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Immunocytochemical localization of β_{II} subspecies of protein kinase C in rat brain

(central nervous system)

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ABSTRACT **The distribution of a subspecies of protein kinase C (PKC) encoded by the β_{II} sequence in rat central nervous tissue was demonstrated immunocytochemically by using antibodies raised against an oligopeptide having a partial sequence specific for the β_{II} PKC. The β_{II} PKC immunoreactivity was widely but discretely distributed in the brain. The distribution of the β_{II} PKC immunoreactivity differed from that of the β_I and γ PKC subspecies. The β_{II} PKC immunoreactivity was found in the perikarya, dendrites, and axons of neuronal cells. Few if any glial cells were stained. Immunoreactive neurons were present in the anterior olfactory nucleus, olfactory tubercle, amygdaloid complex, caudate-putamen, accumbens nucleus, claustrum, dorsal part of the lateral septal nucleus, CA1 region of the hippocampus, subiculum, medial habenular nucleus, cerebral cortex, nucleus of the spinal tract of the trigeminal nerve, nucleus of the solitary tract, and substantia gelatinosa of the spinal cord. In these neurons, the β_{II} PKC immunoreactivity was seen mainly in the form of cytoplasmic dots and, in some cases, diffusely in the cytoplasm. Under electron microscopy, these immunoreactive large dots appeared to be associated with the Golgi complex, suggesting that the β_{II} PKC plays a specialized function at the Golgi complex in certain neuronal cell types.**

Protein kinase C (PKC), which is activated by 1,2-diacylglycerol in the presence of Ca^{2+} and phospholipids, acts as a key enzyme for signal transduction in various physiological processes (1–3). Recent molecular cloning studies have revealed that PKC is a large family consisting of at least seven subspecies (α, β_I, β_{II}, γ, δ, ε, and ζ) with closely related but distinct structures (3). Enzymological studies have shown that PKC can be resolved into three distinct fractions, types I–III, by hydroxyapatite column chromatography (4). Comparison of these fractions with the enzymes expressed in COS-7 cells transfected by the respective cDNA-containing plasmids (5, 6) has indicated that PKC types I, II, and III are products of γ, β, and α genes, respectively. Distribution of these three subspecies has been studied by biochemical and immunocytochemical procedures (7–13). The γ (type I) PKC is found only in the neurons of the central nervous system, whereas the β_I and β_{II} (type II) PKC and α (type III) PKC are detected in both central and peripheral tissues (7, 14–16). The enzymological properties of the δ, ε, and ζ PKC subspecies have not been defined.

The β_I and β_{II} PKC subspecies differ from each other only in short sequences in their carboxyl-terminal regions. A previous report (12) described antibodies against the β_{II} PKC, which were prepared by immunization with a synthetic oligopeptide, representing the specific sequence in this region, conjugated to bovine thyroglobulin with carbodiimide. The antibodies reacted specifically with β_{II} PKC and could distinguish the β_{II} and β_I subspecies, which are not separable by enzymological procedures. However, these antibodies produced only a weak immunoreaction in sections that were fixed with a solution containing glutaraldehyde. We have now prepared antibodies that give a strong reaction specifically with β_{II} PKC in sections fixed with glutaraldehyde and have thereby demonstrated the intracellular localization of the β_{II} PKC in central nervous tissue by light and electron microscopy.

MATERIALS AND METHODS

Production of Antibodies Against β_{II} Subspecies of PKC. The carboxyl-terminal portion of the β_{II} PKC (residues 660–673: Ser-Phe-Val-Asn-Ser-Glu-Phe-Leu-Lys-Pro-Glu-Val-Lys-Ser) was selected as an amino acid sequence specific to the β_{II} PKC. The oligopeptide was synthesized and coupled to bovine serum albumin by glutaraldehyde (17). The product was mixed with complete Freund's adjuvant and injected intracutaneously into rabbits. The antigen, emulsified with incomplete Freund's adjuvant, was injected repeatedly at intervals of 2 weeks, and the rabbits were bled 4–6 days after each booster injection. The titer of antisera was examined by enzyme-linked immunoadsorbent assay (ELISA) as described (18). The crude antisera with the highest titer were purified by affinity column chromatography on Sepharose CL-4B coupled to the β_{II} oligopeptide conjugated to bovine thyroglobulin by glutaraldehyde. The specific antibodies were eluted from the column with 0.2 M glycine buffer (pH 2.2).

