Vol. 150, No. 2, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 29, 1988 Pages 575-582

TRANSFORMING GROWTH FACTOR BETA ACTIVATES PROTEIN KINASE C IN MICROVESSELS ISOLATED FROM IMMATURE RAT BRAIN

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Received November 23, 1987

SUMMARY: We investigated the activation of protein kinase C in microvessels isolated from rat brain. We found that unstimulated kinase activity in microvessels from immature animals is soluble while that from adults is particulate. The tumor promoter, phorbol 12-myristate 13-acetate, and the diacylglycerol analog, 1-oleoyl-2-acetyl-sn-glycerol, caused the redistribution of protein kinase C activity to the membrane fraction in microvessels from immature rats. Exposure to transforming growth factor beta resulted in similar redistribution of kinase activity. To our knowledge, this is the first report of an effect of transforming growth factor beta on protein kinase C. The kinase activity in microvessels from adult animals was unaffected by exposure to these agonists. We suggest that protein kinase C activation promotes differentiation of the brain microvasculature. Transforming growth factor beta may mediate this process.

Endothelial cells in brain microvessels express a number of special features which collectively underlie formation of the blood-brain barrier. These include the continuous tight junctions that seal the endothelial cells together, a paucity of pinocytotic vesicles, an absence of fenestrations, and an asymmetrical distribution of transport carriers capable of mediating transfer of molecules into or out of the brain (1,2). Many of these features are present in immature animals, although important changes occur with development in the competence of the tight junctions (3) and the kinetics of selected transporters (4). In addition, endothelial cells in immature brain are rapidly proliferating. In the rat, cerebral hemisphere mass doubles and the microvascular density increases three-fold during the first month of life (5). Thereafter, the rate of endothelial cell turnover in normal brain is very low. Little is known about the regulatory mechanisms that control the proliferation of endothelial cells in the brain or the expression of specific endothelial properties important to the function of the blood-brain barrier. In several other tissues, activation of a calcium-, phospholipid-dependent kinase, protein kinase C, has a major role in the control of growth and differentiation (6). In this study, we examined the activation of protein kinase C in microvessels isolated from immature and mature rat brain. We found that transforming growth factor type beta , a widely distributed regulatory peptide (7) that promotes endothelial

Abbrevations used: transforming growth factor beta (TGF-β);1-oleoyl-2-acetyl-sn-glycerol (OAG); phorbol 12-myristate 13-acetate (PMA)

0006-291X/88 $1.50

cell differentiation (8,9), produced a translocation of protein kinase C activity from the cytosol to the membrane in brain microvessels prepared from immature rats. To our knowledge this is the first demonstration of a stimulatory effect of TGF-ß upon protein kinase C. In microvessels prepared from adult rat brain, protein kinase C was membrane bound in the unstimulated state and not altered by exposure to TGF-ß.

MATERIALS AND METHODS

Materials

Six day-old Sprague-Dawley rat pups and adult rats were obtained from Harlan (Haslett, MI). [Gamma-^{32}P]ATP (>4000Ci/mmol) was purchased form ICN Biomedicals, Inc. (Irvine, CA). 1,2-diolein was obtained from P-L Biochemicals (Milwaukee, WI). Transforming growth factor type beta, purified from human platelets, was purchased from R & D Systems, Inc. (Minneapolis, MN). Cold ATP, OAG, PMA, phosphatidyl-L-serine, type III-S histone, and all other reagents were obtained from Sigma (St. Louis, MO). Phosphocellulose paper (P81) was purchased from Whatman (Hillsboro, OR).

Isolation of rat brain microvessels

Microvessels were isolated from cortical hemispheres of six day-old Sprague-Dawley rat pups and from adult rats by modification of a technique previously described by Betz and Goldstein (10). The animals were sacrificed by decapitation, the brains rapidly removed and placed in ice-cold Medium 199 buffered with 20mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4 (buffer A). The brainstem, cerebellum, and meninges were discarded and the cortical shells, free of choroid plexus and ependyma, were minced in above buffer. Following homogenization (20 strokes, 390 rpm) in a Teflon/glass homogenizer (0.25mm clearance), a 10% suspension (w/v) was centrifuged at 1000 x g for 10 min. The pellet was suspended to 16% (w/v) in buffer A containing 15% dextran and centrifuged at 4000 x g for 10 min to remove myelin and cellular debris. The resulting pellet consisting of free nuclei and capillary segments was resuspended in buffer A and suctioned gently through a 118µm nylon mesh. To isolate the capillaries from nuclear debris, the suspension was subsequently passed over a 1.2 x 1.5 cm column of 0.25 mm glass beads supported on a 53 µm nylon mesh and washed extensively with the buffer. The capillary segments were retained on the column. Microvessels were recovered by gentle agitation of the glass beads in same buffer, decanted, and collected by centrifugation at 500 x g for 5 min. The quality of each preparation was judged morphologically under phase microscopy (Figure 1).

