Light and Electron Microscopic Localization of β I-, β II-, and γ -Subspecies of Protein Kinase C in Rat Cerebral Neocortex

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We have localized the β I-, β II-, and γ -subspecies of protein kinase C in cerebral neocortex with light and electron microscopic immunocytochemistry using a monoclonal antibody against γ -PKC and polyclonal antisera to β I- or β II-PKC-specific oligopeptides. The γ -PKC-immunopositive cell bodies were seen mostly in layers II, V, and VI, and the vast majority of them were pyramidal cells. The β II-PKC immunoreactive cell bodies were observed in layers II, III, V, and VI, and most of them seemed to be pyramidal cells. Both γ - and β II-PKC were colocalized in some pyramidal cells in layers II, V, and VI. The small number of β I-PKC immunoreactive cell bodies were observed in the neocortex, and many of them were nonpyramidal cells. About 80% of the β I-PKC-immunoreactive cells were shown to be GABAergic neurons. The γ -PKC-immunopositive neuropils were observed in layers I, II, V, and VI, while β II-PKC-immunoreactive neuropils were seen in layers I-III, V, and VI. The distribution of each subspecies is much the same throughout all regions of the neocortex, although with different intensities of immunoreactivity. Electron microscopic studies revealed that, in the perikarya, γ -PKC was distributed throughout the cytoplasm, β I-PKC was just adjacent to the plasma membrane, and β II-PKC was located around the Golgi complex. The immunoreactivity of these 3 subspecies was also seen in dendrites and axons, but no immunoreactivity of these subspecies was found in the presynaptic terminals in the present study. The discrete cellular and intracellular distributions of protein kinase C subspecies imply that each subspecies has a specific role in neuronal activity in the cerebral neocortex.

The physiological importance of protein kinase C (PKC) in cell surface signal transduction is now widely accepted and well documented (for reviews, see Nishizuka, 1984, 1986). The enzyme is distributed ubiquitously in tissues and organs, with the highest level of activity in the brain. Analysis of cDNA clones has revealed that PKC is a large family consisting of at least 7 subspecies (α , β I, β II, γ , δ , ϵ , and ζ) with closely related but distinct structures (for review, see Nishizuka, 1988). PKC purified from rat brain has also been resolved into 3 subfractions,

type I, II, and III by chromatography on a hydroxyapatite column (Huang et al., 1986). Comparison of these fractions with the enzymes expressed in COS-7 cells transfected by the respective cDNA-containing plasmids has revealed that the enzyme encoded by the γ -sequence is found in type I, the 2 subspecies determined by the β I- and β II-sequences are found in type II, and PKC encoded by the α -sequence is contained in type III (Huang et al., 1987b; Kikkawa et al., 1987; Ono et al., 1987).

Several groups have reported immunocytochemical studies of PKC distribution in the brain without showing subspecies-specific distribution (Girard et al., 1985; Wood et al., 1986; Mochly-Rosen et al., 1987). Previously, we have prepared 3 monoclonal antibodies against PKC isolated from the rat brain soluble fraction, named CKI-33, CKI-97, and CKII-90 (Kitano et al., 1987). A mixture of these antibodies, which predominantly recognizes type I PKC, was used to show the entire immunocytochemical map of protein kinase C in the rat brain (Saito et al., 1988) and CKI-97, which reacts specifically with type I PKC, was used to reveal the electron microscopic localization of type I PKC in Purkinje cells (Hashimoto et al., 1988; Kose et al., 1988). Recently, other groups have also reported the distribution of PKC subtypes using subtype-specific antibodies (Huang et al., 1987b, 1988; Hidaka et al., 1988; Stichel and Singer, 1988). We have also prepared 2 polyclonal antibodies specific for the enzymes encoded by β I- and β II-sequences, respectively, and revealed the distinct distribution of β I- and β II-PKC in the rat brain (Ase et al., 1988; Hosoda et al., 1989; Saito et al., 1989).

