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Immunocytochemical Localization of Protein Kinase C in Identified Neuronal Compartments of Rat Brain

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Polyclonal antisera to the phospholipid/Ca^{2+}-dependent protein kinase have been used to study the distribution of the enzyme in identified neurons of several brain regions. The results indicated that the enzyme was concentrated in synaptic terminals of mossy fibers, Golgi II neurons and Purkinje neurons in the cerebellum, and in granule cell terminals in the hippocampus. These synapses have different physiological properties and utilize different neurotransmitters. Electron microscopic results indicated that the enzyme was concentrated in presynaptic terminals. Thus, the protein kinase may play a broad role in Ca^{2+}-related events of the presynaptic terminal during neurotransmission. Light- and electron-microscopic immunocytochemical analysis also indicated that the enzyme was inside the nucleus concentrated in a region adjacent to the inner nuclear membrane, where it may play a role in the regulation of neuronal function.

An interesting question in neurobiology concerns the role of phosphorylation in neuronal function. In view of the importance of calcium to the neuron, it is logical to focus on protein kinase systems, which are regulated in a Ca^{2+}-dependent manner. Two major regulator systems concerned with phosphorylation in brain (and other tissues) are the calmodulin/Ca^{2+} (Cheung, 1980) and the phospholipid/Ca^{2+}-dependent proteins kinase(s) (Kuo et al., 1984; Nishizuka, 1984). The distribution of the calmodulin/Ca^{2+} system in brain has been studied using antisera to calmodulin (Wood et al., 1980) and to calmodulin kinase II (Quimet et al., 1984). We have recently purified phospholipid/Ca^{2+}-dependent protein kinase (PL/Ca-PK, or protein kinase C) from pig brain and raised polyclonal antibodies to the enzyme in rabbits (Girard et al., 1985, 1986). Our preliminary work indicated that the enzyme had a broad distribution in brain and appeared to be present both in neurons and in cells strongly resembling oligodendroglia (Girard et al., 1985, 1986). In this study we have performed a systematic examination of the subcellular distribution of anti-PL/Ca-PK immunoreactivity in identified brain regions. We show that the enzyme is concentrated in presynaptic terminals possibly in association with synaptic vesicles of a variety of neurons that utilize different neurotransmitter types. We further show that a pool of immunoreactivity shown by light microscopy to be in the region of the nucleus (Girard et al., 1985, 1986) is inside the nucleus and is present for the most part subadjacent to the nuclear membrane. These results suggest that the Pl/Ca-PK may also be involved in brain function at the level of regulation of nuclear function in neurons.

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Materials and Methods

The biotin-avidin-peroxidase based immunostaining kit (Vectastain) was from Vector Laboratories (Burlingame, CA). Glutaraldehyde (10%) and paraformaldehyde were from Polysciences, Inc. (Warrington, PA). 3,3′-Diaminobenzidine (DAB) was from Sigma (St. Louis, MO). Acrylamide, bisacrylamide, SDS, nitrocellulose paper, and Zeta-Probe blotting membranes were from Bio-Rad (Richmond, CA). All other chemicals were reagent grade.

Antibody preparation

The purification of antigen and preparation of antibodies are described in detail elsewhere (Girard et al., 1985, 1986). Briefly, the PL/Ca-PK was purified by the chromatographic steps of DEAE-cellulose (1B) and Affi-Gel Blue (Kikkawa et al., 1982), as previously described. The final purificiation was achieved using a polyacrylamide affinity column in which cholesterol and phosphatidylserine were immobilized essentially according to Uchida and Filburn (1984). The ratio of cholesterol to phosphatidylserine was changed to 10:1, and the buffer system used in the affinity chromatography step was 5 mM Tris/HCl, pH 7.5 (Girard et al., 1985, 1986). A typical complete isolation starting from 1 kg of brain tissue yielded 1–2 mg of the enzyme of apparent homogeneity. Antisera were prepared as described (Girard et al., 1985, 1986) using initial intradermal injections of 300–500 μg of purified PL/Ca-PK in complete Freund's adjuvant at multiple sites along the rabbit's back. At 4–6 week intervals, the rabbits were boosted by intramuscular injections of 250–250 μg of the enzyme in incomplete Freund's adjuvant. The antisera were collected after the third boost and subsequently every 3–4 weeks. The antisera were purified in 2 ways. (1) The IgG fraction from the serum was prepared using DEAE-Affi-Gel Blue (BioRad) chromatography eluted with 20 mM potassium diphosphate (pH 8.0) containing 0.02% sodium azide, and (2) affinity-purified antibodies were prepared as described (Girard et al., 1985, 1986; Mazzei et al., 1985) using essentially the method of Olmsted (1981).