Incubation of microvessels

For preincubation with PMA, OAG, or TGF-ß, the microvessels, prepared as described above, were divided into aliquots, centrifuged at 500 x g for 5 min, and resuspended in buffer A containing specified concentrations of agonist. The capillary suspensions were incubated at 37°C for 45 min, unless otherwise noted. The suspensions were then pelleted by centrifugation at 500 x g for 5 min and prepared for protein kinase C assay as described below.

Extract preparation

To prepare the cytosolic and membranous extracts for the assay of protein kinase activity, isolated microvessels were washed twice with ice-cold calcium-free phosphate buffered saline, pH 7.4, sonicated for 15 sec in buffer containing 20mM HEPES, pH7.5, 2mM ethylenediaminetetraacetic acid (EDTA), 2mM ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 0.25M sucrose, and 10mM ß-mercaptoethanol (buffer B), and centrifuged for 1 hr at 40,000 x g. The resulting supernatant and pellet fractions were used for all subsequent experiments. The soluble fractions were adjusted for protein and assayed for protein kinase C activity as described below. The particulate components were solubilized in 0.03% Triton X-100 (in buffer B) in equal volumes as the corresponding supernatants and assayed as described.

Assay for protein kinase C

Protein kinase activity was assayed by a modification of the procedure described by Takai, et al. (11). Kinase activity was determined by measuring the incorporation of radiolabel from [^{32}P]ATP into endogenous cytosolic protein and exogenous lysine-rich histones. In a total volume of 250µl, the standard reaction mixture contained 20mM TRIS-HCl (pH 7.4), 5mM $MgCl_2$, 10µM $CaCl_2$, 5µg phosphatidylserine, 5µg 1,2-diolein (or equivalent volume of ddH_2O), 20µg lysine-rich histones, 0.2nmol unlabeled ATP, and 2 µCi [^{32}P]-ATP (4000Ci/mmol). The

