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Polyclonal antibodies to phospholipid/Ca^{2+}-dependent protein kinase and immunocytochemical localization of the enzyme in rat brain

(protein phosphorylation/subcellular localization/presynaptic terminals)

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ABSTRACT **Antisera against phospholipid/Ca^{2+}-dependent protein kinase (protein kinase C) were raised in rabbits. Immunospecificity of the polyclonal antibodies, as determined by immunoblot and ELISA, was shown by their reactivity to the enzyme but not to other protein kinases or any of many other proteins tested. Immunocytochemical localization of the kinase in rat brains revealed that although the enzyme was distributed broadly in different brain regions, it was highly restricted to the periphery of the nucleus of neurons in cerebral cortex and to axons and cells strongly resembling oligodendroglia in white-matter regions. Initial electron microscopy of cerebral cortex revealed that the enzyme was highly concentrated in the presynaptic terminals, and only rarely were labeled postsynaptic specialization elements seen. It is suggested that the discrete localization of the enzyme, which is distinct from that of the calmodulin/Ca^{2+}-dependent system, may be related to certain biological and functional aspects of brain that are regulated by Ca^{2+} at the level of protein phosphorylation.**

Phospholipid/Ca^{2+}-dependent protein kinase (PL/Ca-PK, or protein kinase C) is a major class of protein-phosphorylating enzyme first found in brain (1) and subsequently shown to occur widely in tissues and phyla of the animal kingdom (2). The biological significance of this Ca^{2+} effector system is further suggested by the presence of its numerous but specific substrate proteins in tissues (for reviews, see refs. 3 and 4), such as myelin basic protein in brain (5, 6), and its key role in transduction of receptor-mediated signals (7, 8), such as in the platelet activation induced by thrombin, platelet-activating factor, phorbol ester, diacylglycerol, and the Ca^{2+} ionophore A23187 (9).

Some significant progress has been made during the last five years on the molecular, pharmacological, and regulatory aspects of this novel protein-phosphorylation system (3, 4). Immunological characterization of the enzyme, however, has not been possible. The major reasons for the lack of progress are the difficulties in the purification of the enzyme in large quantity for immunization, due to its instability, and the low antigenicity of the enzyme, probably because of its common occurrence in tissues. In this laboratory, monoclonal antibodies against the enzyme have been developed recently (10); unfortunately, they are of the IgM class of immunoglobulins and were found to be unsuitable for the intended immunological studies of the enzyme system. We now report the development of polyclonal antibodies against pig brain PL/Ca-PK exhibiting a high immunospecificity toward the antigen and describe their use in immunocytochemical localization of the enzyme in rat brain.

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METHODS

Purification of PL/Ca-PK. The enzyme from the crude extracts of pig brain (1 kg) was purified initially by DEAE-cellulose (11) and Affi-Gel Blue chromatography (12), as previously described. The final step of the purification was affinity chromatography on polyacrylamide on which cholesterol and phosphatidylserine were immobilized, a procedure recently described by Uchida and Filburn (13). We slightly modified their procedure, as follows: The cholesterol/phosphatidylserine ratio was 10:1 instead of 5:1 and the buffer used was 5 mM Tris Cl, pH 7.5, instead of 5 mM Mes [2-(*N*-morpholino)ethanesulfonic acid], pH 6.5. Typically, 1–2 mg of apparently homogeneous enzyme could be prepared. Although no direct comparisons were made for the enzyme purification from the brain extracts, the polyacrylamide-cholesterol-phosphatidylserine affinity step appeared to give a better yield of purified enzyme than affinity chromatography on phosphatidylserine-Affi-Gel 102 (11) or phosphatidylserine-Affi-Gel 10 (14), in which the phospholipid is covalently coupled to the gels via the carboxyl or the amino group, respectively. The purified enzyme was used for immunization and other experiments presented in this report.