Immunocytochemical procedures

The immunocytochemical procedures were exactly as described previously (Wood et al., 1980) and will only be summarized here. Male Sprague-Dawley rats were perfused through the heart for 20 min with a 4.0% paraformaldehyde, 0.1% glutaraldehyde mixture developed by us (Wood et al., 1976), or with the periodate–paraformaldehyde–lysine mixture of McLean and Nakane (1974). Tissue prepared using the latter fixative was kindly provided by Dr. J. McDonald, Emory University. The fixed tissue was sectioned in different planes at 45–75 μm using a Lancer vibratome. The best sections were chosen under a dissecting microscope and used in the immunocytochemical experiments.

All incubation steps in immunoreagents were employed using gentle agitation of the sections. In the final conditions adopted, the sections were treated with primary antiserum or preimmune or normal rabbit serum (1:200, 1:1000, or 1:2000) in PBS for 1 hr at room temperature or for 12, 24, and 48 hr at 0–4°C. No detergents were used in these experiments. The sections were then washed for 2 hr in 6–9 changes of PBS and incubated for 1 hr with biotinylated goat anti-rabbit IgG (Vectastain). The sections were washed for 2 hr, incubated for 1 hr in the avidin DH and biotinylated HRP reagent of the Vectastain kit, followed by an additional 2 hr wash in PBS. The last wash in PBS was at 0–4°C, and the peroxidase was developed for 4–7 min using an ice-cold solution (50 ml) of DAB (22 mg) and 30% hydrogen peroxide (10 μl). The sections were then washed for 1 hr in PBS and processed for light and electron

microscopy exactly as described previously (Wood et al., 1980). Light- and electron-microscopic examination was performed on sections fixed by both procedures outlined above, and the results were equivalent. In all of the EM images reported here, the tissue was not counterstained with heavy metals.

Results

Immunochemistry

The procedures used for immunochemical characterization of the antisera in these studies are described in detail elsewhere (Girard et al., 1985, 1986). We have shown by immunoblot analysis that the antisera are only reactive with a M_r 67,000 polypeptide in brain extracts prepared by homogenization in a standard extraction buffer. When brain tissue was homogenized in 10% trichloroacetic acid to inhibit proteolysis, an M_r 80,000 polypeptide was readily demonstrated in addition to the M_r 67,000 species (Girard et al., 1985, 1986). These 2 polypeptides appear to be immunologically related, since monospecific antibodies eluted from either band immunostain both bands (Girard et al., 1986). The recognized susceptibility of the PL/Ca-PK to proteolysis (Kishimoto et al., 1983) is the most likely explanation for the generation of the M_r 67,000 immunoreactive protein when precautions are not taken to inhibit proteolysis.

Immunocytochemical controls

It has not been possible to perform preabsorption immunocytochemical controls because the enzyme is isolated in very small amounts and is too valuable for other experimental purposes. We have therefore used the affinity-purified antibodies from either the 80 or 67 kDa polypeptide to immunostain brain tissue. The results of these studies (Mazzei et al., 1985) showed that the pattern of staining with whole sera was virtually identical to that seen with affinity-purified antibodies from either the 80 or 67 kDa polypeptide. In view of these results, we used primary antiserum in the present experiments in order to conserve the monospecific antibodies for additional immunochemical characterization of the enzyme. Control experiments in which 1:200 dilutions of preimmune or normal rabbit serum were substituted for immune serum never exhibited specific reaction product for peroxidase (Fig. 2*E*).

Light-microscopic analysis

In these studies we concentrated on 3 specific brain regions for detailed analysis of the distribution of anti-PL/Ca-PK immunoreactivity: the cerebellar cortex, the deep cerebellar nuclei, and the dentate gyrus. Our reason for choosing these areas was that the synaptic connectivity and neurotransmitter type employed by neurons in these regions were relatively well understood, and detailed correlative analysis between light and electron microscopic images was possible.

In the cerebellar cortex, 3 major regions exhibiting positive immunoreactivity with identified neuronal elements were studied. In the granular cell layer, a striking punctate distribution of reaction product was interspersed among the tightly packed granule cells (Fig. 1*B*). On the basis of this distribution pattern, as well as the size of the immunoreactive elements, they were almost certainly components of the synaptic glomerulus formed by mossy fiber and Golgi II neuronal presynaptic elements on granule cell dendrites (Palay and Chan-Palay, 1974). A systematic examination of favorably sectioned profiles indicated that a common pattern of staining was a relatively large centrally placed element surrounded by an immunonegative region, which was in turn surrounded by a punctate ring of labeled profiles (Fig. 1*B*). This pattern strongly suggested that the immunopositive images were the centrally placed mossy fiber and peripherally placed Golgi II neuronal synaptic elements impinging on granule cell dendrites (Palay and Chan-Palay, 1974), and this impression was confirmed at the electron-microscopic level (not shown). Thus, the anti-PL/Ca-PK serum recognized the apparently disparate presynaptic elements of the depolarizing mossy fiber synapse and the hyperpolarizing, probably GABAergic synapse of Golgi II neurons.