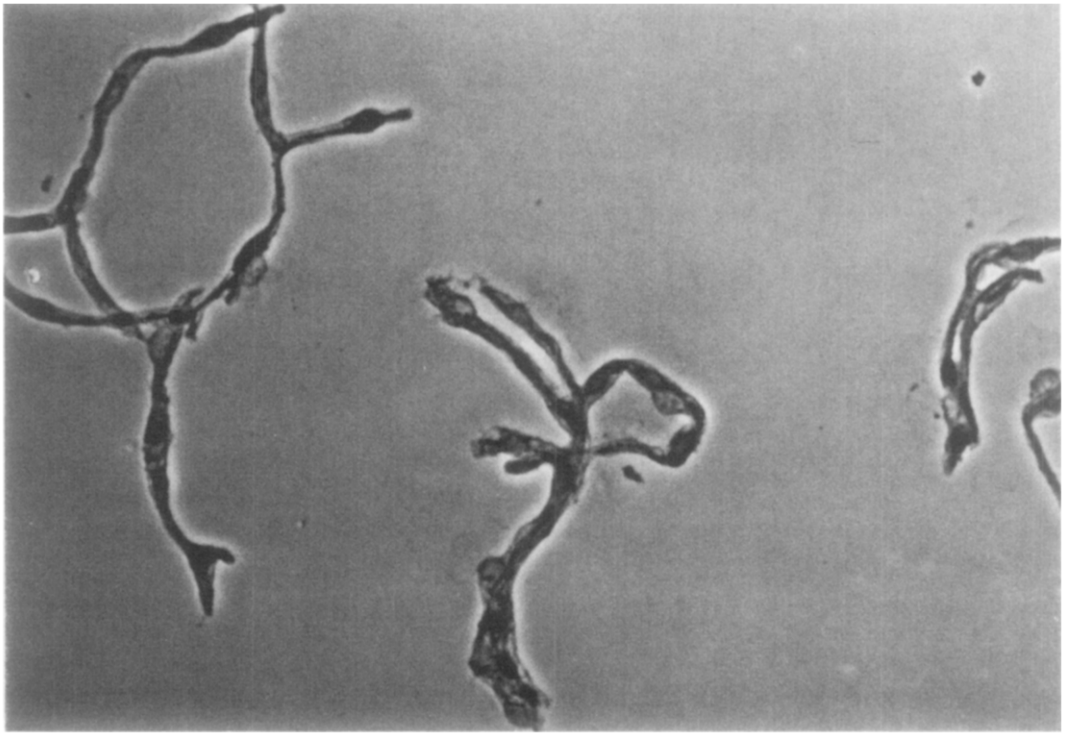

Figure 1. Isolated Immature Rat Brain Microvessels.
Microvessels were isolated from rat cortices as described in Methods. Light phase contrast micrograph indicates segments of microvessels essentially free of debris (x 450).

reaction was initiated by addition of 100µl extract containing 5 - 15µg cytosolic protein, incubated for 5 min at 30°C, and terminated by addition of 75µl of12N glacial acetic acid. Phosphorylated proteins were collected by adsorption on P81 phosphocellulose papers. The filters were washed once in 30% acetic acid, twice in 15% acetic acid, and finally in acetone. The radioactivity was quantitated by Cerenkov counting and protein content determined by the method of Bradford (12). Protein kinase C activity was calculated as the difference in the activity in the presence and absence of 1,2-diolein.

RESULTS

Microvessels purified from brain provide a model system for the study of biochemical events operative in the blood-brain barrier. We investigated the distribution and activation of protein kinase C in microvessels isolated from six day old rats when brain endothelial cells are actively proliferating and from adult brain with quiescent endothelial cells. Figure 2 illustrates that essentially all protein kinase C activity in immature microvessels is found in the soluble fraction with only a trace being localized to the membrane (8.06 ± 1.1 vs 0.15 ± 0.15 pmol/mg/min). In contrast, most of the protein kinase C activity in adult brain microvessels is found in the particulate fraction (6.95 ± 0.13 vs 0.13 ± 0.12 pmol/mg/min in the soluble). To test the reactivity of this enzyme, we exposed brain microvessels to the tumor-promoting phorbol ester, PMA, which is known to activate protein kinase C. As shown in Table 1, incubation with 100nM PMA resulted in a redistribution of protein kinase C activity from the cytosol into the membrane. Exposure of the immature brain microvessels to the diacylglycerol

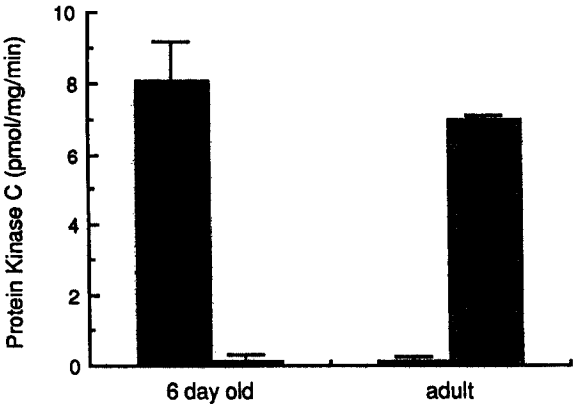

Figure 2. Distribution of Protein Kinase C Activity in Immature and Adult Microvessels.
Protein kinase C activity was measured in soluble and particulate fractions of microvessels isolated from immature and adult rat brain as described in Methods. Solid bars represent soluble kinase activity; hatched bars are particulate activity. Values plotted are the means of three replicates ± s.e.m. Data points without error bars represent s.e.m.<5%.

analog, OAG, (50μg/ml) also produced a translocation of protein kinase C activity into the particulate fraction (Table 1).

TGF-ß, a growth regulatory peptide, inhibits proliferation and induces terminal differentiation of endothelial cells in culture (8,9). When microvessels from immature rat brain were incubated with 20pM TGF-ß, protein kinase C activity was redistributed from the cytosol into the membrane (Table 1). Incubation of these with varying concentrations of TGF-ß, resulted in a dose-dependent translocation of protein kinase C activity, with a threshold of 0.1pM. As illustrated in Figure 3, by 1pM, all of the enzyme activity is redistributed from the

Table 1. Effect of Agonists on Protein Kinase C Activity in Microvessels Isolated from Rat Brain

