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Postnatal Development of a Brain-Specific Subspecies of Protein Kinase C in Rat

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Protein kinase C in the developing rat brain was investigated by a biochemical assay and by light-microscopic immunocytochemistry. The protein kinase was resolved on hydroxyapatite column chromatography into 3 fractions, designated types I, II, and III. Type I, with structure encoded by a γ-sequence, was not detected early postnatally, maintained a low level of activity during the first week, which increased gradually, and reached its maximum around postnatal day 28. This type of enzyme was expressed specifically in nervous tissues, and was not found in any other tissues thus far tested. Type II enzyme activity, a mixture of the 2 subspecies encoded by the βI- and βII-sequences, was found at birth, increased rapidly, and reached a plateau level between postnatal days 14 and 28. This type was the predominant subspecies of protein kinase C in the brain. Type III, its structure encoded by the α-sequence, was also detected at birth, and reached its maximum level on postnatal day 7. Immunocytochemical studies with a monoclonal antibody, which recognized preferentially the type I enzyme, visualized the developmental pattern of type I subspecies in the Purkinje cell, a typical cell having a large quantity of type I protein kinase C.

Protein kinase C is generally accepted as playing pivotal roles in cell-surface signal transduction (for reviews, see Nishizuka, 1984a, b, 1986; Kikkawa and Nishizuka, 1986). Under physiological conditions, this enzyme is activated by diacylglycerol, which may arise transiently in membranes from the receptor-mediated or voltage-dependent hydrolysis of inositol phospholipids (Takai et al., 1979; Kishimoto et al., 1980). Brain tissue contains larger amounts of protein kinase C than other tissues (Kuo et al., 1980; Minakuchi et al., 1981; Kikkawa et al., 1982), and many roles of this enzyme in the modulation of neuronal functions, such as transmitter release and ion conductance, have been suggested (Nishizuka, 1984b, 1986). Immunocytochemical studies using antibodies have confirmed the presence of protein kinase C-like material in many regions of the brain (Girard et al., 1985; Wood et al., 1986; Kitano et al., 1987). Autoradiographic analysis with radioactive phorbol-12,13-dibutyrate has demonstrated the localization of its binding protein in rat brain—possibly protein kinase C (Worley et al., 1986a–c). An earlier study by Kuo and coworkers (Wise et al., 1981; Turner et al., 1984) has shown the ontogenic development of protein kinase C in brain tissues.

Molecular cloning of the cDNA for protein kinase C has recently clarified the existence of multiple subspecies of this enzyme in brain tissues (Coussens et al., 1986; Knopf et al., 1986; Makowske et al., 1986; Ono et al., 1986a, b; Parker et al., 1986; Housey et al., 1987; Ohno et al., 1987). Biochemical studies have suggested some heterogeneity in highly purified protein kinase C from rat brain (Kikkawa et al., 1986; Woodgett and Hunter, 1987). More recently, 3 distinct fractions of this enzyme, types I, II, and III, have been obtained upon chromatography on a hydroxyapatite column (Huang et al., 1986; Kikkawa et al., 1987; Ono et al., 1987). Huang and coworkers (Yoshida et al., 1986) have attempted to clarify the developmental pattern of these 3 fractions. Comparison of each of these fractions with the 4 subspecies of protein kinase C, which were separately expressed in COS (CV1 origin-defective SV40) cells transfected by the cDNAs α, βI, βII, and γ, respectively, identified the primary structures of types I, II, and III protein kinase C (Kikkawa et al., 1987; Ono et al., 1987). Type I has the structure encoded by the γ-sequence; type II is a mixture of the 2 subspecies determined by the βI- and βII-sequences that result from alternative splicing from a single gene; and type III has the structure encoded by the α-sequence. Extending these studies, we wish to describe here the developmental pattern of each of the 3 distinct forms of protein kinase C at an early stage in rat brain. Type I enzyme appears to be located exclusively in the nervous tissue. Morphological support of the development of this species of protein kinase C in cerebellum is also presented.

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Materials and Methods

Materials. Sprague-Dawley rats of various ages were employed for the present studies. The monoclonal antibody designated CKI-97 was prepared as described previously (Kitano et al., 1987). This antibody reacted specifically with type I protein kinase C, as revealed by immunoblotting and immunoprecipitation (see below). Calf thymus H_1 histone was prepared as described previously (Hashimoto et al., 1976). Formaldehyde-fixed, heat-killed *Staphylococcus aureus* (Cowan I) was obtained from Calbiochem. Peroxidase–antiperoxidase complex was obtained from Miles Research Laboratories. Other materials and chemicals were obtained from commercial sources.

