Immunocytochemical localization of the α subspecies of protein kinase C in rat brain

(in situ hybridization histochemistry)

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ABSTRACT The distribution of the α subspecies of protein kinase C (PKC) in rat brain was demonstrated immunocytochemically by using polyclonal antibodies raised against a synthetic oligopeptide corresponding to the carboxyl-terminal sequence of α-PKC. The α-PKC-specific immunoreactivity was widely but discretely distributed in both gray and white matter. The immunoreactivity was associated predominantly with neurons, particularly with perikaryon, dendrite, or axon, but little was seen in the nucleus. Glial cells expressed this PKC subspecies poorly, if at all. The highest density of immunoreactivity was seen in the olfactory bulb, septohippocampal nucleus, indusium griseum, islands of Calleja, intermediate part of the lateral septal nucleus, and Ammon’s horn. A moderately high density of the immunoreactivity was seen in the anterior olfactory nucleus, anterior commissure, cingulate cortex, dentate gyrus, compact part of the substantia nigra, intranuclear nucleus, inferior olive, and olivocerebellar tract. This distribution pattern was consistent with that obtained by in situ hybridization histochemistry. The distribution of α-PKC immunoreactivity was different from that of β₁, β₁II, and γ-PKC immunoreactivity. These findings suggest that α-PKC is involved heavily in the control of specific functions of some restricted neurons.

Protein kinase C (PKC) was once thought to be a single entity, but molecular cloning and enzymatic studies have revealed that the enzyme is a large family with multiple subtypes. At least seven subtypes of PKC (α, β₁, β₂, γ, δ, ε, and η) have been identified from analysis of the rat brain cDNA library (reviewed in ref. 1). Early enzymatic studies have shown that rat brain PKC can be separated into three distinct fractions, types I, II, and III, by hydroxyapatite column chromatography (2), which correspond to γ, β₁ and β₁II, and α-PKC, respectively (3, 4). These PKC subtypes are subtly different from one another in their kinetic properties, mode of activation, and most likely substrate specificity (1, 5–8). Early analysis of the mRNA levels has indicated that α, β₁, and β₁II-PKC are expressed in a variety of tissues, whereas γ-PKC is expressed only in the central nervous system (9–12). Enzymatic and immunochemical analysis has also shown that α-PKC (type III) is most commonly distributed in many tissues and cell types (13, 14). On the basis of these studies, it has been suggested that α-PKC plays a role of crucial importance in the control of common processes in cell functions (1).

Several laboratories have carried out immunocytochemical studies using antibodies specific to each type of PKC (15–19). In earlier reports (20–24), with subspecies-specific antibodies, it was shown that in the rat brain β₁, β₁II, and γ-PKC are differentially distributed in particular cell types, with limited intracellular localization. The present studies were undertaken to identify α-PKC in the rat brain by using immunocytochemistry, and the results show that this PKC subspecies is enriched in particular cell types.

MATERIAL AND METHODS

Preparation of Antibodies Against α-PKC. The carboxyl-terminal portion of α-PKC (residues 662–672; Gin-Phe-Val-His-Pro-Ile-Leu-Gln-Ser-Ala-Val) was selected as a sequence specific to α-PKC. The oligopeptide was coupled to keyhole limpet with m-maleimidobenzoic acid N-hydroxysuccinimide ester. Rabbits were immunized with the immunogen by the method described (23, 24) and were bled 1 week after the third booster administration. The IgG fraction was obtained from the antiserum by affinity chromatography on Sepharose CL-4B coupled to goat anti-rabbit IgG, and the fraction was used as α-PKC-specific antibodies.

