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

(central nervous system)

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ABSTRACT **Polyclonal antibodies were raised against an oligopeptide containing an amino acid sequence specific for β_I protein kinase C (PKC) in order to localize this subspecies of PKC in the rat brain. β_I PKC immunoreactivity was widely distributed but discretely localized in particular brain regions. The immunoreactivity was associated with neurons but not glial cells and was generally weak in the neuropils with the exception of areas such as the triangular septal nucleus, pontine nuclei, superficial layer of the superior colliculus, and gray matter of the spinal cord. The largest number of β_I PKC-immunoreactive cells was seen in the triangular septal nucleus and pontine nuclei. In these areas of dense β_I immunoreactivity, there was no or little γ PKC immunoreactivity. The β_I PKC immunoreactivity was observed in perikarya, dendrites, and axons but not in nuclei or nucleoli. There were various patterns of cytoplasmic immunoreactivity, but the immunoreactivity was seen frequently in the periphery of the perikarya and dendrites. The intraneuronal localization of β_I PKC immunoreactivity differs from that of the subspecies that is coded by the γ gene sequence. The results suggest that β_I PKC may play a role in the regulation of cell membrane responsiveness in particular neurons.**

Protein kinase C (PKC) is thought to be a pivotal mediator of several cellular processes, including secretion, neurotransmitter release, differentiation, and growth (1, 2). Molecular cloning and enzymological analysis have revealed that PKC is a family of multiple subspecies (3). The complete structures of one group of subspecies—namely, α, β_I, β_{II}, and γ PKC, have been identified by molecular analysis of their cDNA clones obtained from brain and spleen libraries of several mammalian species (4–14). The heterogeneity of the enzyme comes from multiple genes (α, β, and γ) (5) and from alternative splicing of a single gene transcript (β_I and β_{II}) (10, 12, 14). PKC isolated from rat brain can be resolved into three major types (I, II, and III) by hydroxyapatite column chromatography (15), and the correspondence of these types to the cDNA clones for α (type III), β_I and β_{II} (type II), and γ (type I) PKC has been determined by comparison with the enzymes that were separately expressed in COS-7 cells transfected with the respective cDNA-containing plasmids (10, 16, 17). Biochemical studies have shown that these three enzyme types differ subtly from one another in their mode of activation and kinetic properties (18–20). More recently, a second group of enzyme subspecies, having δ, ε, and ζ sequence, have been identified (21) that are closely related to, but clearly different from, the first group. The enzymological properties of this group of PKC subspecies have not been defined.

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Immunocytochemical and biochemical studies in this and other laboratories (17, 22–31) have revealed a differential regional and cellular localization of some of these PKC subspecies in the mammalian brain and some other tissues, suggesting that each subspecies has a specialized function in transducing various physiological signals into different cell types. The β_I and β_{II} subspecies, eluted together in the type II fraction, are indistinguishable by conventional enzymatic procedures (10). These subspecies differ from each other only in about 50 amino acid residues at their carboxyl-terminal regions. In the present study a synthetic oligopeptide representing the PKC β_I-specific sequence in this region was employed as immunogen, and the resulting specific antisera were used for immunocytochemical identification of β_I PKC subspecies in brain tissues.

MATERIALS AND METHODS

Preparation of Antisera Against β_I Subspecies of PKC. An oligopeptide corresponding to the carboxyl-terminal end region of β_I PKC (residues 661–671; Ser-Tyr-Thr-Asn-Pro-Glu-Phe-Val-Ile-Asn-Val) was synthesized. One micromole of oligopeptide was coupled to 0.1 μmol of bovine serum albumin by 4 μmol of glutaraldehyde. Four hundred micrograms of the conjugate was emulsified with complete Freund's adjuvant and given to New Zealand White rabbits by multiple intracutaneous injections. The same amount of the conjugate emulsified with incomplete Freund's adjuvant was given to rabbits repeatedly at intervals of 2 weeks, and the rabbits were bled 4–6 days after each booster administration. The antisera were examined by enzyme-linked immunosorbent assay (ELISA) as described (32). The titer was increased after the second booster administration and reached a maximum after the fifth boost. The antisera after the fifth boost were purified and used in all experiments. The crude antisera with the highest titers were purified by affinity column chromatography on Sepharose CL-4B coupled with the β_I oligopeptide conjugated to bovine thyroglobulin by glutaraldehyde. Alternatively, the antisera were purified by incubating the crude antisera with Sepharose CL-4B coupled with bovine serum albumin to remove the antibodies against bovine serum albumin. As the patterns of immunoblot and immunocytochemical staining with these two preparations of antibodies were identical, the latter preparation was routinely used in the present studies.