Assay and resolution of protein kinase C. Protein kinase C was assayed

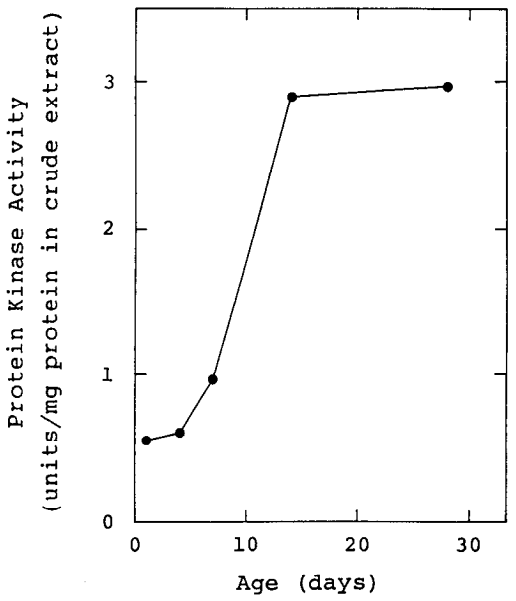

Figure 1. Protein kinase C activity in the developing rat brain after birth. Rat brain tissue of various ages was homogenized, and protein kinase C was assayed as described in Materials and Methods.

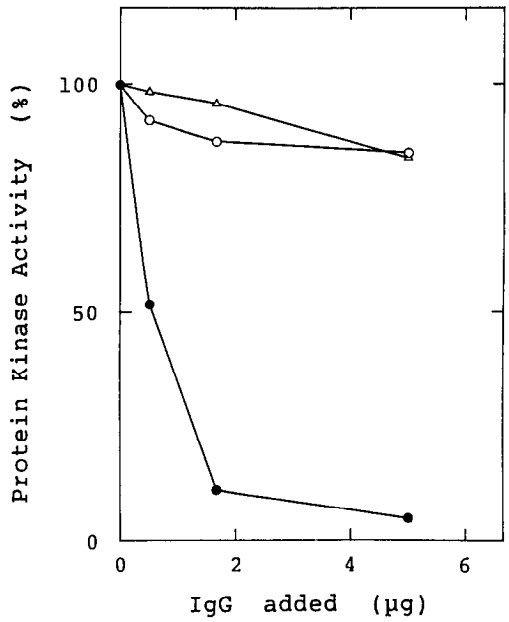

Figure 3. Immunoreactivity of monoclonal antibody CKI-97 with 3 types of protein kinase C. Each type of purified protein kinase C (0.1 μg) was incubated with CKI-97. Other details as described in Materials and Methods. ●——●, Type I protein kinase C; ○——○, type II protein kinase C; △——△, type III protein kinase C.

by measuring the incorporation of ^{32}Pi into calf thymus H_1 histone from γ-^{32}P-ATP in the presence of phosphatidylserine, diolein, and Ca^{2+} under conditions specified earlier (Kikkawa et al., 1986). Basal activity was measured in the presence of 0.5 mM EGTA instead of phosphatidylserine, diolein, and Ca^{2+}. One unit of protein kinase C was defined as the amount of enzyme that incorporated 1 nmol of phosphate/min from ATP into H_1 histone at 30°C. All enzyme-purification procedures were carried out at 0–4°C. Immediately after decapitation, the brain tissue (0.5 gm) was homogenized in a Teflon glass homogenizer with 3 ml of 20 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml leupeptin. The homogenate was centrifuged at 100,000 × *g* for 60 min. The supernatant was employed as crude extract. The crude extract (approximately 10 mg protein) was applied to a DE-52 column (0.9 × 3 cm) that had been equilibrated prior to this with 20 mM Tris-HCl, pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol (buffer A). The column was washed with 6 ml of buffer A, followed by 25 ml of buffer A containing 20 mM NaCl. Protein kinase C was then eluted batchwise with 10 ml of buffer A containing