Cerebral cortex is known to contain large amounts of PKC (Huang et al., 1987a). Some groups have reported the involvement of PKC in neuronal functions in the cortex by using phorbol esters that activate PKC; e.g., modulation of neurotransmitter release such as norepinephrine (Nichols et al., 1987; Shuntoh et al., 1988) and 5-HT (Friedman and Wang, 1989), long-term changes of membrane excitability (Baranyi and Szente, 1987) and down-regulation of muscarinic cholinergic receptors (Jia et al., 1989). Several groups also showed the generation of long-term potentiation (LTP) in cerebral neocortex (Lee, 1982; Artola and Singer, 1987; Bindman et al., 1987), as well as in hippocampus, where PKC are demonstrated to play an important role for triggering LTP. Although these reports indicate that PKC acts on several neuron-specific functions in the cerebral cortex, few studies about the precise localization of PKC subspecies have been documented. In the present study, we have examined in detail the distribution of the γ -, β I-, and β II-PKC in cerebral neocortex under light and electron microscopy to elucidate the functional role of each subspecies of PKC.

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Materials and Methods

Preparation of antibodies against PKC subspecies and against GABA. The monoclonal antibody against γ -PKC was obtained and characterized by Kitano et al. (1987) and Hashimoto et al. (1988). In brief, PKC was purified from the soluble fraction of rat brain, and a monoclonal antibody against PKC (CKI-97), was obtained by a routine method of immunizing mice with the purified enzyme. Immunoprecipitation and Western blot analysis of CKI-97 indicated that this antibody reacts strongly and preferentially with type I PKC, which is encoded by the γ -sequence.

The polyclonal antisera against the β I- and β II-PKC were obtained and characterized as described previously (Hosoda et al., 1989; Saito et al., 1989). In brief, oligopeptides corresponding to the carboxyl-terminal portions of the β I-PKC (residues 661–671; SYTNPEFVINV) and the β II-PKC (residues 660–673; SFVNSEFLKPEVKKS) were synthesized and were coupled to BSA by glutaraldehyde (GA). Then, the conjugates were mixed with complete Freund's adjuvant and injected intracutaneously into rabbits. After 5–7 boosters with the same antigen emulsified with incomplete Freund's adjuvant, antisera were obtained and purified by affinity column chromatography. Western blot analysis revealed that the antibodies reacted specifically with each subspecies.

The monoclonal antibody against GABA was raised and characterized as described (Sakaue et al., 1990). In brief, GABA (1 mmol) was conjugated to BSA (120 mg) by GA (1 mmol) in 0.1 M sodium phosphate buffer (PB, pH 7.4). The product was dialyzed, gel-filtrated, and emulsified with Freund's adjuvant. By immunizing mice with the conjugate, a monoclonal antibody against GABA was obtained using routine methods. ELISA and the spot test revealed this antibody reacted strongly and preferentially with GABA and showed no cross-reactivity against the other amino acids such as L- and D-aspartate, glutamate, glutamine, alanine, glycine, taurine, proline, and leucine, except for weak cross-reactivity against β -alanine.

Preparation of tissues and immunocytochemical staining. The immunocytochemical procedures were performed as described previously (Kose et al., 1988; Saito et al., 1988). In brief, Wistar rats were anesthetized with an intraperitoneal infusion of pentobarbital and perfused through the left ventricle with saline and the fixative which contained 4% paraformaldehyde (FA), 0.5 or 1.0% glutaraldehyde, and 0.2% picric acid (PA) in 0.1 M phosphate buffer (PB). The perfused brain was removed from the cranial cavity, immersed for 2 d in a fixative containing 4% FA and 0.2% PA, and washed for several days with 30% sucrose in 0.1 M PB.

For light microscopic studies, the brain was cut on a cryostat into frontal sections. The sections were incubated for 18 hr at 4°C with the primary antibodies (CKI-97 was diluted to 5 μ g IgG/ml, β I- and β II-PKC antiserum was diluted to 50 ng IgG/ml). After washing with PBS containing 0.03% Triton X-100, the sections were incubated for an additional 4 hr with goat anti-mouse IgG (GAM; Miles) or goat anti-rabbit IgG (GAR; MBL, Nagoya, Japan), diluted 1:1000. The sections were washed and then incubated for 90 min with mouse peroxidase-antiperoxidase (PAP) complex (ICN, Illinois) or rabbit PAP complex (ICN), diluted 1:5000. All antibodies were diluted in 0.1 M PBS containing 0.03% Triton X-100 and 5% normal goat serum. After rinsing 3 times, the preparations were reacted with 0.025% 3,3'-diaminobenzidine (DAB; Sigma), 0.2% nickel ammonium sulfate and 0.05% hydrogen peroxide. The sections were observed and photographed under a Zeiss light microscope.