Characterization of Antisera Against β_I PKC. For ELISA, the β_I-specific oligopeptide, conjugated to glutaraldehyde, was incubated in wells coated with poly(L-lysine). After blocking with lysine and gelatin, the wells were reacted with the antisera (diluted 1:1000) and stained by the peroxidase-antiperoxidase method using *o*-phenylenediamine as sub-

Abbreviation: PKC, protein kinase C.
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strate. The extent of immunoreaction was determined by light absorbance at 492 nm.

The three subtypes of rat brain PKC (I, II, and III) were purified to homogeneity (20). The four subspecies of PKC (α, β_I, β_{II}, and γ) were obtained from the COS-7 cells that were transfected with the cDNAs as described (16). For immunoblotting, samples were fractionated by $NaDodSO_4$/7.5% polyacrylamide slab gel electrophoresis in the discontinuous buffer system of Laemmli (33) and transferred to nitrocellulose paper. The paper was incubated with the PKC-specific antibodies, and immunoreactive bands were visualized by the peroxidase-antiperoxidase method.

Immunocytochemical Staining. Immunocytochemical staining and observation of 20-μm rat brain cryostat sections were performed as described (28). The sections were incubated with the purified antibodies diluted 1:5000, followed by goat anti-rabbit IgG (Miles) diluted 1:1000 and rabbit peroxidase-antiperoxidase (Miles) diluted 1:5000. Then the sections were incubated in 50 mM Tris·HCl (pH 7.4) containing 0.02% 3,3'-diaminobenzidine (Sigma), 0.2% nickel ammonium sulfate, and 0.005% H_2O_2. All sections were observed and photographed under a Zeiss microscope. The pattern of immunostaining was analyzed with an IBAS II image analyzer (Zeiss). The terminology of anatomy used here is that used by Paxinos and Watson (34).

RESULTS

Characterization of Antisera Against β_I PKC. Immunoblot analysis indicated that the antibodies prepared against the oligopeptide described above detected only an 80-kDa protein corresponding to PKC in the crude soluble fraction from rat brain (Fig. 1, lane a). The immunoreactive band was associated with the type II fraction, but not the type I or type III fractions, purified by hydroxyapatite chromatography (lanes b–d). The antibodies reacted specifically with the β_I PKC subspecies expressed in COS-7 cells (lane f). The 80-kDa band was not detected in COS-7 cells expressing cDNA for α, β_{II}, or γ PKC (lanes e, g, and h).

Immunocytochemical Localization of β_I PKC. The β_I PKC immunoreactivity was found throughout the rat brain and was associated primarily with neurons but not glial cells. The immunoreactive neurons, with or without dendritic processes, were widely distributed but discretely localized in the rat brain. In general, the immunoreactivity of the neuropils was weak, with the exception of areas such as the triangular septal nucleus, pontine nuclei, superficial layer of the superior colliculus, and gray matter of the spinal cord (Fig. 2). Even in these areas, the immunoreactive terminal structure was not clearly identified. There was no immunoreaction in most of the white matter except that immunoreactive axons were seen in the middle cerebellar peduncle and cerebellar white matter (Fig. 3). Adsorption of the antiserum with the β_I oligopeptide abolished all immunoreactivity. Normal rabbit serum failed to provide any immunoreactivity.