Production of Antisera and Determination of Antibody Titers. Four young adult rabbits were immunized initially with 300–500 μg of purified PL/Ca-PK in complete Freund's adjuvant by multiple intradermal injections along the back. At 4–6 week intervals, the rabbits were given intramuscular booster injections of 150–250 μg of enzyme in incomplete Freund's adjuvant. The antisera were collected after the third booster injection and subsequently every 3–4 weeks. The serum titers of the antibodies against the enzyme were determined by ELISA, using horseradish peroxidase-conjugated goat anti-rabbit IgG (Fc fragment-specific; Cooper Biomedical, Malvern, PA) as the second antibody and *o*-phenylenediamine as substrate. The color developed was quantified photometrically at 492 nm.

Immunoblotting. Brain extract, purified PL/Ca-PK, and other enzymes were electrophoresed in a $NaDodSO_4$/10% polyacrylamide gel and then transferred electrophoretically to a Zeta-Probe blotting membrane (Bio-Rad). After the membrane was incubated overnight at 50°C with 5% (wt/vol) casein in Tris-buffered saline (Tris/NaCl: 20 mM Tris Cl/150 mM NaCl, pH 7.5) to block nonspecific binding sites, it was incubated for 2–3 hr with the antiserum diluted 1:400 in 1% casein/Tris/NaCl, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Fc fragment-specific). Immunoreactive bands were detected by using 3,3′-diaminobenzidine. Coomassie blue-stained gels containing protein markers were run in parallel to allow determina-

Abbreviations: PL/Ca-PK, phospholipid/Ca^{2+}-dependent protein kinase; A-PK and G-PK, cyclic AMP- and cyclic GMP-dependent protein kinases, respectively; MLCK, myosin light chain kinase; BSA, bovine serum albumin.

tion of the M_rs of the immunostained proteins on the membrane. In addition, as an internal marker on the membrane, bovine serum albumin (BSA) was electrophoresed in the first lane of all gels used for immunoblotting; the BSA subsequently was visualized on the immunoblots by diaminobenzidine staining of horseradish peroxidase-conjugated goat anti-BSA (Cooper Biomedical).

Purification of Antibodies. The IgG fraction from the antiserum was purified by chromatography on DEAE Affi-Gel Blue (Bio-Rad) as follows: The antiserum (12 ml), dialyzed overnight against 20 mM potassium phosphate (pH 8.0) containing 0.02% sodium azide, was applied to a 2.6 × 14.0 cm column of the gel previously equilibrated with the same solution. Elution was with the same solution, and the IgG appeared in the unadsorbed fraction. Monospecific antibodies against PL/Ca-PK were purified by the following two procedures. First, the enzyme (1 mg) was coupled to CNBr-activated Sepharose 4B (0.2 ml, Bio-Rad) and the IgG fraction (0.2 ml) obtained from the above step was applied to the affinity gel. The column was washed with Tris/NaCl, and the antibodies were then eluted with 200 mM glycine·HCl (pH 2.8) and immediately neutralized. Second, purified PL/Ca-PK was electrophoresed on a $NaDodSO_4$/10% polyacrylamide gel and transferred to a blotting membrane, and the position of the enzyme was located as described above for immunoblotting on small strips cut from both sides of the membrane. The region of the membrane containing the enzyme was cut into small pieces, incubated with antiserum (1:5) for 2 hr at room temperature, rinsed with Tris/NaCl, and finally placed in a 3-ml syringe. Elution of the antibodies was carried out by drawing 200 mM glycine·HCl (pH 2.8) into the syringe and mixing for 5 min, a procedure described by Olmsted for other antibodies (15). The eluted antibodies (monospecific) were immediately neutralized with NaOH.

Tissue Preparation and Immunocytochemical Procedures. Male Sprague–Dawley rats (4–8 weeks old) were anesthetized by intraperitoneal injection of 35% chloral hydrate (1 ml/kg of body weight) and perfused through the aorta for 20 min with fixative. The fixative used was either 4.0% (wt/vol) paraformaldehyde and 0.1% glutaraldehyde in 0.12 M Millonig's buffer, pH 7.2 (16, 17), or the periodate/paraformaldehyde/lysine mixture of McLean and Nakane (18). The cerebrum, cerebellum, and brain stem were removed and stored overnight either in 4% paraformaldehyde in 0.12 M phosphate buffer (pH 7.2) or in phosphate-buffered saline (P_i/NaCl: 10 mM potassium phosphate/150 mM NaCl, pH 7.4). The tissue was then sliced in different planes to give 45-μm sections, using a Lancer Vibratome. The best sections were chosen by examination under a dissecting microscope and used in immunocytochemical experiments.