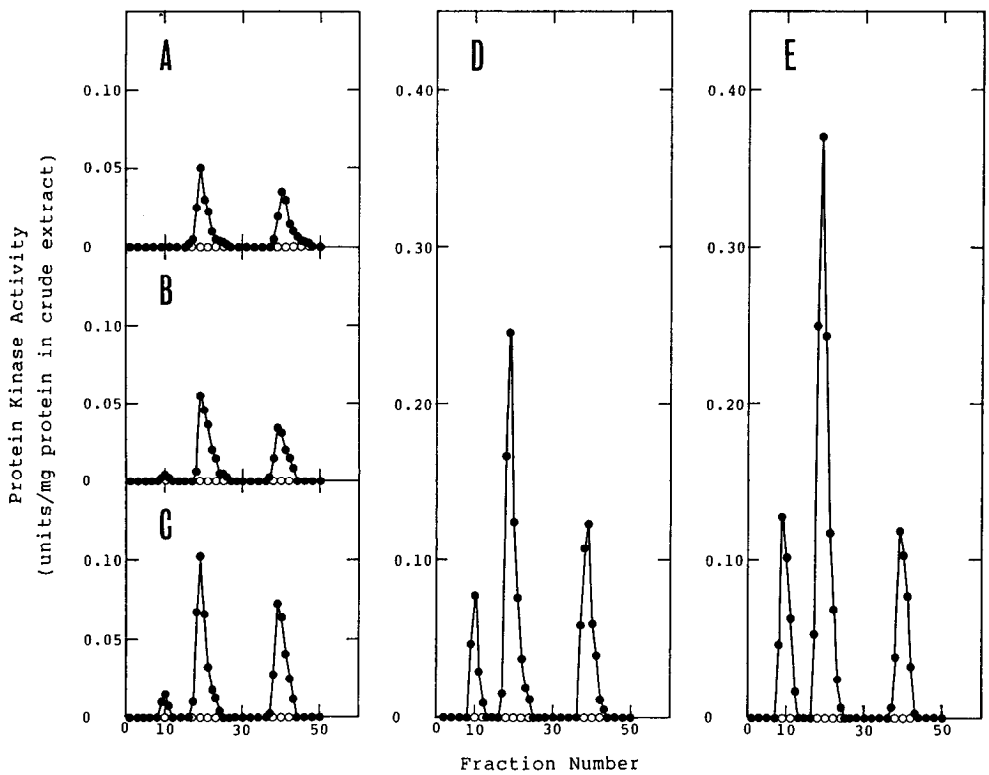

Figure 2. Expression of 3 types of protein kinase C in rat brain. DE-52 fractions from rat brain of various ages were subjected to hydroxyapatite column chromatography and assayed as described in Materials and Methods. ●——●, Protein kinase activity in the presence of 8 μg/ml phosphatidylserine, 0.8 μg/ml diolein, and 0.5 mM $CaCl_2$; ○——○, protein kinase activity in the presence of EGTA instead of phosphatidylserine, diolein, and $CaCl_2$. The peaks that appear around fraction numbers 10, 20, and 40 are type I, type II, and type III protein kinase C, respectively. *A*, The first day after birth. *B*, Day 4. *C*, Day 7. *D*, Day 14. *E*, Day 28.