Immunoblotting Analysis. Specificity of the antibodies was examined by immunoblotting analysis using the three subtypes of rat brain PKC (types I, II, and III) and four subtypes of PKC (α, β₁, β₁II, and γ-PKC). These subtypes were purified from COS-7 cells transfected with the respective cDNA-containing plasmids as described (3, 4). The enzyme samples were subjected to NaDodSO₄/7.5% polyacrylamide slab gel electrophoresis as described by Laemmli (25) and transferred to nitrocellulose paper. The paper was incubated with the α-PKC-specific antibodies, and immunoreactive bands were visualized by the peroxidase-antiperoxidase method.

Immunocytochemical Staining. Frontal sections of the rat brain were prepared as described (21) by using a periodate/lysine/paraformaldehyde fixative containing 0.05% picric acid. The sections were preincubated for 20 min with 0.3% H₂O₂ and 5% normal goat serum in phosphate-buffered saline (pH 7.4) containing 0.03% Triton X-100 (PBS-T), then incubated for 18 hr at 4°C with the purified antibodies diluted 1:500 in PBS-T, washed, incubated for an additional 4 hr with goat anti-rabbit IgG (MBL) diluted 1:500, washed, and incubated for an additional 90 min with rabbit peroxidase-antiperoxidase complex (ICN) diluted 1:500. After three rinses, the sections were developed with 0.02% 3, 3'-diaminobenzidine (Sigma) and 0.2% nickel ammonium sulfate in 50 mM Tris-HCl (pH 7.4) with 0.005% H₂O₂. All sections were observed and photographed under a Zeiss microscope. The pattern of immunostaining was analyzed on an IBAS II image analyzer (Zeiss).

In Situ Hybridization Histochemistry. A synthetic oligodeoxyribonucleotide complementary to the rat α-PKC sequence described by Ono et al. (26) was prepared with an Applied Biosystems DNA synthesizer (model 381A). The probe was

Abbreviation: PKC, protein kinase C.

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buffer (pH 7.4), and the brains were dissected. Frozen sections 15 μm thick were thaw-mounted on poly-l-lysine-coated glass slides. The sections were treated with 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl (pH 8.0) and transferred through graded alcohols (70–100%) and chloroform. After a 3-hr prehybridization in buffer containing 40% (vol/vol) formamide, 10% (wt/vol) dextran, 4× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7), 0.02 M Tris-HCl (pH 7.4), 1× Denhardt’s solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), salmon sperm DNA (20 mg/ml), and yeast tRNA (0.25 mg/ml), hybridization was carried out at 44°C for 24 hr in the same buffer containing 106 cpm of labeled probe. The section was washed three times in 1× SSC/20% formamide at 46°C and exposed to x-ray film for 3–7 days.

RESULTS

Characterization of Antibodies. Purified antibodies against the oligopeptide specific to α-PKC detected a protein in the rat brain soluble fraction that corresponded to PKC with a molecular mass of ~80 kDa (Fig. 1, lane b). It was also shown that the antibodies detected the 80-kDa band only in the fraction of type III but not of type I or II PKC (Fig. 1, lanes c–e). The antibodies stained only α-PKC and not other subspecies encoded by sequence β1, β2, or γ (Fig. 1, lanes f–i).

Immunocytochemical Localization of α-PKC. General distribution of α-PKC immunoreactivity. Fig. 2 shows the patterns of immunostaining of α-PKC that were analyzed

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**Fig. 1.** Immunoblot analysis with antibodies against α-PKC. Samples were subjected to NaDodSO4/7.5% polyacrylamide slab gel electrophoresis. The proteins were transferred to nitrocellulose paper, incubated with antibodies against the α-PKC-specific oligopeptide (1:500 dilution), and visualized by the peroxidase–anti-peroxidase method. Lanes: a, marker proteins; b, crude soluble fraction of the whole rat brain; c, type I PKC; d, type II PKC; e, type III PKC; f, α-PKC; g, β1-PKC; h, β2-PKC; i, γ-PKC. Molecular sizes (in kDa) of marker proteins are indicated on the left.