All steps were performed employing gentle agitation of the sections. The sections were incubated in primary antisera at dilutions from 1:200 to 1:2000 either for 1 hr at room temperature or for 12, 24, or 48 hr at 0–4°C. The sections were washed for 2–4 hr in 6–9 changes of P_i/NaCl and then processed according to the instructions in the Vectastain biotin-avidin-peroxidase kit (Vector Laboratories, Burlingame, CA), except that 2–3 hr washes were used. The last wash step was performed at 0–4°C and the peroxidase was developed for 4–7 min with diaminobenzidine (22 mg) and 30% hydrogen peroxide (10 μl) in 50 ml of ice-cold P_i/NaCl. The sections were then washed for 1 hr in P_i/NaCl and processed for light and electron microscopy exactly as described previously (17, 19). The same staining patterns obtained by using antisera were also observed with either the IgG fraction or the monospecific antibodies prepared by the procedures described above. Immunocytochemical controls included the substitution of normal rabbit serum, or IgG fraction derived therefrom, for the antisera in the first step of the staining. All such controls indicated specificity of immunostaining.

Other Methods. The purified catalytic subunit of cAMP-dependent protein kinase (A-PK) from bovine hearts (20) and the purified cGMP-dependent protein kinase (G-PK) from bovine lungs (21) were kindly provided by D. B. Glass. Myosin light chain kinase (MLCK) was purified from bovine hearts (22). PL/Ca-PK was assayed as described (11).

RESULTS AND DISCUSSION

Properties of Antibodies Against PL/Ca-PK. Antisera collected after the third booster injection showed high titers (up to 1:16,000) of antibodies against the enzyme, as determined by ELISA, in two of the four rabbits immunized. The antiserum from one of these two rabbits was used in the present studies. Immunoblots showed that the antibodies are reactive with PL/Ca-PK (M_r 80,000) but not with the catalytic subunit of A-PK, G-PK, or MLCK (Fig. 1*B*, lanes 3–6) or any of the marker proteins, clearly indicating the immunospecificity of the antiserum. The antibodies reacted with a M_r 67,000 protein in the rat brain extract (Fig. 1*B*, lane 2). This immunoreactive protein presumably is derived from