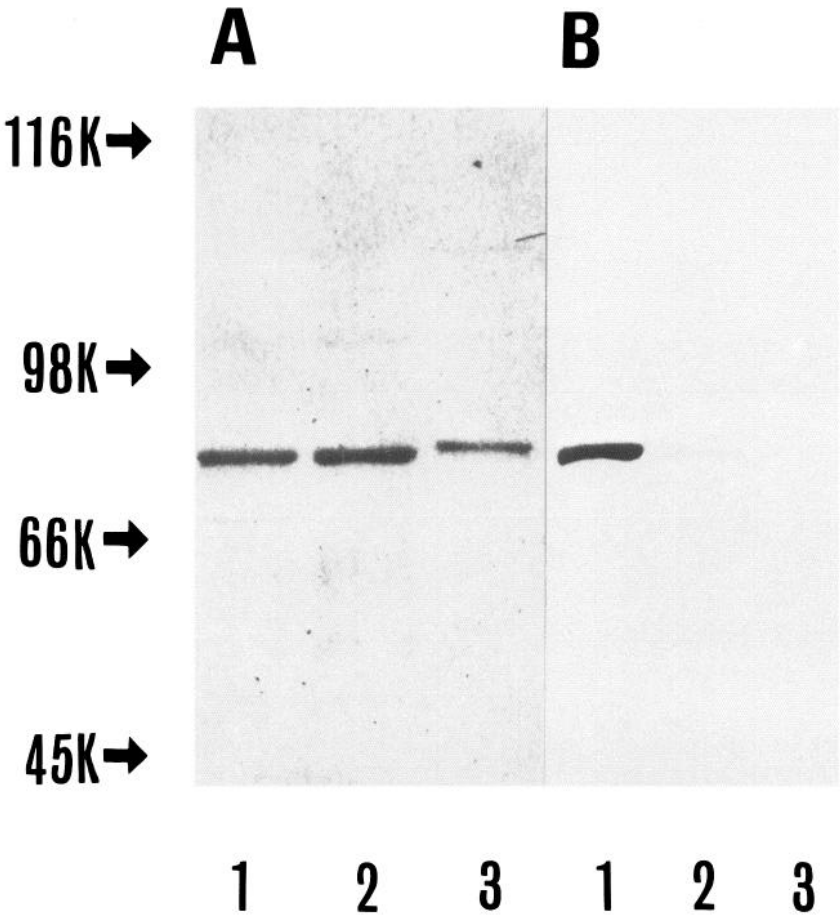

Figure 4. Western blot analysis of 3 types of protein kinase C with monoclonal antibody CKI-97. Each type of protein kinase C (0.2 μg) was subjected to SDS-PAGE, transferred to nitrocellulose, and stained with Coomassie brilliant blue or reacted with the monoclonal antibody (2 μg/ml), as described in Materials and Methods. *A,* Protein staining. *B,* Western blot. Lane *1,* Type I protein kinase C; lane *2,* type II protein kinase C; lane *3,* type III protein kinase C. Molecular-weight markers: 116 kDa, β-galactosidase; 98 kDa, phosphorylase b; 66 kDa, bovine serum albumin; 45 kDa, ovalbumin.

120 mM NaCl. This enzyme fraction was applied to a packed hydroxyapatite column (0.78 × 10 cm; type S, Koken Co., Tokyo), which was connected to a high-performance liquid chromatography (HPLC) apparatus (Pharmacia; FPLC system) and equilibrated with 20 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA, 10% glycerol, and 10 mM 2-mercaptoethanol (buffer B). The column was washed with 50 ml of buffer B, and protein kinase C was then eluted by application of a linear concentration gradient of potassium phosphate (20–215 mM) prepared in 84 ml of buffer B. Fractions of 1 ml each were collected.

Immunoprecipitation and Western blot analysis. Each type of purified rat brain protein kinase C obtained by hydroxyapatite column chromatography (Kikkawa et al., 1987) was incubated with monoclonal antibody CKI-97, and *S. aureus* cells were added as an immunoadsorbant under conditions described previously (Kitano et al., 1987). The supernatant of the immunoprecipitation reaction was subjected to protein kinase C assay. Western blot analysis of each type of protein kinase C was performed with this antibody under conditions specified previously (Kitano et al., 1987).

Protein determination. Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard protein.

Immunocytochemical procedures. Immunocytochemical studies were carried out as described (Saito et al., 1986). Rats of various ages were anesthetized with pentobarbital and perfused via the left ventricle with 0.1 M phosphate-buffered saline, followed by 0.1 M phosphate buffer, pH 7.5, containing 4% paraformaldehyde, 0.5% glutaraldehyde, and 0.2% picric acid. The brain was then removed, postfixed with the phosphate buffer containing 4% paraformaldehyde and 0.2% picric acid, and cut into sagittal sections (50 μm) using a Vibratome. The sections were washed with 0.1 M phosphate-buffered saline containing 0.3% (wt/vol) Triton X-100. The samples were incubated for 18 hr at 4°C with monoclonal antibody CKI-97 (1 μg/ml), and stained by the peroxidase–antiperoxidase method for microscopic observation.

Results

The activity of protein kinase C in rat brain increased as a function of age (Fig. 1). To determine the amount of enzyme in this tissue, interfering materials were removed by DE-52 column chromatography. Most of the activity of protein kinase C was recovered in the eluate by the batchwise elution procedure. The overall level of protein kinase C was relatively low during the early days after birth, increased rapidly between postnatal days 4 and 14, and nearly reached the plateau level after day 14.