Preparation of PKC. Rat brain PKC was resolved into types I–III by hydroxyapatite column chromatography (5, 6). The standard preparations of the α, β_I, β_{II}, and γ subspecies of PKC were obtained from COS-7 cells that were transfected with the respective cDNA-containing plasmids as described (5, 6).

Immunoblotting. For immunoblotting, samples were fractionated in a $NaDodSO_4$/7.5% polyacrylamide slab gel by electrophoresis in the buffer system described by Laemmli (19), and the proteins were transferred to nitrocellulose paper. The paper was incubated with the β_{II} PKC-specific antibodies, and the immunoreactive bands were visualized by the peroxidase-antiperoxidase method.

Immunocytochemical Staining. Frontal sections of rat brain were prepared as described (17), except for the substitution of 1% glutaraldehyde for 0.5% glutaraldehyde in the perfusion fixative. The sections were incubated for 18 hr at 4°C

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Abbreviation: PKC, protein kinase C.

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with the purified antibodies (1:200 dilution). The sections were incubated for an additional 4 hr with goat anti-rabbit IgG (Miles; 1:1000), washed, and incubated for 90 min with peroxidase-antiperoxidase complex (Miles; 1:5000). 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% H_2O_2. All sections were observed and photographed under a Zeiss microscope. The pattern of immunostaining was analyzed on an IBAS II image analyzer (Zeiss).

Electron microscopic studies were performed as described (11). In brief, 40-μm-thick Vibratome sections were stained as described for light microscopy without a detergent in the buffer. After incubation with osmic acid, the sections were embedded flat in Epon, and the ultrathin sections were observed with a Hitachi HS-9 electron microscope. The terminology of anatomy used here is that of Paxinos and Watson (20).

RESULTS

Production and Characterization of Antibodies. The titer of the antisera, as examined by ELISA, was increased after the second booster administration and reached maximal levels after the fourth booster. The antisera obtained were purified as described above and used for all experiments described below.

Immunoblot analysis revealed that the purified antibodies detected an 80-kDa band corresponding to PKC in the rat brain soluble fraction (Fig. 1, lane a) and that the antibodies detected only type II but not type I or type III PKC, which were obtained from the rat brain by hydroxyapatite chromatography (Fig. 1, lanes b–d). Direct evidence that the antibodies react specifically with the β_{II} PKC was provided by immunoblotting of PKC subspecies obtained separately from the respective cDNA-transfected COS-7 cells. The antibodies stained the β_{II} PKC but not the enzymes encoded by the α, β_I, and γ sequences (lanes e–h).

Immunocytochemical Localization of β_{II} PKC. *Distribution of β_{II} PKC immunoreactivity.* The immunoreaction was widely distributed but discretely localized in the brain (Fig. 2). The greatest density of β_{II} PKC immunoreactivity was seen in the olfactory tubercle, caudal part of the caudate-putamen, accumbens nucleus, CA1 region of the hippocampus (strata oriens and radiatum), layer VI of the cerebral cortex, and pars compacta of the substantia nigra. A moderately high density of immunoreactivity was seen in the internal plexiform layer of the olfactory bulb, anterior olfactory nucleus, basolateral and basomedial nuclei of the amygdaloid complex, rostral part of the caudate-putamen, globus pallidus, claustrum, dorsal part of the lateral septal nucleus, CA1 region of the hippocampus (stratum pyramidale), subiculum, dorsal lateral geniculate nucleus, layers I, II, III, and V of the cerebral cortex, layer VI of the cingulate cortex, layer VI of the entorhinal cortex, entopeduncular nucleus, pars reticulata of the substantia nigra, substantia gelatinosa of the spinal cord, fornix, and internal capsule. A moderate density of immunoreactivity was seen in the molecular layer of the cerebellar cortex, pyramidal tract, and corticospinal tract of the spinal tract. Other brain regions showed little or no immunoreactivity. There was no obvious immunoreaction in glial cells.