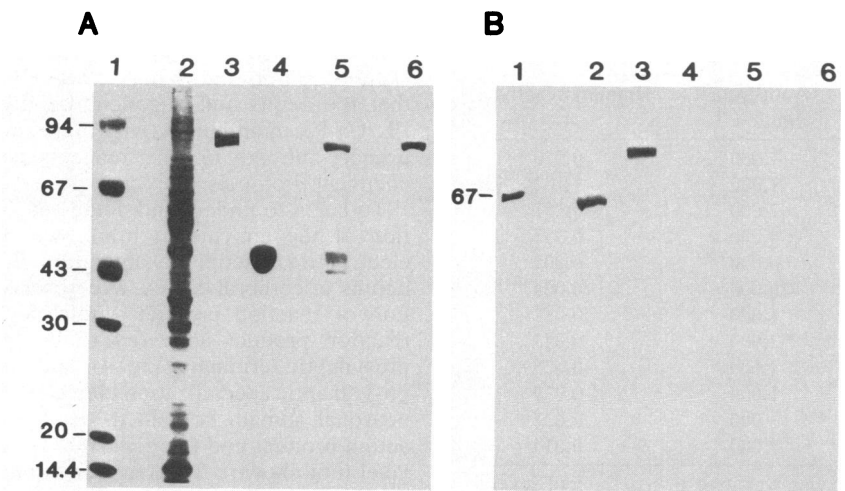

FIG. 1. Immunospecificity of antiserum against PL/Ca-PK. (*A*) Proteins stained with Coomassie blue. (*B*) Immunoblot of the corresponding proteins. Lanes: 1, protein markers ($M_r \times 10^{-3}$ at left); 2, brain extract (150 μg of protein); 3, PL/Ca-PK (5 μg); 4, catalytic subunit of A-PK (5 μg); 5, G-PK (10 μg); 6, MLCK (5 μg). BSA in lane 1 (*B*) was visualized with horseradish peroxidase-coupled anti-BSA.

the native species (M_r 80,000) of the enzyme, as it has been reported that PL/Ca-PK is highly susceptible to proteolysis yielding smaller species of the enzyme (23). This contention is supported by our following findings: An identical immunostain of the M_r 67,000 in the brain extract was also seen when monospecific antibody to the M_r 80,000 enzyme species was used in place of the antiserum and the presence of the M_r 80,000 enzyme species immunostained with the antisera was readily demonstrated when the brain was homogenized in 20% (wt/vol) trichloroacetic acid to inhibit proteolysis (results not shown).

Immunospecificity of the antibodies was further demonstrated by lack of cross-reactivity, as determined by ELISA, for the catalytic subunit of A-PK or MLCK (Table 1) or for any of the marker proteins in Fig. 1 (data not shown).

The antiserum, in contrast to nonimmune serum, was found to be able to slightly inhibit PL/Ca-PK. To ascertain that the effect was not caused by other serum components, we examined the effect of monospecific antibodies prepared from the antiserum. The enzyme activity was inhibited about 60% at the highest amount (4 μg) of the affinity-purified antibodies tested (Fig. 2).

Immunocytochemistry. Low-magnification light microscopy revealed subtle differences in the regional distribution of PL/Ca-PK immunoreactivity in brain (Fig. 3*A*). In general, slightly more reaction product was seen in telencephalic structures than in diencephalic and mesencephalic areas. The white matter appeared lightly stained in low-magnification views (Fig. 3*A*). Since there were no striking differences apparent at low magnifications in the regional distribution of the enzyme, we performed an analysis at higher magnification to test for differences in labeling related to cell type in various brain regions. The staining pattern of cerebral cortex was a laminar distribution of label superimposed on a less dense overall staining (Fig. 3 *B* and *C*); the laminar pattern resembled the characteristic Nissl staining pattern of the various cortical divisions. Higher magnification, however, indicated that the staining was restricted to the periphery of the nucleus of different-sized cells in the various cortical layers rather than staining of the entire cell (Fig. 3*D*). On the basis of size and position within the cortical layers, it is likely that the majority of these cells were neurons. The neuropil surrounding the cells contained a fine punctate distribution of reaction product (Fig. 3*D*). This punctate distribution was also observed in the outer cortical layer which contained a large population of dendrites from cortical neurons lying in deeper regions. The dendrites themselves did not appear to contain reaction product at this level of resolution (Fig. 3*D*).

Table 1. Immunospecificity of antiserum against PL/Ca-PK

Protein kinase	Antiserum dilution^{-1}	Immunoreactivity, A_{492}
None (control)	1,000	0.010
PL/Ca-PK	1,000	1.182
	2,000	0.751
	4,000	0.353
	8,000	0.201
	16,000	0.064
A-PK (catalytic subunit)	1,000	0.082
	2,000	0.043
	4,000	0.025
MLCK	1,000	0.039
	2,000	0.027
	4,000	0.014

One microgram of each enzyme was used to coat each of several wells of a Microtiter plate and subsequently incubated with the indicated dilutions of the antiserum raised against PL/Ca-PK. The immunoreactivity in ELISA was quantified photometrically at 492 nm (see *Methods*).