complementary to bases 2257–2301, present in the 3' non-coding region of α-PKC cDNA. The probe was labeled at the 3' end by using terminal deoxynucletidyl transferase and [α-35S]dATP. Anesthetized rats were perfused with ice-cold fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and the brains were dissected. Frozen sections 15 μm thick were thaw-mounted on poly-l-lysine-coated glass slides. The sections were treated with 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl (pH 8.0) and transferred through graded alcohols (70–100%) and chloroform. After a 3-hr prehybridization in buffer containing 40% (vol/vol) formamide, 10% (wt/vol) dextran, 4× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7), 0.02 M Tris-HCl (pH 7.4), 1× Denhardt’s solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), salmon sperm DNA (20 mg/ml), and yeast tRNA (0.25 mg/ml), hybridization was carried out at 44°C for 24 hr in the same buffer containing 106 cpm of labeled probe. The section was washed three times in 1× SSC/20% formamide at 46°C and exposed to x-ray film for 3–7 days.

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**Fig. 2.** Color images of the distribution of α-PKC immunoreactivity in frontal sections of rat brain [rostral (A) to caudal (G)]. The α-PKC immunoreactivity was localized by peroxidase–anti-peroxidase immunocytochemistry. Relative density of the immunoreactivity is shown according to the color scale (highest density, red; lowest density, black). The highest density of immunoreactivity (red) was seen in the external plexiform layer of the olfactory bulb (A), indusium griseum (B), islands of Calleja (B), intermediate part of the lateral septal nucleus (B), and Ammon’s horn (C). A moderately high density of immunoreactivity (yellow) was seen in the anterior olfactory nucleus (A), anterior commissure (B), dentate gyrus (C), cingulate cortex (C and D), compact part of the substantia nigra (D), interpeduncular nucleus (D), inferior olive (F), and olivocerebellar tract (F). A moderate density of the immunoreactivity (green) was seen in layers II–III of the cerebral cortex (A–D), the dorsal part of the lateral septal nucleus (B), and the substantia gelatinosa of the spinal cord (G). The cerebellum showed relatively low immunoreactivity (E). aca, anterior commissure; Amm, Ammon’s horn; AO, anterior olfactory nucleus; Cg, cingulate cortex; DG, dentate gyrus; EPL, external plexiform layer of the olfactory bulb; ICj, islands of Calleja; IG, indusium griseum; IO, inferior olive; IP, interpeduncular nucleus; LSI, lateral septal nucleus, intermediate part; oc, olivocerebellar tract; sg, substantia gelatinosa; SNC, substantia nigra, compact part.
with an IBAS II image analyzer. The α-PKC immunoreactivity was widely but discretely distributed in both gray and white matter of the brain. The highest density of α-PKC immunoreactivity was seen in the external plexiform layer of the olfactory bulb (Fig. 2A), indusium griseum (Fig. 2B), islands of Calleja (Fig. 2B), intermediate part of the lateral septal nucleus (Fig. 2B), Ammon’s horn of the hippocampal formation (Fig. 2C), and septohippocampal nucleus. A moderately high density of immunoreactivity was seen in the anterior olfactory nucleus (Fig. 2A), anterior commissure (Fig. 2B), cingulate cortex (Fig. 2 C and D), dentate gyrus (Fig. 2C), compact part of the substantia nigra (Fig. 2D), interpeduncular nucleus (Fig. 2D), inferior olive (Fig. 2F), and olivocerebellar tract (Fig. 2F). A moderate density of immunoreactivity was seen in layers II–III of the cerebral cortex (Fig. 2A–D), the dorsal part of the lateral septal nucleus (Fig. 2B), substantia gelatinosa of the spinal cord (Fig. 2G), dorsal hippocampal commissure, and medial raphe nucleus. A weak density of immunoreactivity was seen in the caudate-putamen, corpus callosum, medial forebrain bundle, hypothalamic nuclei, and inferior cerebellar peduncle. Other brain regions such as the cerebellar cortex (Fig. 2E) showed relatively low immunoreactivity.