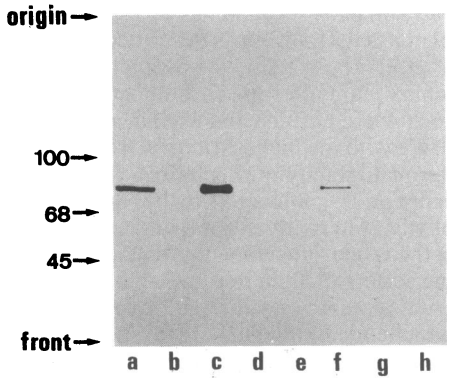

FIG. 1. Immunoblot analysis of the antibodies against β_I PKC. Samples of PKC subspecies were fractionated by $NaDodSO_4$/polyacrylamide gel electrophoresis. After transfer of the proteins to nitrocellulose paper, the paper was incubated with the antibodies against β_I PKC (diluted 1:500), followed by goat anti-rabbit IgG immunoglobulin (diluted 1:1000) and rabbit peroxidase-antiperoxidase complex (diluted 1:2500). Lanes: a, crude soluble fraction of whole rat brain; b, type I PKC; c, type II PKC; d, type III PKC; e, α subspecies; f, β_I subspecies; g, β_{II} subspecies; h, γ subspecies. Molecular sizes (kDa) of standard proteins are indicated at left.

The β_I PKC immunoreactivity was observed in various subcellular compartments of neurons such as the perikarya and dendrites, and there was little or no immunoreactivity in the nuclei and nucleoli. Most of the immunoreactivity was found in the periphery of the perikarya just adjacent to the plasma membrane of the definite neurons in regions such as the cerebral cortex (Fig. 4*A*) and pontine nuclei (Fig. 4*B*). Immunoreactivity was found throughout the cytoplasm in some neurons of regions such as the laterodorsal thalamic nucleus (data not shown), interpeduncular nucleus (paramedian part) (Fig. 5*A*), and raphe nuclei (Fig. 5*B*). The immunoreactivity was seen as variously shaped and sized dots in the cytoplasm in fewer regions, such as the caudate-putamen (Fig. 5*C*) and triangular septal nucleus (Fig. 5*D*). The granular layer of the cerebellar cortex revealed a moderate density of immunoreactivity (Fig. 3).

Distribution of β_I PKC-Immunoreactive Perikarya in Rat Brain. The largest number of cells with β_I PKC immunoreactivity was seen in the triangular septal nucleus (Fig. 5*D*) and pontine nuclei (Fig. 4*B*). A moderately large number of immunoreactive cells was found in the glomerular layer of the olfactory bulb, anterior olfactory nucleus, accumbens nucleus, primary olfactory cortex, cerebral cortex (layers II–VI) (Fig. 4*A*), medial septal nucleus, diagonal band, ventral pallidum, caudate-putamen (Fig. 5*C*), globus pallidus, thalamus (laterodorsal and reticular nuclei), dorsal lateral geniculate nucleus, anterior pretectal area, lateral and anterior hypothalamic area, dorsomedial hypothalamic nucleus, lateral habenular nucleus, interstitial magnocellular nucleus of the posterior commissure, superficial gray layer of the superior colliculus, subiculum, central gray matter, peripeduncular nucleus, interpeduncular nucleus, central gray matter of the pons, ventral cochlear nucleus, prepositus hypoglossal nucleus, external cuneate nucleus, and lateral reticular nucleus.

A small number of immunoreactive cells was recognized in the amygdaloid complex, nucleus of Darkschewitsch, superior colliculus (optic nerve and intermediate gray layers), raphe nuclei (Fig. 5*B*), motor trigeminal nucleus, nucleus of the spinal tract of the trigeminal nerve, medial and lateral vestibular nuclei, facial nuclei, gigantocellular parvocellular reticular nuclei, cuneate and gracile nuclei, nucleus of the solitary tract, and dorsal horn of the spinal cord (substantia gelatinosa).