The most striking immune staining seen in the Purkinje layer was in the periphery of the nucleus of some, but not all, of these cells (Fig. 1, *A, B*). There was very little indication of immunoreactivity in a punctate pattern surrounding the Purkinje cell somata, but the molecular layer was filled with small dots of reaction product (Fig. 1*A*). Many of these dots were adjacent to unlabeled profiles, which were almost certainly sectioned dendrites (Fig. 1*A*). Although the nuclear labeling of Purkinje neurons was the most striking, close inspection of the light micrographs revealed that the nuclei of interneurons in the granular and molecular cell layers were also labeled (Fig. 1, *A, B*). On the basis of size and distribution, the granular layer neurons were probably Golgi II cells and the molecular layer neurons were probably inner (basket) and outer stellate neurons. If the nuclei of the granule cells were labeled at all at the light-microscopic level, the intensity was very low compared to other neurons of cerebellar cortex.

The pattern of immunoreactivity exhibited by the anti-PL/Ca-PK sera in the deep cerebellar nuclei is illustrated (Fig. 1, *C–E*). The most striking features were again labeling of the periphery of the nucleus of large output neurons of the nuclei, as well as a punctate distribution of reaction product around neuronal somata and along identified dendrites from these cells. In addition, there was a distribution of reaction product in the neuropil, which appeared to be made up largely of profiles of presynaptic terminals outlining dendrites of deep cerebellar neurons (Fig. 1, *C–E*). If more concentrated antiserum was used, there was an increase in somal labeling of the deep cerebellar neurons (Fig. 1, *D, E*).

An extensive analysis of anti-PL/Ca-PK immunoreactivity at the light- and electron-microscopic level was performed in the CA3 region of the hippocampus. A light microscopic analysis was also undertaken in the remaining regions of the hippocampal formation. We report here our results from analysis of the CA3 region. Figure 2 illustrates the region immediately adjacent (with some inclusive profiles) to the pyramidal cell layer. It was apparent that the bulk of the label was in large punctate dots adjacent to pyramidal cell apical dendrites coursing towards the stratum radiatum (Fig. 2, *A–C*). In addition, a number of very fine punctate immunopositive profiles filled the region of the stratum radiatum (Fig. 2*B*) and oriens (Fig. 2*C*), and no large labeled profiles were observed in those regions.

Electron-microscopic analysis

Electron-microscopic studies of the CA3 region in areas containing apical dendrites of pyramidal cell neurons revealed a number of large immunopositive presynaptic terminals (Fig. 3*F*)