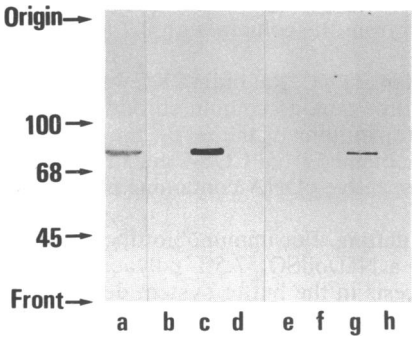

FIG. 1. Immunoblot analysis with the antibodies against β_{II} PKC. Samples of the PKC subspecies were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. The separated proteins were transferred to nitrocellulose paper and incubated with antibodies against β_{II} oligopeptide (1:50 dilution), followed by goat anti-rabbit IgG (1:1000) and rabbit peroxidase-antiperoxidase complex (1:2500). Lane a, crude soluble fraction of whole rat brain; lane b, type I PKC; lane c, type II PKC; lane d, type III PKC; lane e, α subspecies; lane f, β_I subspecies; lane g, β_{II} subspecies; lane h, γ subspecies. Molecular sizes (kDa) of standard proteins are indicated at left.

All immunoreaction was completely abolished by prior incubation of the antibodies with the immunogen (β_{II} oligopeptide). Normal rabbit serum failed to produce any immunoreaction (data not shown).

Characteristics of β_{II} PKC immunoreactivity in the neurons. Perikarya with immunoreactive material in the cytoplasm were seen in restricted areas of the rat brain such as the anterior olfactory nucleus, olfactory tubercle, amygdaloid complex, caudate-putamen, accumbens nucleus, claustrum, dorsal part of the lateral septal nucleus, CA1 region of the hippocampus, subiculum, medial habenular nucleus, layers II, III, V, and VI of the cerebral cortex, nucleus of the spinal tract of the trigeminal nerve, nucleus of the solitary tract, and substantia gelatinosa of the spinal cord.

In the dorsal part of the lateral septal nucleus, intense immunoreactivity was seen in large neuronal perikarya with dendritic processes (Fig. 3*A*). At a higher magnification, the immunoreactive material could be seen in a dotted pattern along the plasma membrane and/or in the cytoplasm of the perikarya and dendrites but not in the nucleus (Fig. 3*B*). The cells showing this type of immunoreactivity were also seen in the nucleus of the solitary tract.

In the hippocampus, which was one of the most densely stained areas, the immunoreaction was seen only in the CA1 area and not in the CA2–4 areas (Fig. 4*A*). In the CA1 area, dendritic trees of the pyramidal cells in the stratum oriens and stratum radiatum were densely stained, and large immunoreactive dots were seen in the pyramidal cell layer (Fig. 4*B*).

Dense immunoreaction was observed in the neuropils throughout the caudate-putamen, but poor or no immunoreactivity was observed in the fiber bundles (Fig. 4*C*). Immunoreactive large dots were more abundant in the caudolateroventral region than in the rostromediodorsal region of the caudate-putamen. Immunoreactive large dots were observed in the perinuclear area of medium-sized cells (20–25 μm) (Fig. 4*D*).

In many other neurons the β_{II} PKC immunostaining in the cytoplasm was seen as several large dots. In the neocortex, the immunoreactive material was seen as large dots mainly in the pyramidal cells through layers II, III, V, and VI, and most frequently in layer VI (Fig. 5).

Electron microscopic observation of the cortical pyramidal cells revealed that the large immunoreactive dots observed under light microscopy were located around the Golgi complex (Fig. 6).