Alternate sections were stained with toluidine blue for study of the cytoarchitecture. Some sections stained with the β II-PKC antiserum by the PAP technique were poststained with 0.1% toluidine blue to elucidate the sizes and shapes of the immunopositive cells. The cytoarchitectonic areas were named according to Zilles and Wree (1985).

For double staining of the γ - and β II-PKC, the sections were incubated with a mixture of monoclonal antibody against the γ -PKC (CKI-97, 10 μ g/ml) and polyclonal antibodies against the β II-PKC (100 ng/ml) for 18 hr at 4°C. The sections were then incubated with Texas red linked donkey anti-rabbit immunoglobulin (Amersham, UK) diluted 1:50 and fluorescein isothiocyanate conjugated goat anti-mouse Fab antibodies (FITC-GAM; ICN) diluted 1:100 at 4°C for 2 hr, and they were mounted on slides with buffered glycerol for fluorescent microscopic observation. As a control for non-cross-reactivity of the secondary antisera, tissue was processed with each primary antibody alone and then with both of the secondary antisera as described above. In each case, the only immunofluorescence observed was from the secondary antiserum directed

against the species-IgG of the primary antibodies used. For double staining of the β I-PKC and GABA, the sections were stained with the β I-PKC antiserum by the PAP method, then incubated with or without monoclonal antibody against GABA at 4°C for 18 hr and stained with FITC-GAM, washed, mounted, and observed as above. No immunofluorescence was observed when the sections were stained without monoclonal antibody against GABA.

For electron microscopic studies, the fixed cerebral cortex was frozen in liquid nitrogen, thawed, and cut on a Vibratome (40 μ m thick). The sections were then incubated for 48 hr with the primary antibodies (identically diluted as for light microscopic studies). The sections were washed with 0.1 M PBS, incubated for 18 hr with GAM (diluted 1:100) or GAR (diluted 1:500), washed, and incubated for 90 min with mouse PAP (diluted 1:200) or rabbit PAP (diluted 1:500). All antibodies were diluted in 0.1 M PBS containing 5% normal goat serum. After rinsing 3 times, the preparations were developed with DAB, nickel ammonium sulfate, and hydrogen peroxide. Control sections were incubated with preimmune serum. The stained sections were postfixed for 1 hr in 2% osmium tetroxide in 0.1 M PBS, dehydrated in graded alcohol, and flat-embedded on siliconized slides in resin (Quetol-812). After polymerization at 60°C for 48 hr, densely stained areas were selected, cut off, and attached to Epon supports for further sectioning on a Reichert-Jung Ultracut E ultramicrotome. Ultrathin sections were cut and mounted on 200 mesh bare grids (MAXTAFORM), stained with 1% uranyl acetate in 50% ethanol, and observed with a JEM 100 SX electron microscope at 80 kV.

Results

Light microscopic localization of γ -, β I-, and β II-PKC

In the neocortex, many neuronal cell bodies with γ -PKC immunoreactivity were distributed in lower half of layer V and the lower half of layer VI (Fig. 1A). There was a smaller number of γ -PKC-immunopositive cell bodies in layer II and only a few in layer III and IV. The γ -PKC-positive cell bodies were not observed in layer I. The vast majority of γ -PKC-immunolabeled neurons in layer V were pyramidal cells, which were 15–20 μ m in size (Fig. 2B). Their long apical dendrites extended vertically across layers IV–II and branched in layer II (Fig. 2A). Most γ -PKC-positive cells in layer VI were modified pyramidal or spindle cells, which had diameters of 9–12 μ m (Fig. 2C). The majority of γ -PKC-positive cells in layer II appeared to be pyramidal cells, but it was difficult to distinguish them into cell types because of their weak immunoreaction and strong immunoreactivity of the neuropils around them. A small number of γ -PKC-positive nonpyramidal cells were distributed in layers II–V. They were weakly or moderately stained multipolar or bipolar cells (data not shown). The distribution pattern of γ -PKC immunoreactivity in each cortical layer was essentially the same in all neocortical regions examined in the present study, but the number of immunopositive cells and intensity of immunoreactivity of the γ -PKC were different between various cortical areas (Fig. 3). For example, layer V of the primary motor area (Fr2) contained less γ -PKC-positive pyramidal cells but more intense immunoreaction in the neuropils (Fig. 3).