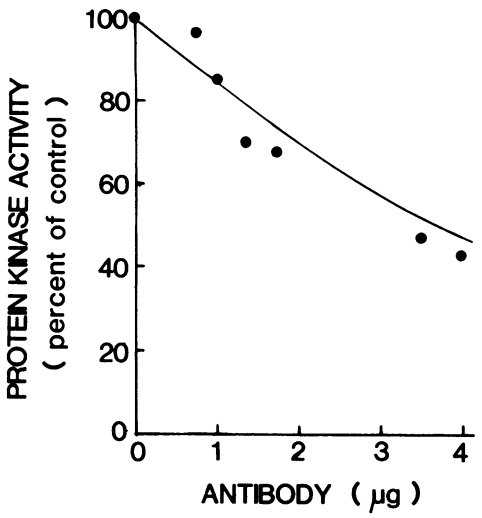

FIG. 2. Inhibition of PL/Ca-PK activity by immunoaffinity-purified (see *Methods*) monospecific antibody. The enzyme was incubated with various amounts of antibody for 45 min at 30°C before the enzyme activity was assayed. The control activity of the enzyme seen in the absence of added antibody, taken as 100%, was 2.72 μmol of phosphate transferred/min. Results similar to those shown here were obtained in three other experiments.

A close inspection of white-matter regions showed an interesting distribution of reaction product. The myelin itself was unstained with these experimental protocols, and the axons were moderately stained (Fig. 3*E*). There were labeled cells throughout the white matter that were characterized by a small oval soma from which fine labeled processes emanated. Although it was not possible to unequivocally identify these cells at the light-microscopic level, their morphological features strongly suggested that they were oligodendroglia. A major substrate for PL/Ca-PK in brain is myelin basic protein (6). Since oligodendroglia play a prominent role in the synthesis of myelin components, these localization results are consistent with a role for the enzyme in myelin function. To further assess the distribution of PL/Ca-PK in white matter (an area where penetration of antibodies is sometimes restricted), we treated some vibratome slices with 0.25% Triton X-100 in P_i/NaCl for 20 min before performing antibody cytochemistry. These slices revealed enhanced immunoreactivity in the axons and in the oligodendroglia (Fig. 3*F*) but no obvious increase in staining of the myelin sheath itself or of grey-matter regions. These results suggest that the axons and oligodendroglia contain pools of anti-PL/Ca-PK immunoreactivity that exhibit restricted access to primary antibody or other reagents used in the immunocytochemical protocols.

To begin to understand details of the subcellular distribution of the enzyme in brain, we performed preliminary electron-microscopic examination of several regions and depths of cerebral cortex. We observed an uneven distribution of reaction product within neuronal processes. The reaction product appeared to be highly concentrated in presynaptic terminals (Fig. 4), and only rarely were labeled postsynaptic specialization elements seen. The cytoplasm of neuronal somata contained small focal depositions of reaction product and the dendrites were essentially devoid of label (not shown). The reaction product within presynaptic terminals tended to associate with various organelles, especially synaptic vesicles (Fig. 4). It was not possible to delineate the exact organelles with which PL/Ca-PK was preferentially associated, since we could not rule out possible