Protein kinase C in the adult rat brain was resolved into 3 peaks, designated types I, II, and III according to the order of elution from the hydroxyapatite column, which was connected to an HPLC system. It is worth noting that the type I enzyme with the γ-sequence was not detected at birth, and was expressed slowly. This subspecies did not reach its maximum level until after 4 weeks (Fig. 2). Type I protein kinase C was found specificially in nervous tissue, and has not been detected in other tissues thus far tested, including liver, kidney, adrenal gland, spleen, fibroblasts, and HL60 cells. The monoclonal antibody CKI-97, which specifically recognized the type I enzyme (see below), could not stain any material in tissues other than brain and spinal cord.

Both types II and III enzymes, on the other hand, were found at birth, increased rapidly, and nearly reached plateau levels at 2 weeks. The type II enzyme was apparently most abundant. This type of protein kinase C seems to be a mixture of 2 sub-

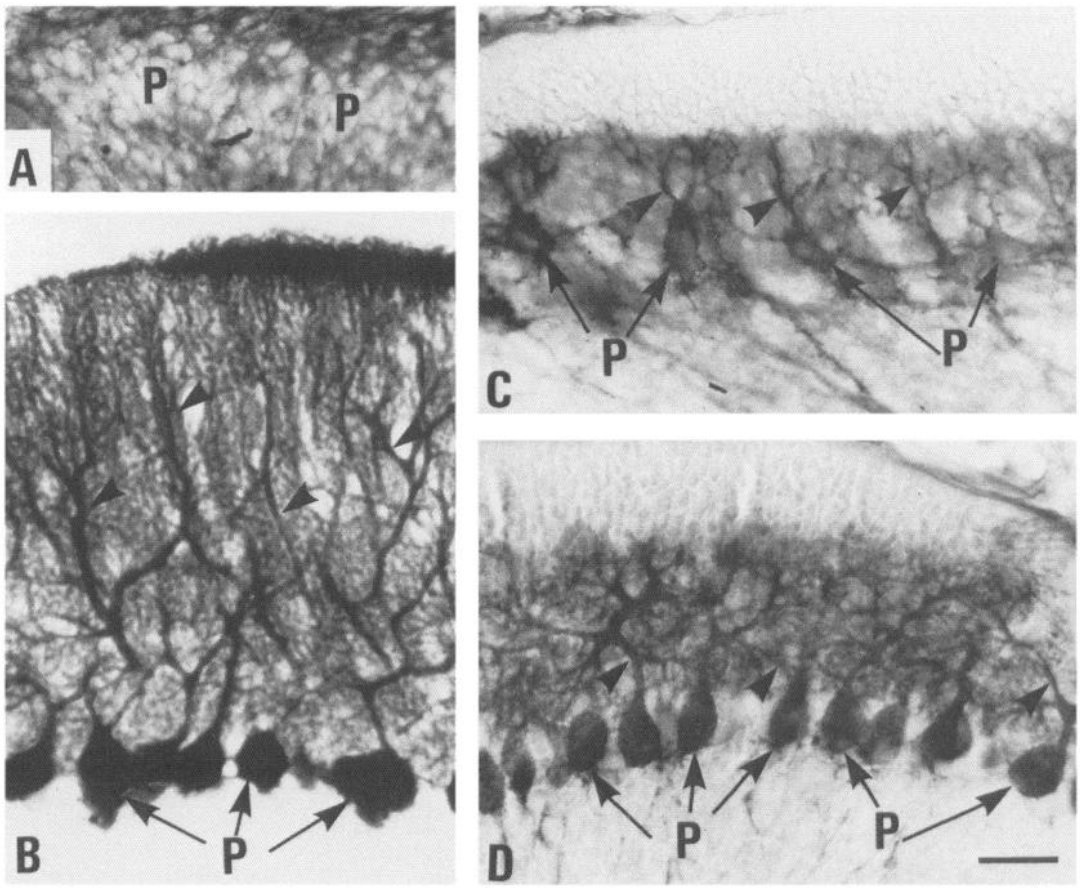

Figure 5. Immunocytochemical localization of protein kinase C in rat cerebellum of various ages. Sections of rat cerebellum were incubated with 1 μg/ml of monoclonal antibody CKI-97, and were stained by the peroxidase–antiperoxidase method, as described in Materials and Methods. *A,* The first day after birth. *B,* Adult. *C,* Day 7. *D,* Day 14. *P,* Perikarya of Purkinje cells; *arrows,* dendrites of Purkinje cells. Same magnification for *A–D.* Calibration bar, 100 μm.