In the white matter, nerve fibers were stained densely in the internal capsule and fornix, and moderately in the corpus callosum, pyramidal tract, and corticospinal tract of the spinal cord. There was, however, no or very weak immunoreaction in other nerve tracts such as the cranial nerve and optic tract.

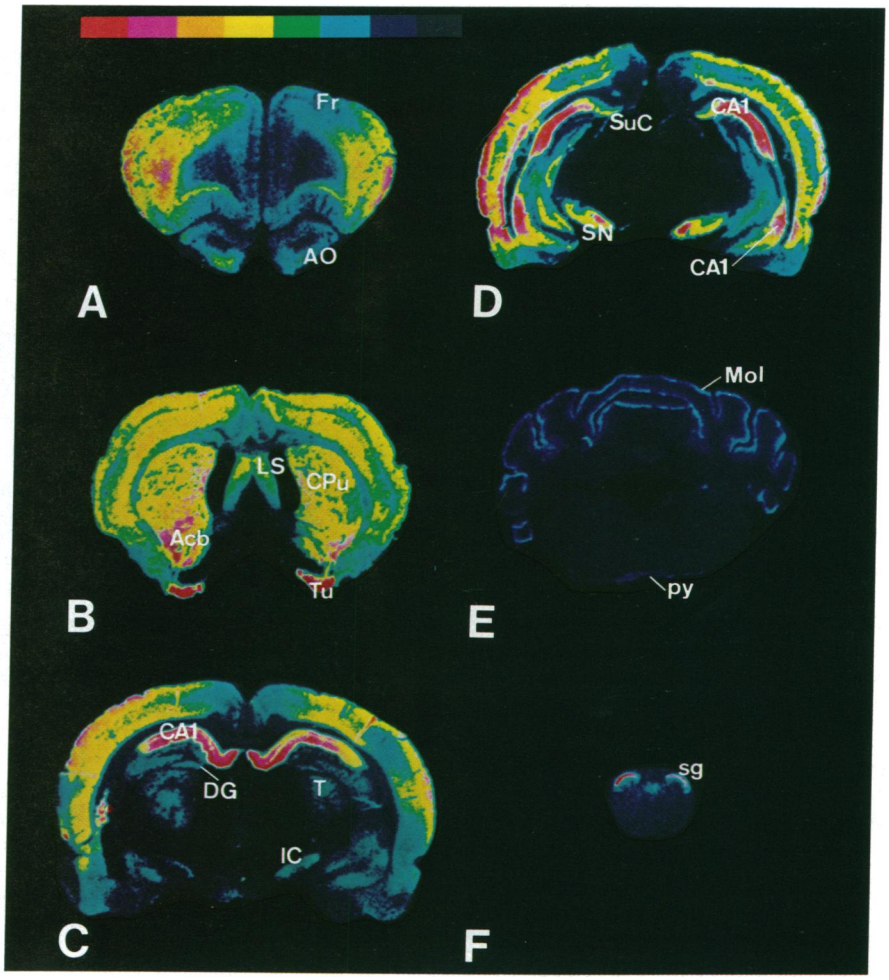

FIG. 2. Distribution of β_{II} PKC immunoreactivity in frontal sections of rat brain [rostral (*A*) to caudal (*F*)]. Immunoreactivity was localized by peroxidase-antiperoxidase immunocytochemistry. Strong immunoreaction with β_{II} PKC is seen in the olfactory tubercle, caudate-putamen, accumbens nucleus, CA1 region of the hippocampus (strata oriens and radiatum), neocortex, and substantia nigra. Relative density of immunoreactivity is shown according to the color table (highest density, red; lowest density, black). Acb, accumbens nucleus; AO, anterior olfactory nuclei; CA1, CA1 region of the hippocampus; CPu, caudate-putamen; DG, dentate gyrus; Fr, frontal cortex; IC, internal capsule; LS, lateral septal nucleus; Mol, molecular layer of the cerebellar cortex; py, pyramidal tract; sg, substantia gelatinosa of the spinal cord; SN, substantia nigra; SuC, superior colliculus; T, thalamus; Tu, olfactory tubercle.