DISCUSSION

In the present studies we have prepared an antiserum that can recognize β_I PKC but not α, β_{II}, or γ PKC. The δ, ε, and ζ subspecies do not contain the amino acid sequence of the oligopeptide employed as immunogen (21). Theoretically, therefore, the antibodies should not react with these subspecies.

The β_I PKC immunoreactivity is widely distributed but discretely localized in various brain regions. The distribution of β_I PKC immunoreactivity differs from that of the subspecies coded by the γ sequence. The immunoreactive areas of γ PKC in the rat central nervous system (28) correspond roughly to the dense areas observed in a [^{3}H]phorbol 12,13-dibutyrate autoradiogram (35). In contrast, only a few phor-

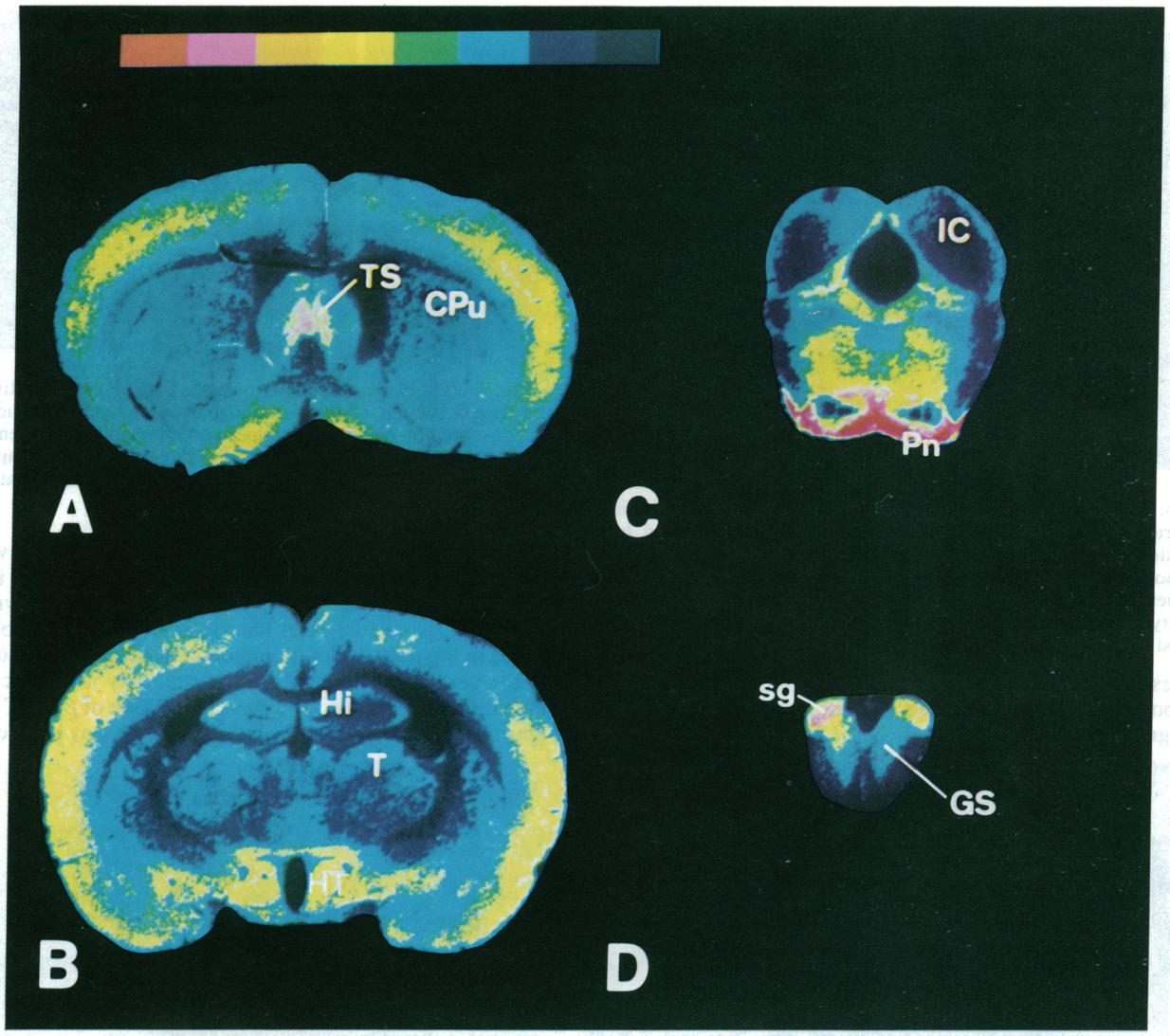

FIG. 2. Color images of the distribution of β_I PKC immunoreactivity in frontal sections of rat brain. The β_I PKC immunoreactivity was localized by peroxidase-antiperoxidase immunocytochemistry. Strong immunoreaction with β_I PKC is seen in the triangular septal nucleus (*A*), pontine nuclei (*C*), and substantia gelatinosa of the spinal cord (*D*). The hippocampus and thalamus revealed weak immunoreactivity (*B*). Relative density of the immunoreactivity is shown according to the color table (highest density, red; lowest density, black). CPu, caudate-putamen; GS, gray matter of the spinal cord; Hi, hippocampus; HT, hypothalamus; IC, inferior colliculus; Pn, pontine nuclei; sg, substantia gelatinosa of the spinal cord; T, thalamus; TS, triangular septal nucleus. [Rostral (*A*) to caudal (*D*).]

bol ester-binding sites are found in the dense β_I PKC-immunoreactive areas, such as the triangular septal nucleus, anterior and lateral hypothalamic area, pontine nuclei, granular layer of the cerebellar cortex, middle cerebellar peduncle, and gray matter of the spinal cord. This finding may be attributed to the lower content of β_I PKC compared with other subspecies. The