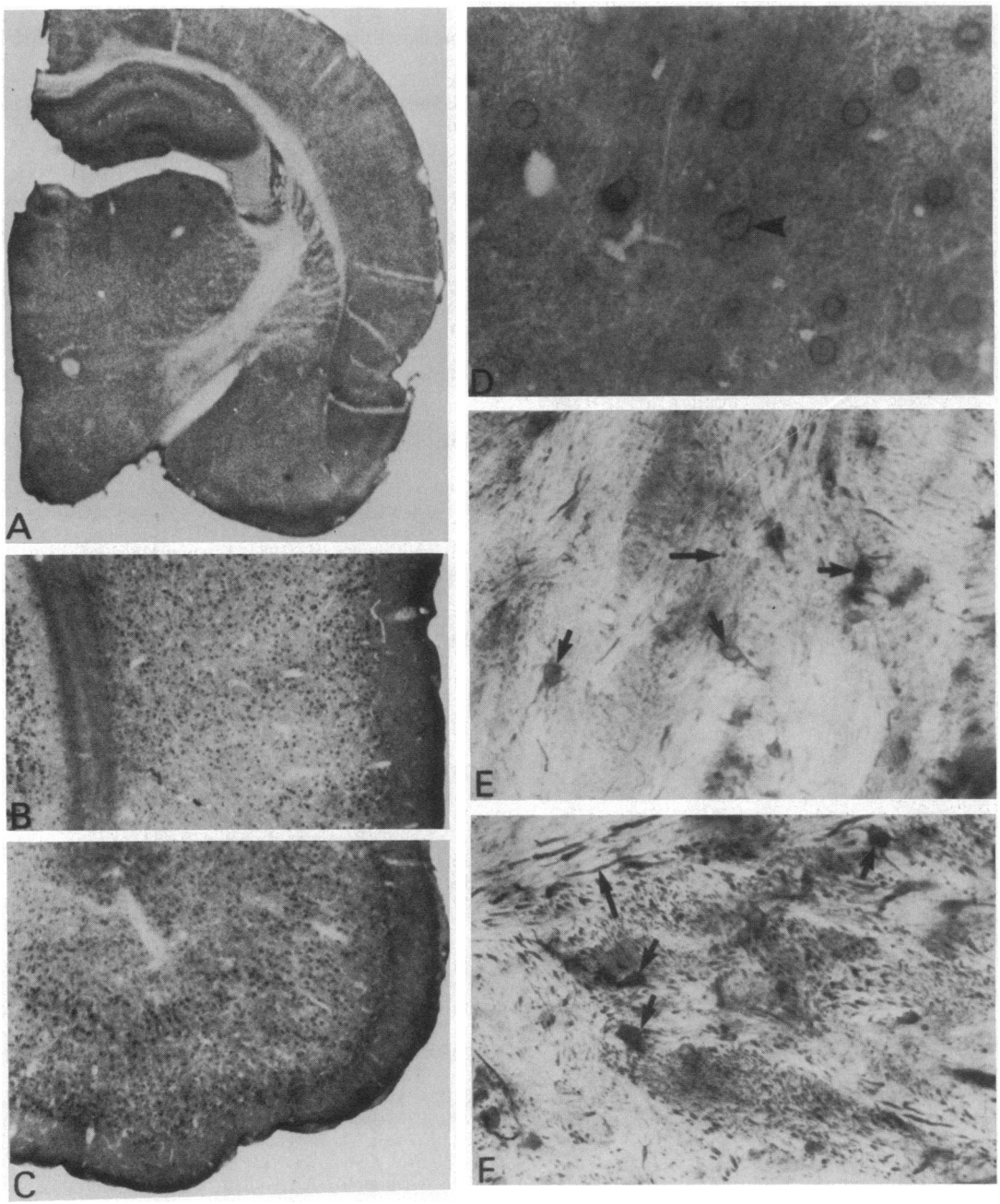

FIG. 3. Light micrographs of coronal sections of the rat brain indicating the distribution of PL/Ca-PK immunoreactivity. (*A*) Immunoreactivity is broadly distributed throughout the grey matter, whereas the white matter appears lightly stained at this magnification. (×10.) (*B* and *C*) Higher-magnification views of neocortex (*B*) and entorhinal cortex (*C*) which illustrate that the pattern of immunoreactivity reflects the characteristic cellular distribution of neurons in these regions. (×40.) (*D*) Cellular immunoreactivity is due to staining of the periphery of the nucleus (arrowhead) of neurons rather than of the cytoplasm. There is also a fine, punctate distribution of reaction product in the neuropil that is better visualized in the electron micrographs. (×408.) (*E* and *F*) Regions of white matter showing the distribution of immunoreactivity without (*E*) or with (*F*) prior treatment of the section with Triton X-100. In both cases, cells closely resembling oligodendroglia (short arrows) are labeled as are profiles that are axons (long arrows) sectioned at different angles. Immunoreactivity is enhanced after Triton treatment. (×240.) The myelin sheath is unstained and cannot be seen in these micrographs.

artifacts of diaminobenzidine diffusion. Nonetheless, these results are consistent with a major role for the enzyme in presynaptic function, perhaps related to the phosphorylation of proteins such as synapsin (24).

