKC-γ and synaptogenesis. Significant differences, observed in the expression of PKC isoforms of freshly isolated microvessels, primary cultures and immortalized cerebral endothelial cell line, may warn us to use the *in vitro* preparation with caution. However, the heterogeneity of the cells in the microvessel preparation could also give rise to some of the differences in results.

Previous studies have shown by using hydroxylapatite chromatography that the major PKC isoforms in rat cerebral microvessels are PKC-α and-β (Kobayashi *et al.*, 1991). However the sensitivity of the used method does not enable the detection of low enzyme levels. The presence of the novel isoforms, which do not require Ca^{2+} for their function, in brain microvessels and endothelial cells may confer activability to these cells

even at basal concentrations of calcium. PKC can be activated in the cerebral microvessel fraction through different type of receptors. Preliminary experiments, designed to investigate the effect of factors known to induce or re-induce BBB-like phenotype such as cAMP or astrocyte conditioned medium on the expression pattern of PKC isoforms, have led so far to negative results. Further experiments are being done to check this important point in co-cultures.

As regards possible function of PKC in the CEC, phorbol esters were shown (Drewes *et al.*, 1988) to increase hexose uptake. Fluid-phase endocytosis within primary cultures of brain microvessel endothelial monolayers was demonstrated to be significantly stimulated by nanomolar concentrations of phorbol myristate acetate (Guillot *et al.*, 1990). This effect was not mediated by prostaglandins. Since PKC is a prime target for actions of phorbol esters, its involvement in the activation of BBB opening seems to be established. The assumption that endothelial PKC takes part in permeability regulation was substantiated by our finding (Joó *et al.* 1989) showing that H-7, a potent inhibitor of PKC, prevented brain oedema formation after ischemic brain injury. Disruption of tight junctions elicited by low extracellular calcium concentration could be inhibited with protein kinase inhibitor H-7 (Citi, 1992) in epithelial cells cultures from Madin Darby canine kidney suggesting that PKC and probably protein phosphorylation are involved in the regulation of tight junction permeability. First indications for the operation of such a molecular mechanisms have been published, recently (Berthoud *et al.*, 1992). Another important function of PKC in the CEC may be related to the regulation of cell growth. Namely, different activators of PKC were found (Daviet *et al.*, 1989) to stimulate bovine brain capillary endothelial cell growth through high-affinity receptors for tumor-promoter phorbol esters in a dose-dependent manner. The identification of *in vivo* phosphorylation substrates is of primordial importance in understanding PKC function. Inspite of the importance of PKC in basal cellular processes such as regulation of gene expression only a few substrates have been characterized so far (MARCKS, neuromodulin or neurogranin). In the cerebral microvessel fraction several proteins with different

molecular weight (40, 45, 50, 55, 58 kDa) were phosphorylated with different time course by the addition of purified PKC in *in vitro* studies (Oláh *et al.*, 1988).

So far, no studies have been carried out to elucidate the function of different PKC isoforms, mainly because of the lack of specific inhibitors. Since different isoforms have different cellular localization and sensitivity to activators, substrates of each isoform may have their specific role in cellular signalling. Changes in intraendothelial calcium concentrations have been detected already (Revest *et al.*, 1991) in a number of conditions. It is tempting to assume that the variety of expression pattern of PKC family members in endothelial cells of different type may have consequences in the functional responsiveness to environmental stimuli. It is well known that PKC isoenzymes show differences in subcellular localization, Ca^{2+} dependence and responsiveness to phospholipid metabolites, therefore the presence of different isoforms may confer a sophisticated intracellular regulatory mechanism to the brain endothelial cells. Further experiments are warranted to explore the details of this important aspect.

8. PERSPECTIVES

For many years, the cerebral endothelial cells have been regarded as a passive cellular layer with the only function to separate mechanically the brain from the circulation. In the past two decades, however, we have learned that these cells are equipped with a sophisticated signal transduction system coupled to different receptors through which the function of the blood-brain barrier can be modulated. The deeper understanding of the molecular mechanisms working in the cerebral endothelial cells will open us the way to manipulate the blood-brain barrier, to improve or impair the transport of different drugs to the brain. The rapid development of the molecular biology and

biotechnology however may open new perspectives to study and therapeutic use of endothelial cells. We are now at the dawn of a new era, the gene therapy, which will make possible the treatment of today still untreatable diseases. However, one of the most important issues to be resolved is how to deliver functionally active genes to the CNS which is isolated from its environment by the blood-brain barrier.

Endothelial cells due to their location at the interface between blood and brain are good candidates to become substrates for gene therapy. The *in vitro* introduction of foreign genetic material into the endothelial cells is already a routine procedure. These genetically modified cells then can be transplanted into the CNS where they express the gene introduced for therapeutical purposes.The first step in this direction has already been made by the introduction of a potentially therapeutic gene, the nerve growth factor (NGF) into the cerebral endothelial cells. Preliminary experiments show that these cells can survive, integrate into the host cerebral vasculature and release biologically active NGF. (Couraud *et al.*, 1995)

Future experiments shall decide if cerebral endothelial cells could become vehicles for gene therapy.

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