DISCUSSION

In the present studies we have prepared an antiserum that recognizes the β_{II} PKC but not the α, β_I, 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 distribution of the β_{II} PKC subspecies is considerably different from that of γ PKC (type I) (10, 22) or β_I PKC (17). However, the densely immunoreactive β_{II} PKC areas also show γ PKC immunoreactivity and phorbol ester binding (23). The findings presented here do not perfectly correspond to the immunostaining patterns of the PKC antibodies prepared by other workers (7, 9, 24–26). Their antibodies appear to recognize other subspecies of PKC or both the β_I and β_{II} subspecies. For example, the present results confirmed that, in the rat cerebellum, the β_{II} PKC is present in the molecular layer, while β_I PKC is present in the granular layer, as described previously (12, 17). Huang *et al.* (7) have shown the presence of type II PKC in the granular layer, suggesting that their antibodies recognized β_I PKC. Hidaka *et al.* (9) have reported that, in the rabbit cerebellum, immunoreaction with type II PKC is detectable in the granular and molecular layers, suggesting that their antibodies react with both β_I and β_{II} PKC.

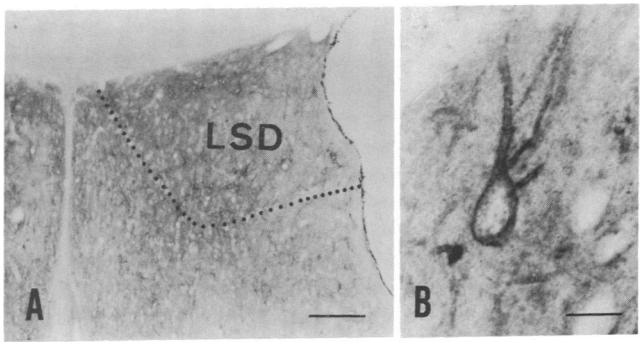

FIG. 3. Photomicrographs showing β_{II} PKC immunoreactivity in a frontal section of the septal nucleus as demonstrated by peroxidase-antiperoxidase immunocytochemistry. (*A*) Lower magnification of the septal nucleus. Dorsal part of the lateral septal nucleus contains immunoreactive neurons and relatively high immunoreactivity. (*B*) Higher magnification of the septal nucleus. A large neuron in this nucleus shows immunoreactivity along the plasma membrane and in the cytoplasm. LSD, dorsal part of lateral septal nucleus. (Bar = 50 μm in *A* and 25 μm in B.)

