during fetal and post-natal development, PKC activity in hind limb muscles is determined by innervation. In these muscles, we previously showed[8] that this activity increased during two crucial periods of muscle development – the polyinnervation phase (E18), and at day 5 after birth, when regression of polyinnervation takes place. In the present study, we observed that during the same periods, PKC activities in SM only displayed one peak, at birth, in both the cytosol and membrane fractions. By E18, it was already higher in SM than in limb muscles, and increased until birth instead of decreasing. We concluded that the level of PKC activity in SM is determined by changes in muscle innervation as in limb muscles. This was supported by the results obtained after SM denervation. The drastic decrease in the PKC activity of the membrane fraction of the neural part of denervated SM muscle showed that most of this activity was due to the presence of the nerve ending. Thus, the discrepancy we observed between the patterns of PKC activity in growing SM and limb muscles might be attributable to differences between these muscles with regard to the times at which innervation is acquired and stabilized during development. Innervation might mature earlier in the SM, which is involved in suckling, than in limb muscles. The shift of most of the PKC activity from the cytosol to the membrane fractions observed in 14-day-old and adult SM can be interpreted as follows. In tissue in which PKC is physiologically active its activity is mostly membranous[15]. Enzyme translocation from the cytosol to the membrane fraction is considered to be PKC activation[16]. Thus, in our experiments, stronger PKC activity in the membranous fraction of 14-day-old and adult SM might signify permanently stabilized muscle innervation. The high level of PKC activity observed during the neonate period, i.e. during intensive muscular differentiation and polyinnervation, probably corresponded, on the contrary, to a phase of intense synthesis of PKC which then accumulated principally in the cytosol.

Acknowledgments. This work was supported in part by PAN (grant CPBP 04-12), by INSERM (grant 876015) and by ATP Franco-Polonaise (n° 2C15).

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0014-4754/91/030241-05$1.50 + 0.20/0


## Subtypes of protein kinase C in rat cerebral microvessels

H. Kobayashi, T. Mizuki[a], Y. Koda, M. Okazaki[a], A. Kuroiwa[a] and F. Izumi

*Department of Pharmacology and the [a] Second Department of Internal Medicine, University of Occupational and Environmental Health, School of Medicine, Kitakyushu 807 (Japan)*
*Received 28 May 1990; accepted 3 September 1990*

*Summary.* Protein kinase C in rat cerebral microvessels was characterized. By hydroxyapatite column chromatography, protein kinase C in the soluble fraction was resolved into two major peaks corresponding to type II and III enzymes, in the proportions of 57% and 38%, respectively. Since each subtype is considered to have a distinct role, the high proportion of type II enzyme found in this study suggests that this type may be involved in specific functions of the cerebral microvessels.
*Key words.* Cerebral microvessel; protein kinase C; subtype.

Elucidation of the mechanisms that regulate the physiological function of the cerebral microvessels is important for a better understanding of cerebral disorders, and may give us a clue to methods for their clinical treatment. Several biological phenomena occur through protein phosphorylation and dephosphorylation. The presence

of the protein phosphorylating system in the cerebral microvessels has been reported [1-3], but its detailed characteristics have not been analyzed.

Protein kinase C is a $Ca^{2+}$/phospholipid-dependent enzyme that mediates a variety of physiological and pathological cell functions [4-6]. Protein kinase C is composed of several isoenzymes, and at least three distinct subtypes (types I, II, III) were separated by hydroxyapatite chromatography [7,8]. The fact that the three subtypes differ in their tissue distribution and enzymatic properties suggests that each subtype has its own distinct functional role [6]. To understand the physiological function of protein kinase C in a tissue, it is important to know the predominant subtype.

In this study, subtypes of the enzyme were analyzed in order to characterize the protein kinase C in the cerebral microvessels.

*Methods*

Cerebral microvessels were isolated from male Wistar rats weighing about 200 g (Charles River) by the albumin flotation and glass bead filtration technique [9]. Their purity was checked by phase-contrast microscopy and by the measurement of γ-glutamyltranspeptidase tissue activity as described previously [9].

