Distinct cellular and regional localization of immunoreactive protein kinase C in rat brain

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ABSTRACT Monoclonal antibodies raised against highly purified protein kinase C were used to localize protein kinase C in the rat brain. Using various monoclonal antibodies, at least three distinct antibody-staining patterns were found. One monoclonal antibody exclusively labeled astroglial elements, including astrocytes, tanyocytes, and cerebellar radial glia. Another monoclonal antibody exclusively labeled neural cells, including cortical and hippocampal pyramidal dendrites and Purkinje cells of the cerebellum. A third monoclonal antibody (which inhibited protein kinase C activity) intensely stained more limited brain regions, particularly thalamic neurons, and also stained astroglial structures in brain, spinal cord, and cerebellum. The possibility that the three staining patterns reflect the differential regional and cellular localization of related, but distinct, enzymes of protein kinase C is discussed.

Protein kinase C is a mediator of signal transduction for hormones that induce phosphatidylinositol breakdown (1). This enzyme is the receptor for tumor-promoting phorbol esters (2) and has been implicated in a variety of processes, including hormone release (3), regulation of ion channels (4) and of cAMP production (5), control of metabolic processes (6), and control of cell growth and differentiation (7). Although protein kinase C is highly abundant in the central nervous system (8, 9), its regional distribution has not been fully characterized. A detailed description of the distribution of protein kinase C in the brain would provide important information toward understanding the diverse effects of protein kinase C. To this end, Snyder and coworkers (10, 11) who mapped phorbol ester binding sites and Kuo and his coworkers (12, 13) who localized protein kinase C with polyclonal protein kinase C antisera suggested that it is widely distributed in rat brain. In this paper, using monoclonal anti-protein kinase C antibodies, we report that there are at least three different distribution patterns of immunoreactive protein kinase C in rat brain.

MATERIALS AND METHODS

Protein Kinase C and Antibody Preparation. The purification of protein kinase C, preparation of the anti-protein kinase C monoclonal antibodies, CK 1.4, CK 1.3, and CK 1.12, and their characteristics are described elsewhere (ref. 14; D.M.-R. and D.E.K., unpublished data). The inhibitory effect of anti-protein kinase C antibodies on the enzyme's activity was assayed by preincubating the antibodies with intact and trypsin-digested protein kinase C preparations followed by assay of kinase activity (ref. 14; D.M.-R. and D.E.K., unpublished data). The removal of the antigen–antibody complex was not required when using these monoclonal antibodies (15).

Immunohistochemical Analysis. Adult male rats were deeply anesthetized with sodium pentobarbital at 60 mg/kg and perfused intracardially with 0.1 M phosphate-buffered saline (PBS; 150 mM NaCl in 50 mM sodium phosphate, pH 7.4), followed by fixation in PBS/4% (wt/vol) paraformaldehyde. The brain and spinal cord were removed and post-fixed in the same solution for 3 hr. Fifty-micrometer serial Vibratome sections through the brain and various spinal levels were collected into PBS.

Sections were immunostained according to the peroxidase-antiperoxidase (PAP) protocol of Sternberger (16), or using an avidin–biotin kit (Vector Laboratories, Burlingame, CA). The sections were first blocked by incubating for 1 hr in 3% (vol/vol) normal goat serum (Cappel Laboratories, Cochranville, PA). Without an intervening wash, the sections were next incubated for 24–48 hr at 4°C with the CK 1.4 ascitic fluid (diluted from 1:200 to 1:5000 and containing 0.3% Triton X-100) and then washed several times in PBS containing 1% goat serum. The tissue was then sequentially incubated in bridge antiserum (goat anti-mouse IgG; Cappel Laboratories) and the PAP complex (Cappel Laboratories), or in biotinylated goat anti-mouse IgG followed by the avidin–biotin complex. Diaminobenzidine was the chromogen.

The staining patterns of the anti-protein kinase C monoclonal antibodies CK 1.3 and CK 1.12 were studied by immunofluorescence. Twenty-micrometer slide-mounted cryostat sections were blocked, as above, and then incubated overnight in hybridoma medium diluted 1:1 with PBS and 0.3% Triton X-100. After washing, the sections were incubated in fluorescein-conjugated goat anti-mouse IgG (Antibodies, Inc.; diluted 1:100). The sections were washed, coated with 1,4-diazabicyclo[2.2.2]octane (Dabc; Aldrich) and coverslips were added.