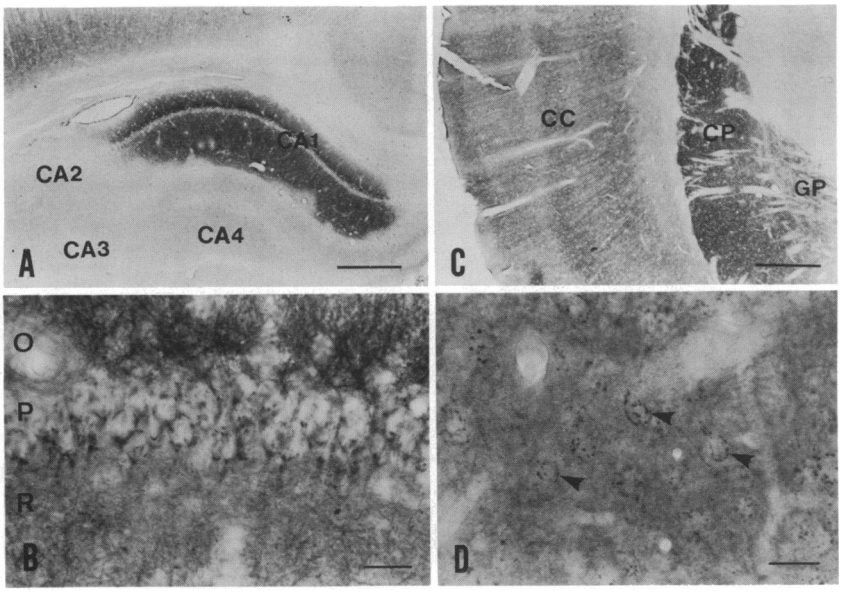

FIG. 4. Photomicrographs showing β_{II} PKC immunoreactivity in a frontal section of the hippocampus and caudate-putamen as demonstrated by peroxidase-antiperoxidase immunocytochemistry. (*A*) Lower magnification of the hippocampus. Immunoreaction is observed only in the CA1 region and not in the CA2, CA3, or CA4 region. (*B*) Higher magnification of the CA1 region. Dense immunoreaction is located in the neuropils of the strata oriens and radiatum. Large immunoreactive dots are seen reflecting the profile of the pyramidal cells. (*C*) Lower magnification of the cerebral cortex, caudate-putamen, and globus pallidus. Dense immunoreactivity is found in the caudate-putamen, and relatively dense immunoreactivity is seen in the cerebral cortex and globus pallidus. (*D*) Higher magnification of the caudate-putamen. The large immunoreactive dots are scattered among the immunoreactive neuropils. The large immunoreactive dots are seen reflecting a profile of the neuron (arrowheads). CA1–4, regions CA1–4 of the hippocampus; CC, cerebral cortex; CP, caudate-putamen; GP, globus pallidus; O, stratum oriens of the hippocampus; P, stratum pyramidale of the hippocampus; R, stratum radiatum of the hippocampus. (Bar = 500 μm in *A* and *C* and 25 μm in *B* and *D*.)

Brandt *et al.* (27) have identified the RNA transcripts of the β subspecies in the granular layer and those of the γ subspecies in the Purkinje cell layer in the rat cerebellar cortex by *in situ* hybridization histochemistry. Their results, together with the β_{II} PKC immunoreactivity in the molecular layer shown in the present studies, suggest that β_{II} PKC may be present in the parallel fibers which are the axons of the granule cells.

Both β_I and β_{II} PKC are present in regions such as the caudate-putamen, hippocampus, neocortex, laterodorsal thalamic nucleus, and substantia gelatinosa of the spinal cord, as described earlier (17). The present results, however, indicate that β_I and β_{II} PKC show different cellular expressions. For example, the β_I PKC immunoreactive cells (30–40 μm) in the caudate-putamen are apparently larger than the β_{II} PKC immunoreactive cells (20–25 μm). In the hippocampus, β_{II} and γ PKC colocalize in the pyramidal cells of the CA1 region (10). In contrast, the very few β_I PKC-immunoreactive cells recognized in the hippocampus are apparently nonpyramidal cells (17).

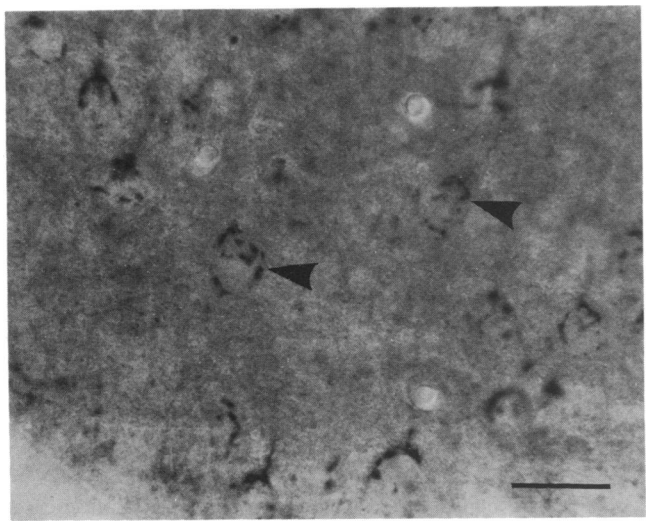

FIG. 5. Photomicrograph showing β_{II} PKC immunoreactivity in a frontal section of layer VI of the cerebral cortex as demonstrated by peroxidase-antiperoxidase immunocytochemistry. Large immunoreactive dots are observed along the profile of the neuron. (Bar = 50 μm.)