relative enzyme activities in whole brain cytosol are roughly 16% (α), 8% (β_I), 55% (β_{II}), and 21% (γ) (11, 26). The distribution of B_I PKC immunoreactivity does not correspond to that of inositol 1,4,5-trisphosphate-binding sites, which are present at a high level in regions such as the molecular layer of cerebellar cortex, caudate-putamen, and the CA1 region in the hippocampus (36).

The distribution of PKC subspecies has been most extensively studied in the cerebellar cortex. The γ subspecies is present in the Purkinje cells, β_{II} in the neuropils of the molecular layer, and β_I in the granular layer (30). These results are similar to the distribution of type I and type II PKC in the cerebellar cortex detected by immunocytochemical studies of other investigators (17, 27). The type II-specific antibody prepared by Huang *et al.* (17) detects the presence of this enzyme in the granular, but not the molecular, layer in the rat cerebellum. Their antibody probably recognizes β_I PKC but not β_{II} PKC. Hidaka *et al.* (27) reported that, in the rabbit cerebellum, type II PKC is present in both molecular and granular layers but not in the Purkinje cells. Since their antibody against type II PKC (MC-2a) recognizes the regulatory domain, a region common to both β_I and β_{II} PKC, MC-2a may recognize both subspecies. Brandt *et al.* (37) identified the β subspecies in the granular layer by *in situ* mRNA hybridization procedures. From the present studies, it is not clear whether the β_I PKC immunoreactivity in the granular layer is present in the granule cell itself or in other components associated with it. In addition to the granular layer, the neurons of the pontine nuclei and the nerve fibers in the middle cerebellar peduncle showed β_I immunoreactivity, suggesting that the pontocerebellar pathway, a mossy fiber system, may contain β_I PKC. Electron microscopic study is needed to identify these immunoreactive tissues.