In all neocortical areas, β II-PKC-immunopositive cells were found in layers II/III, V, and VI but not in layers I and IV, and the number of them was much greater than that of γ - or β I-PKC-positive cells (Fig. 1B). The β II-PKC-immunoreactive products were seen as large dots. In layer VI, there were many cell bodies with very strong β II-PKC immunoreactivity, which were 9–14 μ m in size (Fig. 2F). Many moderately stained cells were found in layers II and III, and they had diameters of 11–16 μ m (Fig. 2D). The β II-PKC-positive cells of 11–20 μ m in layer V contained smaller dots than those in the other layers (Fig. 2E). In the preparation poststained with toluidine blue, the majority of these β II-PKC-positive neurons appeared to be

Figure 1. Immunohistochemical localization of PKC subspecies in somatosensory cortex (Par I) as demonstrated by peroxidase-antiperoxidase technique. *A*, γ -PKC immunoreactivity in layers I, II, V, and VI. Immunopositive cells are seen in layer II, deep layer V, and deep layer VI. Most of them are pyramidal cells with long apical dendrites. *B*, β II-PKC immunoreactivity in layers I–III, V, and VI. A large number of neurons with large immunoreactive dots are seen in layers II, III, V, and VI. Most of them are pyramidal cells, but immunoreactivity of the apical dendrites is weak and discontinuous. *C*, A small number of β I-PKC-immunoreactive perikarya are distributed in layers I–VI. Most of them are nonpyramidal cells. Scale bar, 200 μ m.

pyramidal cells in layers II, III, and V, and modified pyramidal or spindle cells in layer VI (data are not shown). Weak β II-PKC immunoreactivity was occasionally seen in vertically extending dendrites (Fig. 2*E*). The β II-PKC-positive neuropils were intensely stained in layers I, II/III, the superficial part of V and VI. Layer IV was the most weakly stained. The pattern of distribution of the β II-PKC immunoreactivity was much the same in all neocortical regions (Fig. 4).

Double staining of γ - and β II-PKC revealed that almost all of the γ -PKC-immunoreactive neurons contained the β II-PKC-immunoreactive materials (Fig. 5). In layers V and VI of the parietal cortex, medium-sized pyramidal cells were both γ - and

β II-immunopositive (Fig. 5, *A*, *B*), but smaller neurons were only β II-PKC immunoreactive (Fig. 5*B*).

The β I-PKC-immunoreactive neuronal cell bodies were sparsely distributed in layers I–VI (Figs. 1*C*, 6). The greatest number of β I-PKC-immunoreactive perikarya was observed in layer V. The dominant form of β I-PKC-positive cells was multipolar cells in a variety of shapes and sizes (10–20 μ m) and which were seen in layers I–VI (Fig. 7*A*). In layer I, some β I-PKC-positive cells were horizontal cells with spindle-shaped cell bodies, which were 7–9 $\times$ 15–18 μ m in size (Fig. 7*B*). They had horizontally extending dendrites emerging from both ends of elongated cell bodies. A small number of bipolar cells in layers