The microvessels were homogenized by sonication in a buffer composed of 0.5 mM EDTA, 0.5 mM EGTA, 10 mM 2-mercaptoethanol and 20 mM Tris-HCl pH 7.5. After centrifugation at 100,000 × g for 30 min, the supernatant was used as a soluble fraction. The resulting precipitate was solubilized in 1% Triton X100 in buffer A, diluted to 0.05% of Triton X100 and centrifuged at 100,000 × g for 30 min. The resulting supernatant was used as a membrane fraction. Subtypes of protein kinase C were separated by a hydroxyapatite column (Type S, 7.8 × 100 mm, KOKEN, Tokyo, Japan), using a linear gradient of potassium phosphate as described previously [10]. Protein kinase C activity was measured using H1 histone as a substrate [10]. Protein concentrations were determined by the method of Bradford [11].

*Results*

The kinase activity of the soluble fraction was stimulated by the low concentrations of $Ca^{2+}$ in the presence of phosphatidylserine and diolein, reaching a plateau value at $10^{-6}$ M of $Ca^{2+}$ (fig. 1). In the absence of phospholipids, $Ca^{2+}$ did not increase the kinase activity, indicating that our assay system specifically detects the protein kinase C activity of the cerebral microvessels. In the membrane fraction, phospholipids stimulated the kinase activity only to a small extent, and the kinase activity was increased even in the absence of phosphatidylserine by increasing the $Ca^{2+}$ concentration, which suggested that the activity of protein kinase C was low, and that a Ca-dependent kinase other than the protein kinase C was present in this fraction.

Subtypes of protein kinase C of the cerebral microvessels were separated by chromatography on a hydroxyapatite column. The protein kinase C of the soluble fraction of microvessels was resolved into two major fractions corresponding to types II (57 ± 6%, mean ± SE, n = 4) and III (38 ± 5%), with a minor fraction of type I (5 ± 1%, fig. 2). The subtypes in the membrane fraction were similar to those in the soluble fraction but the activity was considerably lower than that of the soluble fraction.

*Discussion*

In this paper, we confirmed the presence of protein kinase C in the cerebral microvessels and found that the major subtype was type II. The protein kinase C found in this study was apparently located in the capillaries of the microvessels, since our preparations were composed almost exclusively of capillaries. However, the preparation also contained a very small quantity of precapillary vessels. Since smooth muscle expresses the type III enzyme [12], the possibility that a part of the type III enzyme

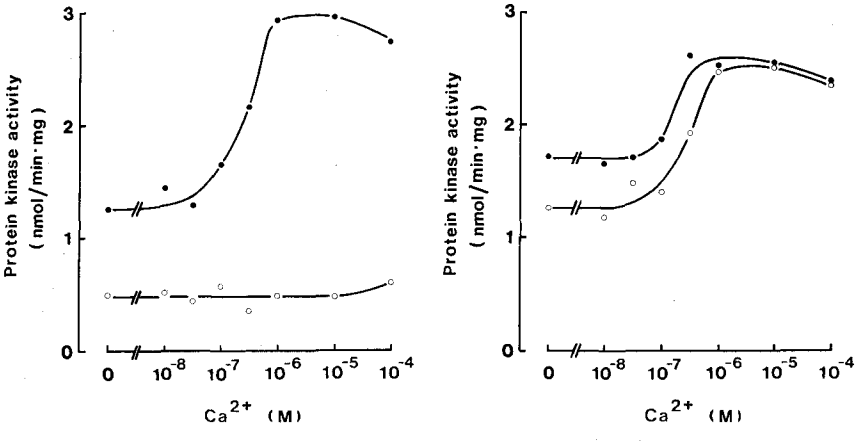


Figure 1. Ca-dependency of protein kinase C activity in soluble (left panel) and membrane (right panel) fractions of rat cerebral microvessels. The various $Ca^{2+}$ concentrations in the assay mixture were made in $Ca^{2+}$-EGTA buffer and indicated in abscissa. The reaction was carried out at 30 °C for 3 min. Protein kinase activity in the presence of 8 μg/ml phosphatidylserine and 0.8 μg/ml diolein (●) or absence of phospholipid (○). Data are representative of 5 separate experiments and are means of duplicate determinations.