Immunocytochemical studies of PKC have been reported from several laboratories (22–24). The staining patterns observed with the monoclonal antibody CK1.12 of Mochly-Rosen *et al.* (24) are similar to those of type I PKC in our study (28). The findings presented above, however, do not

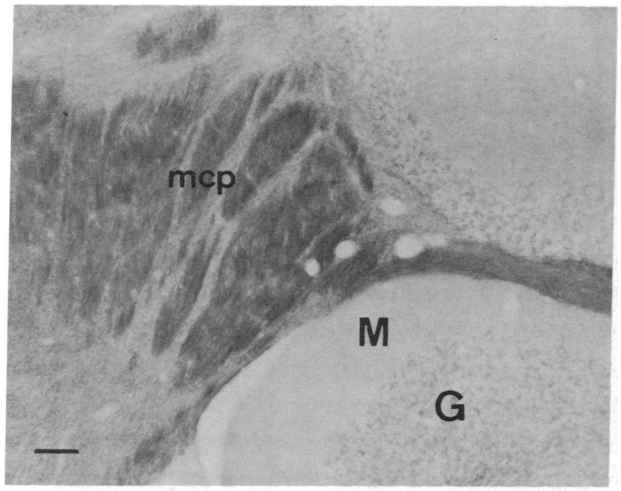

FIG. 3. Photomicrograph showing β_I PKC immunoreactivity in the frontal section of the cerebellar cortex as demonstrated by peroxidase-antiperoxidase immunocytochemistry. Dense immunoreaction occurred in the middle cerebellar peduncle (mcp) and cerebellar white matter. The granular layer (G) revealed a moderate density of immunoreactivity, and the molecular layer (M) revealed almost no immunoreactivity. (Bar = 100 μm.)

correspond to any of the immunostaining obtained with PKC antibodies by these workers. Their antibodies probably recognize other PKC subspecies.

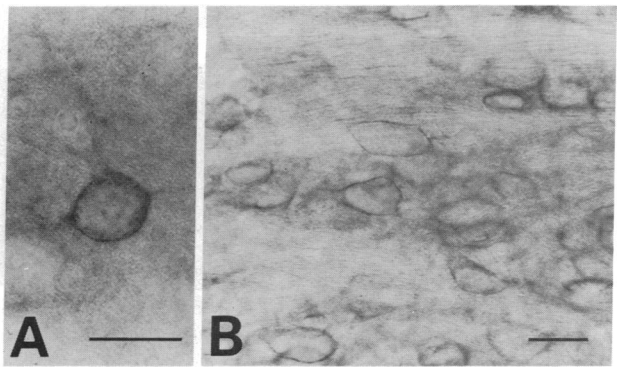

FIG. 4. Photomicrographs showing β_I PKC immunoreactivity in the frontal section of the cerebral cortex and pontine nuclei as demonstrated by peroxidase-antiperoxidase immunocytochemistry. (*A*) Layer V of cerebral cortex. (*B*) Pontine nuclei. Dense immunoreaction occurred in the periphery of the neurons just adjacent to the plasma membrane. (Bar = 25 μm.)

Electron microscopic study of the Purkinje cells revealed that γ PKC is located on the cell membrane and in the cytoplasm, dendrite, axon, and axon terminal (synaptic vesicles) (31). On the other hand, under light microscopy, β_I PKC, in the majority of immunoreactive neurons, is located in the perikarya and dendrites, particularly just adjacent to the plasma membrane. The close apposition of β_I PKC to the plasma membrane suggests that its function may be integrally