Figure 2. Microphotographs of γ - and β II-PKC immunoreaction in somatosensory cortex (Par 2) were stained with CKI-97 (*A–C*) or with the anti-serum against β II-PKC (*D–F*) using peroxidase–antiperoxidase technique. *A*, In layer II, a few neurons have moderate or weak γ -PKC immunoreaction. *B*, In deep layer V, medium-sized pyramidal cells have strong γ -PKC immunoreaction. Stronger immunoreaction of neuropils is observed in superficial layer V than in deep layer V. *C*, In deep layer VI, many modified pyramidal cells or spindle cells have moderate γ -PKC immunoreactivity. Horizontal cells in the white matter are also stained (*arrows*). *D*, In layer II, a smaller number of β II-PKC-immunopositive neurons are observed than in layer III. *E*, In layer V, small- or medium-sized pyramidal cells have smaller β II-PKC-immunoreactive dots with moderate intensity. *F*, In layer VI, many modified pyramidal cells or spindle cells have strong β II-PKC-immunoreactive dots. Scale bar, 50 μ m.

Figure 3. γ -PKC immunoreactivity in various cortical areas as demonstrated by peroxidase–antiperoxidase technique. *A*, Primary motor (*Fr 2*); *B*, primary auditory (*Te 1*); and *C*, primary visual (*Oc 1*) cortex. In all areas, most of the γ -PKC-immunoreactive neurons are localized in layers II, V, and VI, and immunopositive neuropils are seen in layers I, II, V, and VI. Scale bar, 200 μ m.

II and III were also β I-PKC immunopositive (Fig. 7C). They had ovoid cell bodies, which are 7–9 $\times$ 13–19 μ m in size, with vertically extending dendrites from both poles. A few β I-PKC-positive neurons in layer V resembled pyramidal cells in shape, but apical dendrites with immunoreactive products were not observed (Fig. 7D). β I-PKC-immunopositive neurons of each type described here were observed in all of the cortical areas. About 80% of β I-PKC-immunoreactive neurons contained GABA immunoreactivity in the double-staining study using the β I-PKC antiserum and the monoclonal antibody against GABA, while 30–40% of GABA-immunopositive neurons were β I-PKC-immunopositive (Fig. 8). The β I-PKC-immunoreaction of the neuropils was weak throughout the neocortex.

Electron microscopic localization of γ -, β I-, and β II-PKC

Electron microscopic analysis of both parietal and frontal cortices revealed that the dense immunoreactivity of γ -PKC was present in the cytoplasm (Fig. 9A). Weak immunoreactivity was

sometimes seen on the plasma membrane. Moderate immunoreaction was found in the nucleoplasm, but there was no or faint immunoreactivity in the nucleoli and on the nuclear membrane. At higher magnification, immunoreactive products were observed throughout the perikaryal cytoplasm except for inside the cell organelles (Fig. 10A). Immunoreactivities in the perikarya were associated with free ribosomes and the outer membrane of mitochondria. Weak staining was observed along the outer membrane of the endoplasmic reticulum (Fig. 10A).

In the dendrites of the pyramidal cells, dense γ -PKC immunoreactivity was seen in the cytoplasm, especially along the microtubules, but not within the organelles. The cytoplasmic membrane of dendrites was weakly stained when dense immunoreactivity was seen in the cytoplasm just adjacent the cytoplasmic membrane. Stained dendritic spines were seen to form synaptic contacts with immunonegative terminals, but no or only a faint immunoreactive product was seen on postsynaptic density (Fig. 10B). γ -PKC-immunopositive myelinated axons

Figure 4. β II-PKC immunoreactivity in various cortical areas as demonstrated by peroxidase–antiperoxidase technique. *A*, Primary motor (*Fr 2*); *B*, primary auditory (*Te 1*); and *C*, primary visual (*Oc 1*) cortex. No or faint immunoreactivity is seen in layer IV throughout neocortex. Scale bar, 200 μ m.

were also seen (Fig. 10C). We could detect no or faint γ -PKC-immunopositive terminals in the cortical preparations examined.