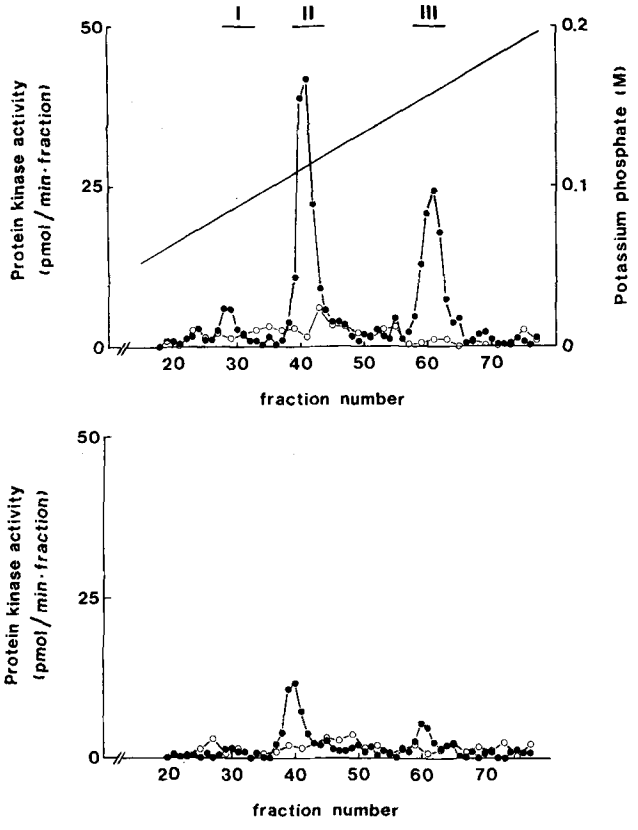


Figure 2. Hydroxyapatite column chromatography of protein kinase C of rat cerebral microvessels. The soluble (upper panel) or membrane (lower panel) fraction of the rat cerebral microvessels was applied to a hydroxyapatite column and protein kinase C was separated by linear concentration gradient of potassium phosphate buffer. Protein kinase activity in the presence of 8 μg/ml phosphatidylserine, 0.8 μg/ml diolein and 0.3 mM $CaCl_2$ (●) or 1 mM EGTA (○). From the hydroxyapatite column used in this study, type I, type II and type III enzymes of the rat brain were eluted at about 80 mM, 110 mM and 155 mM potassium phosphate, respectively, as indicated by bars. Data are representative of 4 (soluble fraction) and 3 (membrane fraction) separate experiments.

of the cerebral microvessels locates on precapillaries cannot be neglected.

The protein kinase C characterized in this study may be involved in growth and differentiation of the endothelial cells, because tumor-promoting phorbol esters, which are known as activators of the protein kinase C, stimulate capillary endothelial cell proliferation in bovine brain [13], capillary-like tube formation in vitro [14], and in vivo angiogenesis [15].

It is interesting to note that cytosolic protein kinase C of the cerebral microvessels translocates completely to the membrane upon stimulation by phorbol ester [1, 16]. So it is likely that all types of the cytosolic enzyme translocate to the membrane as a result of stimulation in the cerebral microvessels.

The type III enzyme is the most widely distributed subtype, whereas distribution of the type II enzyme is restricted to certain types of tissues [6]. Their different tissue distributions and enzymatic properties suggest that the functional roles of the subtypes are different. The large proportion of type II enzyme found in the present study may be involved in physiological functions specific to the cerebral microvessels.

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