It is of some interest to compare the distribution, as currently understood, of the PL/Ca-PK system to that of the calmodulin/Ca^{2+}-regulated kinase systems in brain. The immunocytochemistry of the calmodulin/Ca^{2+} system has been studied by using antibodies to calmodulin itself (25, 26) and to calmodulin kinase II (24). Calmodulin kinase II (24) and calmodulin (unpublished observations) exhibit a regional variation in their localization, whereas PL/Ca-PK is more broadly distributed (present results). Similar subcellular distributions for the calmodulin/Ca^{2+} system are revealed with antibodies to calmodulin (25) or to calmodulin kinase II (24). The system (as defined by presence of immunoreactive elements) is highly concentrated in neuronal somata and dendrites and is specifically associated with postsynaptic densities (24, 27) and dendritic neurotubules (25). The present results suggest that the PL/Ca-PK is concentrated in axons and presynaptic terminals and is sparsely distributed in neuronal cell body cytosol and dendrites. Although negative

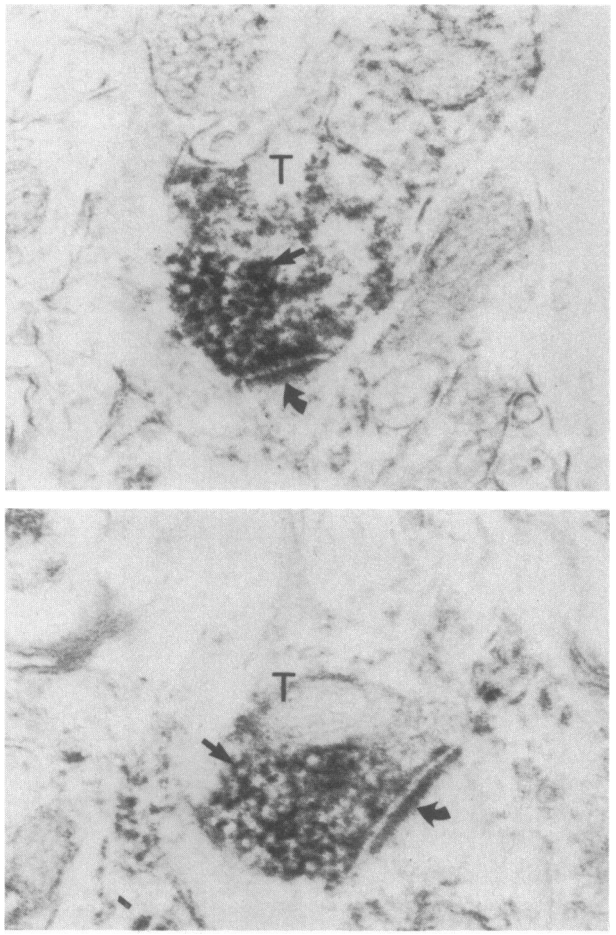

FIG. 4. Electron micrographs showing the PL/Ca-PK immunoreactivity in presynaptic terminals (T) of rat cerebral cortex. Reaction product is particularly prominent around synaptic vesicles (straight arrows). The postsynaptic densities (curved arrows) are inherently electron-dense but do not contain reaction product. (×44,800.)

results in immunocytochemical experiments must be viewed with caution, this apparent complementary distribution of the two major Ca^{2+} effector systems (activated by phospholipid or calmodulin, respectively) in brain suggests that at least a part of the functional role of the systems may be determined by their compartmentalization within the neuron.

The present results indicate two additional major pools of PL/Ca-PK that may play roles in nervous system function. As stated earlier, the presence of the enzyme in cells strongly resembling oligodendroglia is consistent with the suggestion that this kinase may play a role in the process of myelinogenesis, and perhaps, demyelination (6). The presence of a pool of immunoreactivity in the periphery of the nucleus is intriguing, as it suggests a role for the kinase at the level of nuclear function in the cell.

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