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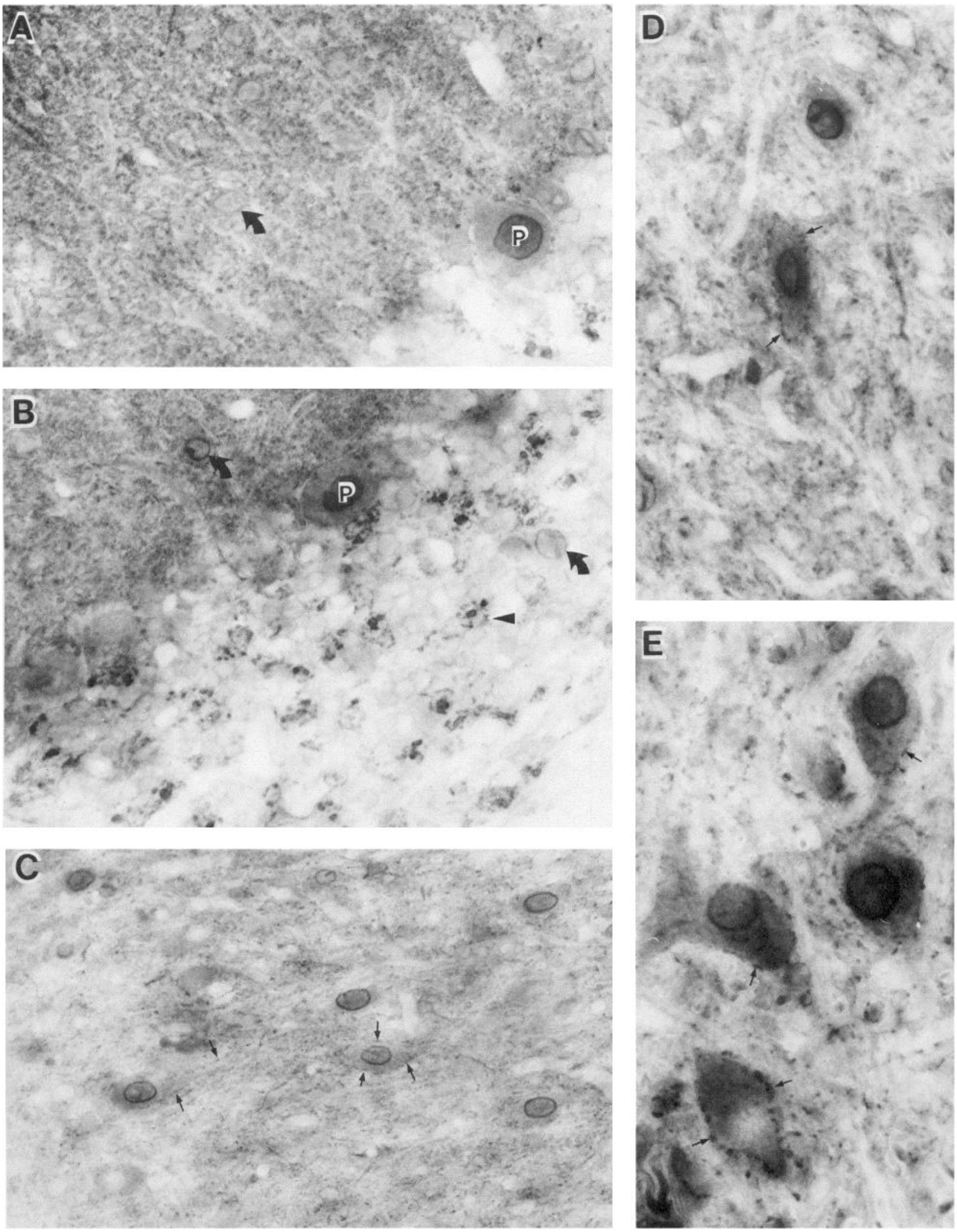
Figure. 1. Light micrographs of anti-PL/Ca-PK immunoreactivity in cerebellum. *A* and *B*, Images of cerebellar cortex in which the Purkinje neurons (*P*) run obliquely. The molecular layer is to the *left* of the Purkinje layers and exhibits a fine punctate distribution of reaction product. The periphery of the nucleus of Purkinje neurons (*P*) is labeled, and some label appears in the nucleus itself. In addition, staining is seen in the periphery of the nucleus of interneurons (*curved arrows*) and in the molecular layer and the granular layer which lies to the *right* of the Purkinje layer in these images. A striking punctate distribution is seen throughout the granular layer (*arrowhead*). This staining pattern is almost certainly composed of a central positive mossy fiber terminal surrounded by an immunonegative region containing granule cell dendrites, which are in turn



surrounded by punctate profiles of presynaptic terminals primarily from Golgi II neurons. The images in *C–E* are all of deep cerebellar nuclei. The tissue in *C* was treated with antiserum at a 1:1000 dilution, and the tissues in *D* and *E* were treated at 1:200 dilution. In all 3 images the staining at the periphery of the nucleus is prominent, and the cells and their dendrites are surrounded by a punctate distribution of reaction product in structures resembling synaptic boutons (*small arrows*). Tissue treated with more dilute antisera shows little staining of the neuronal cytoplasm at the light-microscopic level, whereas some staining of somata is apparent using more concentrated antisera. It is expected that some antigen would be present in the somata, since this is the site of synthesis of neuronal macromolecules. *A* and *B*, ×400; *C*, ×235; *D*, ×500; *E*, ×530.