	Protein Kinase C Activity (pmol/mg/min)			
	Immature		Adult	
Agonist	Soluble	Particulate	Soluble	Particulate
none	8.06 ± 1.10	0.15 ± 0.15	0.18 ± 1.2	10.9 ± 3.1
PMA	1.30 ± 0.93	8.32 ± 0.23	0.00 ± 1.9	11.8 ± 1.3
OAG	0.00 ± 1.50	12.85 ± 0.41	0.00 ± 2.0	11.0 ± 1.0
TGF-ß	0.35 ± 0.35	12.65 ± 0.85	0.25 ± 0.75	10.8 ± 1.7

Microvessels were incubated with either 100nM PMA, 50μg/ml OAG, 20pM TGF-ß, or serum-free medium without additions for 45 minutes at 37°C. Protein kinase C activity was measured in soluble and particulate fractions of microvessels isolated from six day old and adult rat brain as described in Methods.

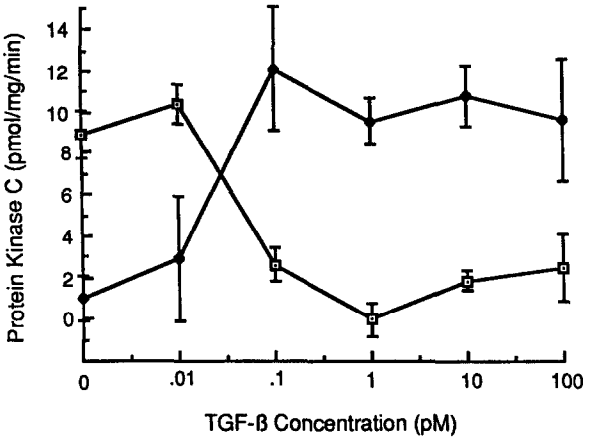

Figure 3. Effect of Varying Concentrations of TGF-ß upon Protein Kinase C Activity in Immature Rat Brain Microvessels.

Microvessels were incubated with varying concentrations (0 - 100pM) of TGF-ß for 45 min at 37°C. Protein kinase C activity was measured in the soluble (□) and particulate (■) fractions as described in Methods. Values plotted are the means of three replicates ± s.e.m.

cytosol into the membrane. Figure 4 shows the time-dependence of translocation of kinase activity in response to 1pM TGF-ß. By 15 minutes, all the activity was recovered from the particulate fraction. In contrast to the immature microvessels, all of the protein kinase C activity in microvessels isolated from adult rat brain was recovered in the particulate fraction. Exposure of adult brain microvessels to PMA, OAG, or TGF-ß had no significant effect on the distribution of protein kinase C activity between the cytosol and membrane (Table 1).

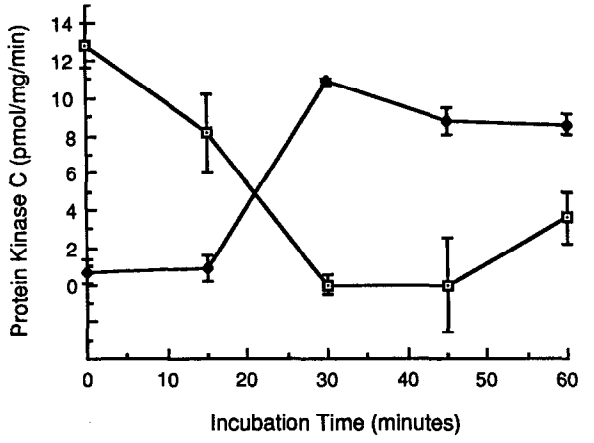

Figure 4. Effect of Incubation Time upon TGF-ß Stimulated Protein Kinase C Activity in Immature Rat Brain Microvessels.

Microvessels were incubated for varying times (0 - 60 min) with 10pM TGF-ß at 37°C. Protein kinase C activity was assayed in the soluble (□) and particulate (■) fractions as described in Methods. Values plotted are the means of three replicates ± s.e.m. Data points without error bars represent s.e.m.<5%.

DISCUSSION

During postnatal development, microvascular density of the brain increases several fold (5). Using in situ ^{3}H-thymidine labeling, maximal postnatal endothelial cell proliferation in rat brain occurs between 4 and 9 days of age and is approximately 40 times greater than in adult brain (13). The formation of brain microvessels is a complex process involving both endothelial cell proliferation and differentiation of the new cells into functional tubules with blood-brain barrier properties (14). Endothelial cells of established vessels form proliferating sprouts from which new capillaries are constructed in response to angiogenic stimuli (15). Specific signals from brain tissue, most likely generated by the astrocytes, cause the new microvessels to express the anatomic and metabolic features of the blood-brain barrier (16,17). The biochemical mechanisms regulating microvascular growth and differentiation in brain are not known.