species having the structures of the βI- and βII-sequences, which are determined by alternative splicing (Ono et al., 1987). The relative ratio of the 2 subspecies in this fraction is not known. Type III protein kinase, having the α-sequence, was at its maximum at 2 weeks. The differential postnatal development of the 3 types of protein kinase C did not appear to be simply an artifact of the purification procedures, and the elution profiles were highly reproducible. The enzymes of these fractions showed apparently identical molecular weights of about 80 kDa upon SDS–PAGE (see Fig. 4). Proteolytic modification may presumably be excluded, since the extraction buffer contained high concentrations of EGTA, EDTA, PMSF, and leupeptin. The primary protein structure of each subspecies was identified by comparison with the protein kinase that was separately expressed in COS cells transfected by the plasmids containing the respective cDNAs; types I, II, and III enzymes were shown to correspond to the γ-, βI-, plus βII-, and α-sequences, as described previously (Kikkawa et al., 1987; Ono et al., 1987).

The monoclonal antibody CKI-97, previously raised to a mixture of these subspecies of protein kinase C (Kitano et al., 1987), was subsequently found to react preferentially with the type I enzyme, having the γ-sequence. Figure 3 shows that this antibody bound specifically to the type I enzyme. Types II and III enzymes react weakly, if at all, with this antibody. Figure 4 illustrates the immunoblotting of these subspecies, and again shows that the CKI-97 antibody specifically recognizes type I protein kinase C. In agreement with these results, type I enzyme-positive-immunoreactive material was not found after birth in the Purkinje cell layer of cerebellar cortex (Fig. 5*A*), which is seen to contain a large amount of type I protein kinase C in the mature rat brain (Fig. 5*B*). This immunoreactive material was increased in the perikarya, axons, and dendrites of Purkinje cells in a manner consistent with the biochemical analysis. The profile of Purkinje cells, with axons and poorly arborized short dendrites, was seen on day 7 (Fig. 5*C*), and a unique feature of the cells was clearly stained arborized dendrites, which had not yet fully matured on day 14 (Fig. 5*D*).

Discussion

Kuo and coworkers (Wise et al., 1981; Turner et al., 1984) observed that protein kinase C is detectable in prenatal embryonic rat brain tissue, and increases rapidly after birth. Huang and coworkers (Huang et al., 1986; Yoshida et al., 1986) subsequently separated this enzyme into 3 distinct fractions, and briefly presented different patterns of postnatal development for each fraction. Earlier reports from our laboratories (Kikkawa et al., 1987; Ono et al., 1987) have clarified the structure of each fraction of the enzyme by comparison with the 4 subspecies of protein kinase C that were separately expressed in COS cells transfected by the respective cDNAs. By using a high resolution

of these fractions of protein kinase C by HPLC, together with a monoclonal antibody specific to the enzyme having the γ-sequence, the present study demonstrates that this type of protein kinase C is expressed only after birth, and specifically in nervous tissues. This subspecies is most abundant not only in perikarya, but in dendrites and axons of Purkinje cells and many other neuronal cell types, such as pyramidal cells in the hippocampus, as has been described in detail (Saito et al., 1988). The immunoreactive material was not found in any tissues other than brain and spinal cord, implying a specific role of the type I enzyme in the control of neuronal functions. Brandt et al. (1987) have recently shown the patterns of expression of mRNAs of several subspecies of protein kinase C in rat brain by *in situ* hybridization histochemical analysis. The mRNA of "PKC-I," which corresponds to the γ-sequence, is shown to be abundant in Purkinje cells, while the granular cell layer of cerebellum is labeled with the DNA probes of "PKC-II" and "PKC-III," which have βI- and βII-sequences, respectively. The distinct distributions of types II and III protein kinase C have been shown by biochemical analysis in various rat brain regions (Shearman et al., 1987). The morphological studies in which protein kinase C was localized using radioactive phorbol-12,13-dibutyrate binding (Worley et al., 1986a, b) provide a picture of the total distribution of all 3 types of enzyme, but it is necessary to investigate the distribution and development of types II and III enzymes with specific antibodies against these subspecies of protein kinase C when they become available.

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