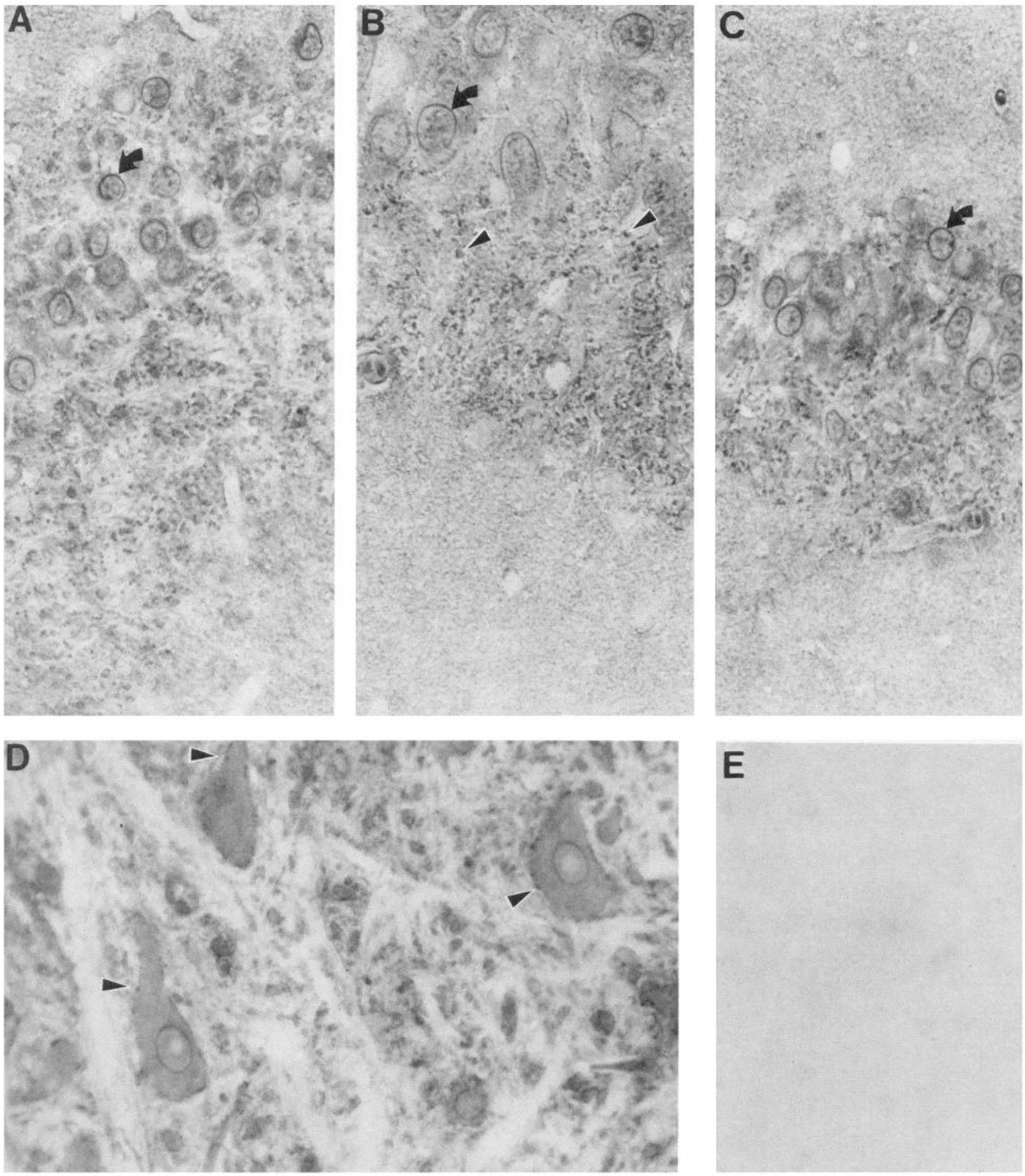


Figure 2. Light micrographs of several areas in the CA3 region of hippocampus. *A, B, C,* The nuclear periphery of pyramidal neurons is stained (*curved arrows*) and some fine staining is apparent in the rest of the nucleus. A number of large immunopositive profiles (*arrowheads*) are apparent in the region of pyramidal cell apical dendrites in all 3 images. A distribution of much smaller profiles is also present in the stratum radiatum and stratum opticum regions. *D,* Example of the generality of our results to other brain regions by showing a punctate distribution of reaction product around motor neurons in the spinal cord (*arrows*). There is also a punctate distribution of reaction product in the neuropil as well as a perinuclear staining within the motor neurons. *E,* Indicates specificity of the immunocytochemical procedures in which preimmune serum was substituted for immune serum to stain cerebellar slices adjacent to those shown in Figure 1. The Purkinje cell layer is in the middle of the micrograph. No specific reaction product for peroxidase is observed. *A,* ×450; *B,* ×500; *C,* ×410; *D,* ×510; *E,* ×400.

that correlated with the light-microscopic images shown in Figure 2. These terminals have been extensively characterized as mossy fiber terminals of axons coming from the hippocampal granule cells (Swanson et al., 1978). The reaction product was consistently seen as a rim of label around synaptic vesicles (Fig. 3*F*). In contrast to the heavy label within presynaptic terminals, the dendrites and postsynaptic densities (PSD) were unlabeled at moderate to high dilutions of antisera (Fig. 3*F*). Experiments

using more concentrated primary antisera (1:200) resulted in the apparent labeling of PSDs in the most superficial ultrathin sections (not shown). The most consistent pattern of synaptic labeling using a range of dilutions of antisera, however, was a labeled presynaptic terminal and unlabeled PSD.