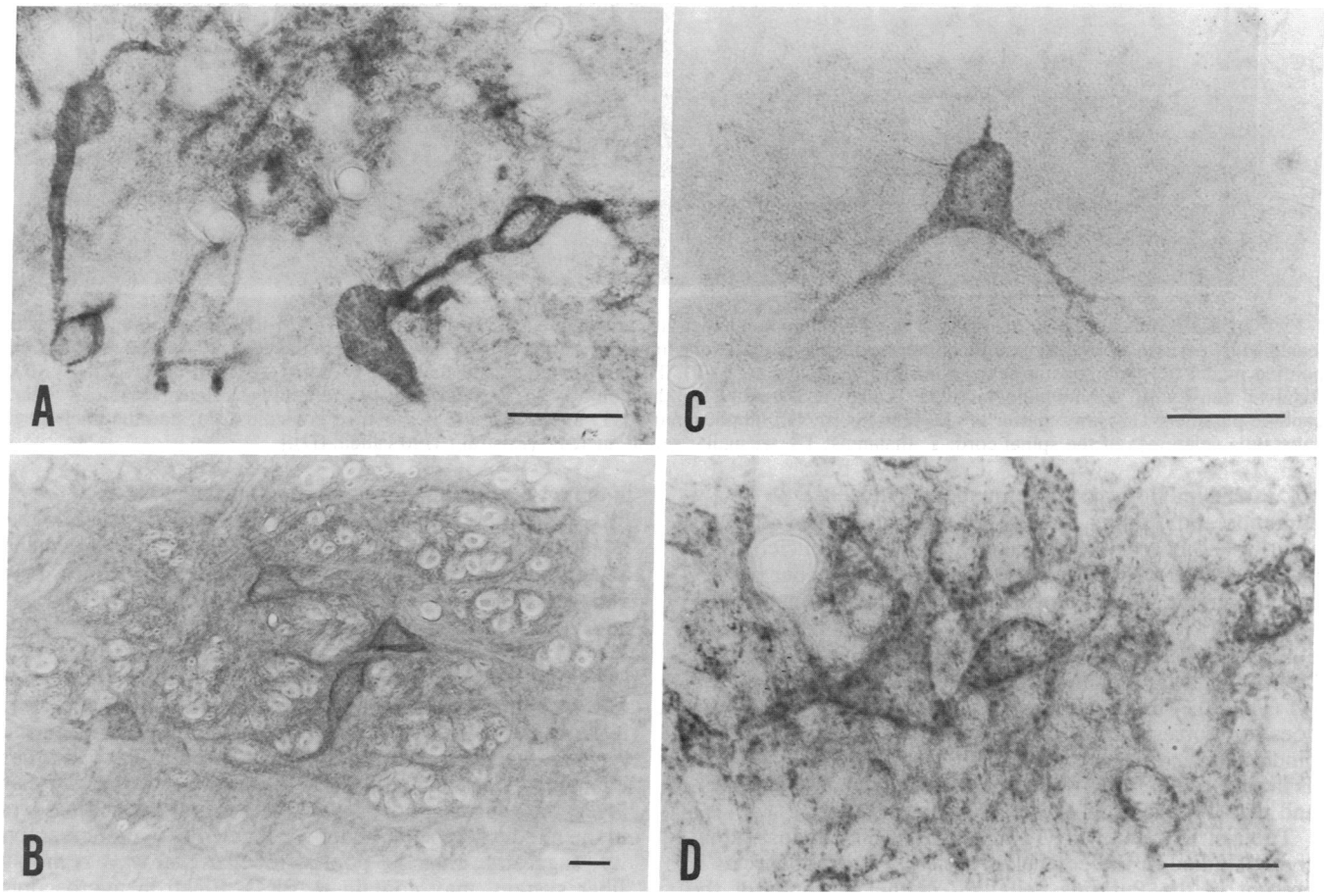

FIG. 5. Photomicrographs showing β_I PKC immunoreactivity in the frontal sections of the interpeduncular nucleus (paramedian part), raphe magnus nucleus, caudate-putamen, and triangular septal nucleus as demonstrated by peroxidase-antiperoxidase immunocytochemistry. In the interpeduncular nucleus (paramedian part) (*A*) and raphe magnus nucleus (*B*), β_I PKC immunoreactivity is seen in the periphery of the neurons just adjacent to the plasma membrane and throughout the cytoplasm of the neurons. In addition to the findings as mentioned in *A* and *B*, small immunoreactive dots are seen on the neurons of the caudate putamen (*C*) and triangular septal nucleus (*D*). (Bars = 25 μm in *A*, *C*, and *D*; bar = 50 μm in *B*.)

related to the cell membrane responsiveness of particular neurons.

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