All immunoreaction was completely abolished by prior incubation of the antibodies with the immunogen. The use of the same concentration of nonimmune rabbit serum as was used for the first antibodies failed to produce any immunoreaction (data not shown).

Cellular localization of α-PKC immunoreactivity. In the gray matter, the α-PKC immunoreactivity was associated mainly with neurons, with or without dendritic processes, and the immunoreactivity of the neuropils was generally weak. The α-PKC immunoreaction was observed in the perikaryon, dendrite, and axon, but little was seen in the nucleus.

At low magnification of the hippocampal formation, the α-PKC immunoreactivity was distributed through Ammon’s horn; the highest concentration was in CA2 as well as fasciola cinereum and indusium griseum (Fig. 3A). Weak immunoreactivity was seen in the proximal third of the molecular layer of the dentate gyrus as an immunopositive band.

At higher magnification, the pyramidal cells of CA1 and CA3 were stained with fine dots along the plasma membrane (Fig. 3B), whereas coarse immunopositive dots were seen in pyramidal cells of CA2 (Fig. 3C). In the stratum oriens through the whole Ammon’s horn, dendritic processes were seen among the intense staining of the neuropils (Fig. 3 B and C). In the stratum radiatum, immunoreactive fiber tufts were observed running perpendicular among the moderate immunopositive neuropils. A considerable number of immunoreactive cell bodies were found in the neuropil of the strata oriens and radiatum (Fig. 3C). Large triangular or fusiform neurons were seen on the dentate-hilus border, but little immunoreactivity was seen in the granular cells of the dentate gyrus (Fig. 3D).

Many neurons in the intermediate part of the lateral septal nucleus were densely stained with long dendrites, while few, if any, immunopositive neurons were seen in the dorsal part of the nucleus (Fig. 4A). At closer inspection of the stained neurons, dense immunoreaction was seen along the plasma membrane (Fig. 4B).

The inferior olive was moderately stained in the hindbrain where little immunoreactivity was seen (Fig. 5A). Most neurons, if not all, showed dense immunoreaction, which was seen along the plasma membrane and as fine dots within the weakly stained cytoplasm (Fig. 5B).

Most of the other immunopositive neurons were similar to these neurons found in the intermediate part of the lateral septal nucleus and inferior olive. The neurons with immunoreactive dots along the plasma membrane were observed in layers II–III of the cerebral cortex as well as the stratum pyramidale of the Ammon’s horn.

Many immunoreactive fibers were clearly recognized in the white matter such as the anterior commissure, olivocerebellar tract, dorsal hippocampal commissure, corpus callosum, median forebrain bundle, and inferior cerebellar peduncle. Immunoreactive fibers were seen to run through the olivocerebellar tract (Fig. 5C), which originates from the inferior olive (Fig. 5A).

There was no obvious immunoreaction in glial cells in either the gray or white matter.

**Distribution of α-PKC mRNA.** In situ hybridization histochemistry of the rat brain revealed that α-PKC mRNA was expressed in areas corresponding to the areas where α-PKC-immunoreactive neuronal cell bodies were observed. For example, α-PKC mRNA was localized abundantly in the
Fig. 4. Photomicrographs showing α-PKC immunoreactivity in a frontal section of the septal nucleus. Many neurons in the intermediate part of the lateral septal nucleus were densely stained (A). The dense immunoreaction was seen along the plasma membrane of the neurons. Many fine dots were seen in the cytoplasm (B). (A, bar = 200 μm; B, bar = 20 μm.)

Many neurons in the hippocampus, especially in the CA2 region (Fig. 6), where the densest immunoreaction was seen in the hippocampus (Fig. 2 C and D). Fig. 6B shows that α-PKC mRNA was also moderately expressed in the compact part of the substantia nigra and the cingulate cortex, where moderately high immunoreactivity was seen (Fig. 2D). The autoradiographic signals were completely abolished when the sections were hybridized in the presence of either nonlabeled oligonucleotide or the oligonucleotide complementary to the labeled probe.