An extensive analysis of neuronal somal labeling at the electron-microscopic level revealed a gradation of reaction product partially related to neuron type and partially to the depth of immunocytochemical analysis in tissue sections. Purkinje neurons exhibited a dense band of reaction product adjacent to the inner nuclear membrane and a sprinkling of reaction product throughout the remainder of the nucleus (Fig. 3, *A, D*). There were also focal depositions of reaction product throughout the cytoplasm (Fig. 3*A*). Neurons in the dentate gyrus showed essentially the same staining pattern (Fig. 3, *B, C, E*), but the nuclear product was frequently more restricted to the sites immediately adjacent to the inner nuclear membrane.

Other brain regions

In order to test the generality of these results, we performed a light-microscopic analysis of anti-PL/Ca-PK immunoreactivity in other brain regions. In every case the basic pattern consisted of a punctate distribution of reaction product surrounding neurons and their dendritic processes, as well as a rim of reaction product at the periphery of the nucleus. These results are illustrated by an example from the spinal cord ventral horn shown in Figure 2*D*.

Discussion

These studies were undertaken in order to examine the detailed distribution of anti-PL/Ca-PK immunoreactivity in neurons. The brain regions we have chosen to examine have the advantage that considerable information is available concerning their synaptic architectonics, much of which can be identified at the light-microscopic level.

It is clear from the light-microscopic examination of cerebellar cortex, deep cerebellar nucleus, and hippocampus that the PL/Ca-PK is present in structures strongly resembling synaptic boutons. Further, the results indicate that the enzyme is present in synapses of diverse physiological properties that utilize different neurotransmitter types. The Golgi II presynaptic terminals of the cerebellar granule cells are inhibitory and probably use GABA as a neurotransmitter, whereas the mossy fiber presynaptic terminals are excitatory and do not use GABA as a neurotransmitter (see Wood et al., 1976, for references). Many of the presynaptic terminals on deep cerebellar neurons are also inhibitory GABAergic terminals of Purkinje cell axons. The mossy fiber terminals of the hippocampus are excitatory glutamate terminals. In addition, we have recently demonstrated the presence of the PL/Ca^{2+}-PK in cholinergic neuromuscular junctions of skeletal muscle and in neuromuscular synapses of the heart (unpublished observations). The electron-microscopic studies of this and our previous study (Girard et al., 1985, 1986) indicate that immunoreactive sites to anti-PL/Ca-PK sera are present in presynaptic terminals in apparent association with synaptic vesicles. The peroxidase procedures used in these studies, however, are inadequate to provide proof that the enzyme is associated with synaptic vesicles, since the possibility of some artifactual rearrangement of reaction product cannot be excluded (Novikoff, 1980). The widespread distribution of the kinase in presynaptic terminals is consistent with a fundamental role for this enzyme in presynaptic function. Since the major Ca^{2+}-dependent event in presynaptic terminals is neurotransmitter release/exocytosis from synaptic vesicles, the possible association of the immunoreactive label with synaptic vesicles may be particularly significant. The exact role of the PL/Ca-PK system in presynaptic function, however, must await the identification of specific substrates for the kinase in presynaptic terminals.

In order to understand the potential function of the various Ca^{2+}-dependent protein kinase systems in neuronal functions, it is necessary to understand their compartmentalization within the neuron. Two major Ca^{2+}-dependent protein kinase systems in brain are the PL/Ca^{2+}-PK and the calmodulin/Ca^{2+}-PK(s). It is clear that we do not yet fully understand all details of the distribution of the systems in neurons, but several observations, based on immunocytochemical analysis, deserve comment. The immunocytochemistry of the calmodulin/Ca^{2+} system has been studied using antibodies to calmodulin (Wood et al., 1980) and to calmodulin kinase II (Quimet et al., 1984). The immunocytochemical distribution of the calmodulin/Ca^{2+} system is similar in using antibodies to calmodulin or calmodulin kinase II and is concentrated in neuronal somata and dendrites (Quimet et al., 1984; Wood et al., 1980). The PL/Ca-PK immunoreactivity, on the contrary, is concentrated in axons and presynaptic terminals. As we have emphasized earlier (Wood et al., 1976, 1980) and reiterate here, immunocytochemistry is a powerful but imperfect tool, and negative results cannot be taken as proof that an antigen is not present in a particular compartment. In particular, these results do not rule out the possibility that functionally significant pools of the PL/Ca-PK may exist at postsynaptic sites in a form or at a level that precludes immunocytochemical visualization. Indeed, we have been able to show that positive immunoreactivity is present in cell somata at higher concentrations of antisera. In this regard, it may be that the low levels of calmodulin kinase II seen in presynaptic terminals, for example, could perform highly significant functions related to the phosphorylation of synapsin (Quimet et al., 1984). Nonetheless, the positive immunocytochemical results indicating an apparent complementary distribution of the 2 major Ca^{2+} effector systems in brain, however, suggest that the physiological role of these systems may, at least in part, be determined by their compartmentalization within the neuron.