Electron microscopic analysis of the β II-PKC-immunopositive pyramidal cells showed the dense β II-PKC immunoreactivity in the area of the Golgi complex in somata and in primary dendrites (Figs. 9B, 11A). Staining was mainly observed in the cytoplasm around the *trans* compartment of the Golgi complex and on its membrane, but the inside of the Golgi complex was not stained. Most, if not all, the Golgi complex was associated with the immunoreactive products in the β II-PKC-positive cells. No or only a faint immunoreactivity was observed within the mitochondria, endoplasmic reticulum, nuclei, and nucleoli. In immunopositive neuropils, the β II-PKC immunoreaction was observed in the branched dendrites but not on the postsynaptic densities (Fig. 11B). In primary dendrites, the β II-PKC im-

munoreaction was observed just adjacent to the cytoplasmic membrane (data are not shown). Immunopositive dendritic spines were not observed. Immunoreaction was also seen in the myelinated axons (Fig. 11C) but no or only a faint immunoreactivity in presynaptic terminals.

Electron microscopic analysis of the β I-PKC-positive neurons showed the strong immunoreactivity to be in the perikarya just adjacent to the plasma membrane (Fig. 9C). The fringy staining consisted of clustered immunomaterial which was observed on and just inside of the plasma membrane. They were not always associated with the postsynaptic density (Fig. 12, *A, B*). In some cells, dense but small immunoreactive products were seen around a subpopulation of the Golgi complex (Fig. 9C). No or only a faint immunoreactivity was observed on the mitochondria and endoplasmic reticulum. Weak staining was sometimes seen in the nuclei. Around the β I-PKC-immunopositive perikarya,

Figure 5. Double staining of γ - and β II-PKC immunoreactivity in cerebral neocortex. *A*, γ -PKC immunoreactivity in layer V of parietal cortex as demonstrated by the indirect FITC immunofluorescent technique. *B*, β II-PKC immunoreactivity in layer V of parietal cortex as demonstrated by the indirect Texas red immunofluorescent technique. Some pyramidal cells contained both β II- and γ -PKC immunoreactivity (arrows); the other pyramidal cells have only β II-PKC immunoreaction (asterisks). Scale bar, 20 μ m.

Figure 6. β I-PKC immunoreactivity in various cortical areas as demonstrated by peroxidase–antiperoxidase technique. *A*, Primary motor (*Fr 2*); *B*, primary auditory (*Te 1*); and *C*, primary visual (*Oc 1*) cortex. β I-PKC-immunoreactive neurons are sparsely distributed throughout the neocortex. Scale bar, 20 μ m.

Figure 7. Higher-magnification photomicrographs of β I-PKC-immunopositive neurons as demonstrated by peroxidase–antiperoxidase technique. *A*, Multipolar medium-sized cells in layer V; *B*, horizontal cell in layer I; *C*, bipolar cell in layer II; and *D*, pyramidal cell in layer V. Scale bar, 20 μ m.

stained dendrites (Fig. 12*B*) and myelinated axons (Fig. 12*C*) were seen, but no or only a faint immunoreaction was observed in the presynaptic terminals.

Discussion

Cellular distribution of β I-, β II-, and γ -PKC

In the present study we have localized β I-, β II-, and γ - subspecies of PKC in the rat cerebral neocortex by light and electron microscopic immunocytochemistry.

Immunocytochemical localization of PKC isozymes was reported by Huang et al. (1988) using isozyme-specific monoclonal and polyclonal antibodies. Their results concerning type I (corresponding to γ -PKC) and type II (corresponding to β I- and β II-PKC) in the neocortex were quite different from our results. With their antibodies, both type I and type II PKC immuno-

reactivities are localized in the pyramidal cells and granule cells throughout layers II–VI, and no immunoreactivity was seen in layer I. In our study, the γ - and β II-PKC were distributed mostly in pyramidal cells and showed distinct laminar patterns. Although the reasons for the differences between our and their results are not clear, the laminar pattern of the PKC subspecies in the neocortex was very similar to the result obtained by *in situ* hybridization (Brandt et al., 1987). Our data do not coincide completely with the result by *in situ* hybridization in detail, but the distributions are considered to be basically consistent because the resolution of X-ray film is not sufficient to distinguish the cortical layers precisely. The present findings also agree with the immunocytochemical study using a monoclonal antibody which reacts with both type II and type III PKC (Stichel and Singer, 1988). The staining patterns and distribution of their

Figure 8. Double staining of β I-PKC and GABA immunoreactivity in cerebral neocortex. *A*, β I-PKC-immunopositive cells in layers I and II in the parietal cortex (Par 1) were shown by peroxidase-antiperoxidase technique. *B*, GABA immunoreactivity in the same place as *A* was demonstrated by indirect FITC immunofluorescent technique. Many β I-PKC-immunoreactive cells contained GABA immunoreaction (arrows), but some β I-PKC-positive cells did not show GABA immunoreaction (arrowheads). Scale bar, 50 μ m.