We used microvessels isolated from brain as an in vitro model to investigate these mechanisms and chose to study protein kinase C activation because of its recognized role in regulating growth and differentiation in other tissues (6). In brain microvessels isolated from 6 day old rats, protein kinase C was in a basal state with all of the activity located in the cytosol and none bound to cellular membranes. By contrast, the protein kinase C activity measured in microvessels isolated from adult rats was entirely bound to cellular membrane (Figure 2).

In most tissues, protein kinase C translocates from the cytosol to the membrane when activated after an appropriate agonist binds to receptors located on the surface of the cell. The transduction mechanism includes stimulation of a phosphodiesterase that hydrolyzes polyphosphoinositides and releases diacylglycerol (18). The diacylglycerol binds to protein kinase C and in turn causes the enzyme to attach to cellular membranes and become capable of phosphorylating proteins that may control growth and differentiation (6). Protein kinase C is thought to be the cellular receptor for tumor promoting phorbol esters (19). These agents bypass the cell surface receptors and transduction processes to directly activate protein kinase C by binding to the diacylglycerol site (20). Phorbol esters induce differentiation in both large-vessel and capillary endothelial cells. Following exposure to phorbol myristate acetate, cultured endothelial cells grown on three-dimensional collagen gels form capillary-like tubular structures and invade the extracellular matrix (21, 22).

The reactivity of protein kinase C in our model system was confirmed when we found that the phorbol ester, PMA, and the diacylglycerol analog, OAG, were effective in translocating cytosolic protein kinase C to the membrane fraction of immature brain microvessels (Table 1). By contrast, the kinase activity in adult brain microvessels was membrane bound and presumably active prior to exogenous addition of stimulatory agents. This difference in the distribution of protein kinase C may relate to the difference in growth rate between immature and mature brain microvessel. At 6 days of age, endothelial cells in rat brain are at the peak of their proliferative activity while the endothelial cells in adult brain are quiescent (13). This is puzzling since adult brain is highly enriched with endothelial cell mitogens (23,24). The distribution and state of protein kinase C activation thus reflects and

may in part determine the responsiveness of brain endothelial cells to mitogens. The observation that ß-phorbol 12,13-dibutyrate, a protein kinase C activator, suppresses the proliferation and DNA synthesis of bovine capillary endothelial cells in response to the angiogenic agent, human hepatoma-derived growth factor is consistent with this idea (25). Such desensitization may be critical in the regulation of microvascular growth. We suggest that activation and translocation of protein kinase C in brain microvessels causes phosphorylation of proteins that limit their sensitivity to mitogens.

Further support for this hypothesis was obtained when we found that TGF-ß caused a dose- and time-dependent translocation and activation of protein kinase C in immature brain microvessels (Figures 3 and 4). TGF-ß, although originally characterized by its ability to induce phenotypic transformation in fibroblastic cells (26,27), is now known to be in a family of peptides that regulate and usually inhibit cell growth. This factor and its cellular receptors have a ubiquitous distribution (7). TGF-ß blocks growth either by antagonising cellular mitogens (28,29) or as a more direct inhibitor of cell division (7,30,31). Exposure to TGF-ß can produce terminal differentiation in several cell types including endothelial cells (7-9). Protein kinase C activity in nonreplicating brain microvessels isolated from adult brain was already located in the membrane fraction and its activity was not changed by exposure to TGF-ß.

Our results suggest a role for protein kinase C activation in terminating endothelial cell growth during brain development. It remains to be determined if TGF-ß or a related peptide is present in brain tissue at the developmental stage when endothelial cells stop proliferating and express blood-brain barrier characteristics. In addition to inhibiting cellular growth, TGF-ß induces the synthesis of extracellular matrix products as well as affecting receptors for cell to matrix attachment (32). The microvascular basement membrane which lies between endothelial cells and astrocytes, may be important for their mutual differentiation. The thickening of the basement membrane, which occurs during postnatal development (10), would be consistent with the presence of a TGF-ß-like factor orchestrating growth arrest and differentiation of the cells that form the blood-brain barrier.

In summary, we propose that activation of protein kinase C in immature brain microvessels inhibits endothelial cell proliferation and promotes differentiation of the blood-brain barrier. Furthermore, TGF-ß may mediate these processes.

ACKNOWLEDGEMENTS: This research was supported by grants EY03772 and ES02380 from the National Institutes of Health.

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