As stated earlier, PL/Ca-PK localization in presynaptic terminals is consistent with a role for the enzyme in presynaptic function. PL/Ca-PK, however, is not restricted to neurons or even to the nervous system (Kuo et al., 1980, 1984), so this enzyme would be expected to play a broader role in phosphorylation of substrates in the nervous system and in non-neural tissue. This hypothesis is supported by our previous observations that PL/Ca-PK is present in cells of the nervous system strongly resembling oligodendroglia (Girard et al., 1985, 1986), where it might play a role in the phosphorylation of myelin basic protein (Turner et al., 1982, 1984). The present results showing localization of PL/Ca-PK subadjacent to the inner nuclear membrane suggest another potential site for the enzyme to play a role in the regulation of nerve function. It is of some interest to identify specific nuclear substrates for PL/Ca-PK. One obvious possibility is histone H_1, which is a particularly good substrate for the enzyme *in vitro*. In view of the emerging evidence of a role for PL/Ca-PK in the transduction of a variety of receptor-mediated biological signals (Nishizuka, 1984), as well as tumorigenesis (Helfman et al., 1983), we believe that a systematic examination of possible substrates for the PL/Ca-PK specifically associated with nuclear function should be undertaken prior to discussion of the mechanisms by which the PL/Ca-PK affects neuronal function at the level of the nucleus. The electron-microscopic immunocytochemical results presented here would suggest that substrates most likely to be significant should be subadjacent to the inner nuclear membrane.

One additional aspect of the immunocytochemical localization of the PL/Ca-PK should be noted. We consistently observed an uneven distribution of reaction product in neurons that was partially, but not completely, related to cell type. The nuclear staining, when observed, always consisted of a heavy rim of reaction product, but staining within the nucleus was variable. Purkinje cells consistently exhibited more reaction product within