“class 1” and “class 2” neurons in the rat visual cortex are quite similar to the β II- and β I-PKC-immunopositive cells, respectively, obtained in the present study.

There were some differences in the number of immunopositive cell bodies and in the intensity of immunopositive neuropils throughout the neocortex, but the staining pattern of each PKC subspecies was essentially the same. The left cerebral hemisphere was reported to have more PKC activity than the right hemisphere (Ginobili de Martines and Barrantes, 1988), but in this study no laterality was seen in the distribution of the γ -, β I-, and β II-PKC immunoreactivity.

Relationship between PKC subspecies and neurotransmitters

The vast majority of γ - or β II-PKC-containing cells are pyramidal cells, which are excitatory glutamatergic/aspartatergic neurons. The γ -PKC always colocalizes with the β II-PKC in the pyramidal cells of layers II, V, and VI, although with different intracellular localization. As distinct modes of activation of each PKC subspecies have been documented (Sekiguchi et al., 1988; Shearman et al., 1989), it may be possible that the γ - and β II-subspecies are involved in the function of different intracellular components by different activation processes in the same neuron.

The vast majority of β I-PKC-containing cells are nonpyramidal cells and about 80% of the β I-PKC-immunoreactive neurons contained GABA, a major inhibitory neurotransmitter in CNS. These results indicate that β I-PKC may have a special function in a subpopulation of cortical GABA neurons. However, other regions of the brain do not always show colocalization of the β I-PKC and GABA. For example, in the cerebellum, Purkinje cells, which are known to be GABAergic neurons, contained γ -PKC, while the pontine nuclei, which have strong β I-PKC immunoreactivity, do not contain GABA.

LTP is known to correlate with learning and memory, and the involvement of PKC in LTP in the hippocampus has been well discussed. NMDA receptors are considered to provide a natural basis for triggering LTP. LTP is also observed in the adult rat neocortex (Lee, 1982; Bindman et al., 1987), and blockade of NMDA receptors prevents LTP in the rat visual cortex (Artola and Singer, 1987). Interestingly, there is a high density of NMDA receptors in the layers I and II of the neocortex (Maragos et al., 1988) in which both β II- and γ -PKC immunoreactivity is observed in the postsynaptic sites of the neuropils as well as in the CA1 region of the hippocampus. These findings lead to the suggestion that PKC in the postsynaptic components of the neocortex and in the hippocampus may be related to LTP.

Figure 9. Electron micrographs of perikarya in cerebral neocortex. *A*, γ -PKC-immunopositive pyramidal cell in layer V. *B*, β II-PKC-immunopositive pyramidal cells in layer V. Intense immunoreaction around the Golgi complex is observed (arrows). *C*, β I-PKC-immunopositive nonpyramidal cell in layer IV. A small amount of immunoreaction around the Golgi complex is observed (arrows). *D*, Control section stained by the same method with preimmune serum. Scale bars, 2 μ m.

Figure 10. Electron micrographs of γ -PKC immunoreactivity. *A*, Perikarya of pyramidal cell in layer V. Immunoreactive products were observed in the cytoplasm and nucleus (*n*), on the outer membrane of mitochondria (*m*), and on the membrane of endoplasmic reticulum. No immunoreactivity was seen in nucleus of glial cell (*ng*). Scale bar, 1 μ m. *B*, Dendrite and its spine in layer I. No immunoreaction is seen on postsynaptic density (arrow). Scale bar, 500 nm. *C*, γ -PKC immunoreactivity in axoplasm of myelinated axon in layer V. Scale bar, 500 nm.