The distribution of β_{II} PKC in the hippocampus is very similar to that of the inositol 1,4,5-trisphosphate receptor, which is predominantly located in the strata oriens and radiatum in the CA1 region (28). It is also remarkably similar to the localization of the *N*-methyl-D-aspartate-sensitive L-glutamate receptor (29), which is considered to provide a basis for triggering long-term potentiation of synaptic transmission in the hippocampal CA1 region (30). The F1/B50 protein is a neuron-specific substrate of PKC that has been implicated in long-term modulation of synaptic efficacy (31, 32). Immunohistochemical studies have shown that this protein is also densely localized in the CA1 region of the hippocampus (33). By *in situ* hybridization, the selective expression of F1/B50 mRNA has been shown in the CA1 to CA4 pyramidal cells but not in the granule cells in the hippocampus (34). These findings, taken together with the present results, indicate that the F1/B50 protein may be contained with the γ and β_{II} PKC subspecies in the CA1 pyramidal cells and with the γ PKC in the CA3 pyramidal cells. However, PKC and the F1/B50 protein differ in their intraneuronal localization: PKC is located more densely in the perikarya, whereas the F1/B50 protein is located in the nerve terminal (33).

Electron microscopic observations demonstrated that the β_{II} PKC-immunoreactive material is frequently located around the Golgi complex in particular neurons. The location of the β_{II} PKC in the Golgi area suggests the involvement of this enzyme in Golgi function such as intracellular vesicle-

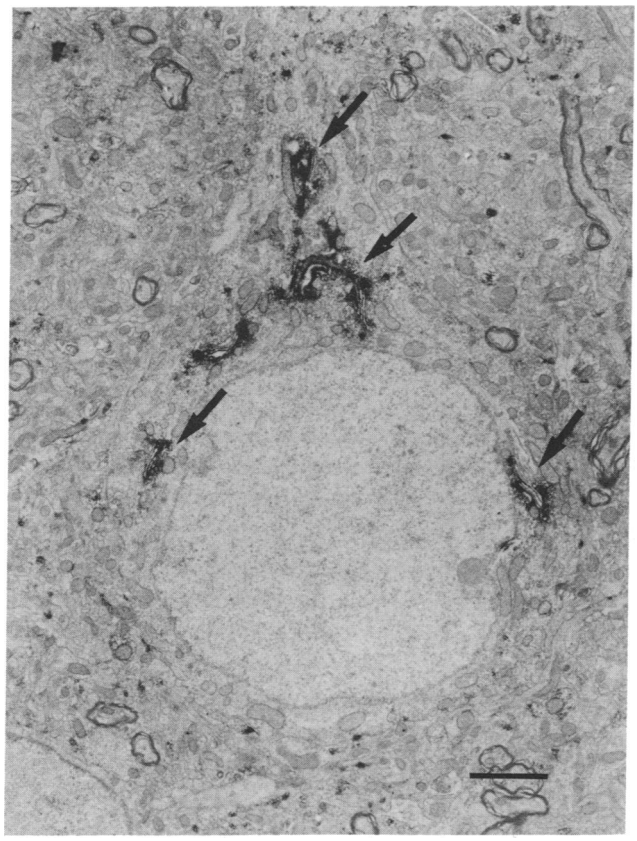

FIG. 6. Electron micrograph of a neuron in the cerebral cortex shows β_{II} PKC immunoreactivity as demonstrated by peroxidase-antiperoxidase immunocytochemistry. Immunoreaction is seen within the cytoplasm and located around the Golgi complex (arrows). (Bar = 2 μm.)

traffic regulation. The present observation also suggests that the β_{II} PKC is associated with a variety of neuronal functions in the central nervous system. Further biochemical and electron microscopic studies are needed to understand the precise functions of this PKC subspecies.

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