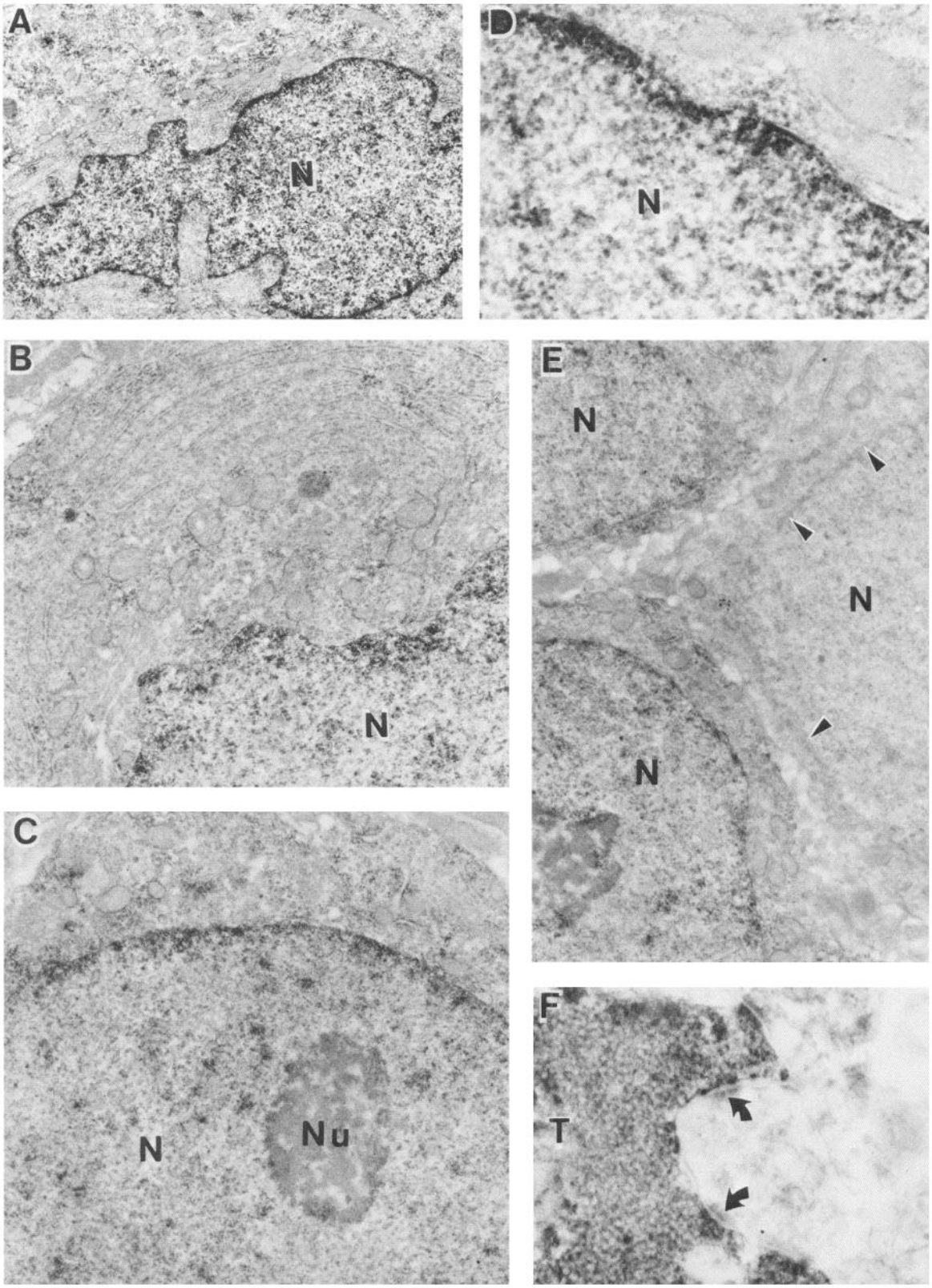


Figure 3. Electron micrographs of cerebellar cortex (*A, D*) and hippocampus (*B, C, E, F*) illustrating the fine structural distribution of anti-PL/Ca-PK immunoreactivity. A striking distribution is seen at the nuclear envelope of Purkinje neurons (*N*) and a sprinkling of reaction product is found throughout the nucleus (*A, B*). Higher-magnification views reveal that reaction product (*arrows*) is concentrated against the inner nuclear membrane (*D*). Reaction product is also seen in small deposits throughout the Purkinje cell cytoplasm (*A*). *B, C,* and *E,* Examples of staining adjacent to the inner membrane of nuclei (*N*) of cells located in the pyramidal cell region in the hippocampus. Nucleolus (*Nu*) appears unlabeled, but there is a fine distribution of product throughout the rest of the nucleus, and at small focal points in the cytoplasm (*B, C*). *E,* Pyramidal neuron with an unlabeled inner nucleus membrane that may be used as an internal control to gauge the intensity of staining seen in panels *A–C* and *D. F,*

the nucleus than did hippocampal pyramidal cells, for example. The nuclei of some neurons such as the cerebellar granule cell did not exhibit immunoreactivity for PL/Ca-PK at all. Since many factors can influence immunocytochemical results, it is difficult to assess the meaning of these observations to PL/Ca-PK function in neurons. Of perhaps greater potential interest, we also observed qualitative differences in the distribution of immunoreactivity within given neuronal types. It was not at all uncommon to observe Purkinje neurons, for example, with completely unlabeled nuclei, even though they were cut at essentially the same plane of section as adjacent cells with heavily labeled nuclei. We have not been able to discern a pattern of organization of labeled compared to unlabeled neurons, but we cannot rule out that a pattern exists. Further qualitative differences were observed in neurons that stained with more concentrated antisera and that exhibited somal labeling. Again, many examples of differential intensity of immunolabeled neurons of the same morphological type were observed. This result was very similar to that obtained in our earlier studies localizing anti-calmodulin immunoreactivity in brain (Wood et al., 1980). At this stage, the meaning of these observations is simply not clear, and one explanation is that they reflect problems of uneven fixation or penetration of the antisera into cells. Another possibility that intrigues us, however, is that the results may reflect different physiological states among populations of related neurons. This possibility should be testable by subjecting populations of neurons to defined physiological stimulation prior to fixation for immunocytochemical analysis.

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Immunopositive labeling in a probable mossy fiber terminal (*T*) contacting a pyramidal cell dendrite. Synaptic vesicles in the terminal are surrounded by a rim of reaction products. The prominent postsynaptic densities (*curved arrows*) are unlabeled. *A,* ×6500; *B* and *C,* ×380; *D,* ×37,800; *E,* ×11,700; *F,* ×33,600.