Figure 11. Electron micrographs of β II-PKC immunoreactivity. *A*, β II-PKC immunoreactivity is seen around the *trans* compartment of the Golgi complex (*G*). The membrane of the Golgi complex is also stained. *n*, nucleus. Scale bar, 500 nm. *B*, Immunoreactivity in branched dendrite in layer II. No immunoreaction is observed on postsynaptic density. Scale bar, 500 nm. *C*, Axoplasm of myelinated axon contains the β II-PKC immunoreactivity. Scale bar, 500 nm.

Figure 12. Electron micrographs of β I-PKC immunoreactivity. *A*, Strong immunoreactivity of β I-PKC is in the periphery of the perikarya just adjacent to the plasma membrane. Some strong immunoreaction is seen in the postsynaptic area (arrow), but the other immunoreaction has no relationship to the synaptic contact (arrowheads). Scale bar, 250 nm. *B*, Sometimes the β I-PKC immunoreactivity is observed near the postsynaptic density in dendrites (*d*) but no immunoreaction is observed on postsynaptic density (arrows). Scale bar, 400 nm. *C*, β I-PKC immunopositive myelinated axon. Scale bar, 400 nm.

GAP-43 (F-1, B-50) is a neuron-specific substrate of PKC, and its phosphorylation correlates highly with the degree of LTP that results from high-frequency stimulation of the perforant pathway (Akers and Routtenberg, 1985; Lovinger et al., 1985). An immunohistochemical study has shown that GAP-43 is localized in layers I, Va, and VI of the cerebral neocortex (Benowitz et al., 1988) and the distribution was quite similar to that of γ -PKC. However, GAP-43 is present in the presynaptic terminals in the hippocampus (Gispén et al., 1985), while γ -PKC is localized postsynaptically in the present study, so the relationship between LTP, including the phosphorylation of GAP-43, and γ -PKC remains to be clarified.

Subcellular localization of PKC subspecies and their functions

The β I-PKC immunoreaction is mainly just adjacent to the plasma membrane, while the β II-PKC is located only around the Golgi complex in the neocortex. However, the β II-PKC was located just adjacent to the plasma membrane and around the Golgi complex in the lateral septal nucleus and β I-PKC showed the same intracellular distribution in the spinal cord (unpublished observations). So the subcellular localization of β I- and β II-PKC may be basically the same throughout the brain.

γ -, β I-, and β II-PKC are found in the dendrites, but the regional distribution of each subspecies is not exclusively asso-

ciated with any one neurotransmitter system that is known to be coupled to phosphatidylinositol turnover. The subcellular distribution of γ - and β -PKC in dendrites is different. γ -PKC is homogeneously distributed throughout the dendrites and dendritic spines, while β I- and β II-PKC are located mainly in the branched dendrites but not in the dendritic spines. It suggests that γ -PKC may play a different role from β I- and β II-PKC in postsynaptic signal transduction, especially in dendritic spines. PKC has been shown to phosphorylate many proteins, including several ion channels and receptors. Reportedly, phorbol esters increase EPSP and decrease IPSP in rat neocortical neurons (Baranyi and Szente, 1987) and cause a rapid down-regulation of muscarinic cholinergic receptors in the cortex (Jia et al., 1989). These works suggested that some PKC subspecies control the neuronal response postsynaptically.

The involvement of PKC in the K^+ -evoked release of norepinephrine from guinea pig brain cortical synaptosomes (Nichols et al., 1987; Shuntoh et al., 1988) and that of 5-HT from the rat brain cortical slice (Friedman and Wang, 1989) have been reported, but γ -, β I-, and β II-PKC immunoreactivities are not observed in the presynaptic terminals, although they are observed in the axons. Negative results cannot be taken as proof that an antigen is not present in the presynaptic terminals. Moreover, other subspecies of PKC such as α , δ , ϵ , and ζ may be involved in presynaptic functions.

The present observation shows the distinct patterns of cellular and intracellular distribution of β I-, β II-, and γ -PKC subspecies and suggests that each subspecies may play a special function in postsynaptic site of neocortical neurons. Further studies using antibodies specific for the other subspecies of PKC are necessary for understanding the total distribution and function of PKC in the neocortex.

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