Discussion

The antibodies employed in the present studies specifically recognize type III PKC, which chromatographically corresponds to the enzyme-encoded α sequence, α-PKC. Although other PKC subspecies so far isolated do not cross-react with the antibodies, the possibility cannot be excluded that type III PKC is still a mixture of several thus far unidentified subspecies having sequences closely related to α-PKC. However, the enzyme distribution obtained with antisera raised against another part of the α-PKC sequence was essentially similar to that presented here (data not shown). In addition, the pattern of in situ hybridization histochemistry with an α-PKC-specific oligonucleotide probe matches well that obtained by the immunocytochemical procedure described above. Theoretically, the antibodies presently used should not react with δ-, ε-, and γ-PKC, because these subspecies do not contain, in any part of the molecules, the amino acid sequence employed as antigen (1). Perhaps, the histochemical pattern presented in this paper represents ipso facto type III PKC having the α sequence, which was clarified by molecular cloning analysis.

The distribution of α-PKC, nevertheless, does not always coincide with the immunostaining pattern of type III PKC reported from other laboratories, although the reason for some of these discrepancies remains unknown. Huang et al. (15) have proposed the existence of type III PKC in other neurons in addition to those observed in the present studies. We have also prepared antibodies against type III PKC by Olmsted's method (27). The antibodies thus obtained have shown immunostaining patterns identical with those given in this paper. Hidaka et al. (18) have described a monoclonal antibody against type III PKC (MC-3a) that stains only oligodendroglia but not neurons in the rabbit cerebellum. The present studies reveal, however, that the α-PKC immunoreaction is more heavily localized in neuronal cells. In the...
monkey brain, we have observed many α-PKC-immunopositive glial cells, in addition to neuronal cells (N.S., T.T., and C.T., unpublished data). Stichel and Singer (19) have described localization of type II/III PKC in the rat brain, which is compatible with that presented in this paper. The distribution of α-PKC does not seem to correspond to any particular neurotransmitter or receptor distribution. For example, the pyramidal cells of Ammon’s horn and neuronal cells of the inferior olive are typical excitatory neurons, which utilize glutamate and aspartate, respectively, as neurotransmitters (28, 29). On the other hand, some immunopositive neurons located in the dentate-hilus border, strata oriens, and radiatum are known to contain γ-aminobutyric acid, which is an inhibitory neurotransmitter (28). These findings imply that α-PKC is not related to a specific neurotransmitter or receptor system.

The hippocampus is known as a principal site for long-term potentiation. Although it is controversial whether, at the presynaptic or postsynaptic side, PKC is involved in long-term potentiation (30–32), the electron microscopic analysis reveals that both βγ- and γ-PKC are observed in the postsynaptic but not presynaptic side (33). α-PKC appears to be abundant also at the postsynaptic side, although it has not been confirmed by electron microscopy.

The distribution of α-PKC differs significantly from those of βγ-, βε- and γ-PKC reported earlier (20–24). However, several PKC subspecies are coexpressed in some neuronal cells, such as the pyramidal cells of the hippocampal CA1 region. Electron microscopic analysis has shown that in these cells γ-PKC is localized diffusely in the cytoplasm, whereas βγ- and γ-PKC is frequently associated with the Golgi complex (33) and α-PKC seems to be localized around the Golgi complex. Enzymatic studies have indicated that these PKC subspecies exhibit subtly different modes of activation and kinetic properties (1, 5–8). It is plausible, therefore, that various PKC subspecies may play distinct functions in the processing and modulation of various physiological and pathological cellular responses to external signals.

Biochemical analysis has shown that α-PKC is expressed in all tissues and cell types thus far examined (1, 9–14). The present results, however, demonstrate that α-PKC is enriched more heavily in specific cell types than in others. Presumably, the involvement of this PKC subspecies in the regulation of cellular functions greatly varies with the cell type.

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