RESULTS

Preparation and Properties of Monoclonal Antibodies. To obtain specific markers for protein kinase C, monoclonal antibodies against the highly purified enzyme were generated (ref. 14; D.M.-R. and D.E.K., unpublished data). Of particular interest was monoclonal antibody 1.4 (CK 1.4), one of several antibodies that inhibited the enzymatic activity of protein kinase C (14). Protein kinase C was activated with phosphatidylserine at 24 μg/ml, diacylglycerol at 0.8 μg/ml, and 1 mM Ca2+ for 5 min; the inhibitory effect of 1 μg of CK 1.4 was tested after a 30-min incubation with antibody and either nonactivated or activated enzyme. CK 1.4 inhibition was significantly greater when antibody was incubated with activated rather than nonactivated enzyme (65 ± 3% vs. 50 ± 3%, respectively; P < 0.02). Removal of the regulatory domain of protein kinase C by limited trypsin digestion released the catalytic fragment. CK 1.4 inhibited the activity

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of the catalytic fragment by only 15 ± 5% (see also ref. 14). Under these conditions the catalytic fragment activity was independent of phospholipids and calcium (14, 17). Importantly, CK 1.4 had no effect on cAMP-dependent kinase (from rabbit muscle), its catalytic subunit (from bovine heart), and calcium/calmodulin-dependent protein kinase type II (from rat brain). Under the same conditions, however, an antibody specific for the latter kinase (Ab 4A11, M. B. Kennedy and N. E. Erondu, personal communication) inhibited its activity by 91%.

Immunocytochemical Localization of Protein Kinase C. Since CK 1.4 specifically inhibited protein kinase C activity, it is a positive marker of the enzyme. We, therefore, used this antibody to localize protein kinase C-like immunoreactivity in the rat brain. The most intense protein kinase C immunoreactivity was found in thalamic nuclei (Figs. 1 and 2A). Specific sensory (dorsolateral and medial geniculate and ventralis posterior) and motor (ventralis lateralis and ventralis anterior) thalamic relay nuclei contained the most densely stained neuronal cell bodies. Heavily labeled varicosities were also found throughout the thalamic neuropil. The intralaminar and anterior (i.e., more limbic related) thalamic nuclei also contained highly immunoreactive cell bodies and varicosities.

The following regions of the thalamus were either unstained or only weakly stained: the habenula, the ventrolateral geniculate, the zona incerta, and the nucleus reticularis thalami (Figs. 1 and 2A). With the exception of some densely stained cells and astrocytic processes (tanyocytes) in the caudal part of the arcuate nucleus (see below and Figs. 1 and 2), there was no staining of hypothalamic structures.

CK 1.4 binding in cortical structures was generally weak (Fig. 1). Pyramidal neurons and their apical dendrites were only lightly stained with CK 1.4. Caudate and putamen were unstained and the globus pallidus was very lightly labeled. Light staining was found in several subnuclei of the amygdala. Of particular interest was the hippocampus, where the staining was restricted to a nonpyramidal, possible basket cell, population of the pyramidal layer (Fig. 2B). Scattered, lightly labeled cells were also located in the stratum oriens.

![Fig. 1. Protein kinase C immunoreactive staining at the level of the caudal diencephalon using monoclonal antibody CK 1.4. There is dense staining in the major thalamic relay nuclei, e.g., the somatosensory relay in the ventroposterior nuclei (vp) and in the visual relay in the dorsolateral geniculate (dlg). In contrast, staining is minimal or absent in the ventrolateral geniculate, in the thalamic reticular nucleus (rt), and in the medial and lateral habenula (hb). In the hippocampus (hi), staining is limited to a subpopulation of cells (see Fig. 2). Immunoreactivity in the amygdala (am) and cerebral cortex is also minimal. With the exception of some dense staining of cells and presumed tanyocytes in the arcuate nucleus (arc and see Fig. 2), the hypothalamus is unlabeled. The asterisk overlies a region of dense astrocyte labeling along the border of the lateral ventricle (ic denotes internal capsule). (Bar = 1 mm.)](image-url)
With the exception of dense cellular and fiber staining in the area postrema, there was almost no neuronal labeling in the pons, medulla, and spinal cord. Structures, morphologically similar to astrocyte cell bodies and their processes, were densely stained by CK 1.4 in several regions of the brain and spinal cord. The pattern of staining was comparable to that seen with antisera directed against glial fibrillary acidic protein (18, 19). Most notably, the spinal cord, which showed no neuronal labeling, had dense, radially oriented, stained fibers in the white matter, particularly around the periphery of the cord (Fig. 2D). Radially oriented Bergman glial fibers were stained in the molecular layer of the cerebellum (Fig. 2E). As described above, the medial basal hypothalamus contained densely labeled tanyctye processes (Fig. 2C) that contacted the third ventricle. Some levels of the hippocampus had dense astrocyte cell body staining, as did the grey matter surrounding the lateral margin of the lateral ventricle (Fig. 1, asterisk). The ventromedial portion of the caudate nucleus was perforated by rostrocaudally directed fibrils that we presume to be astrocyte in origin.

Several controls were used to assess the specificity of the staining. Since there was a limited amount of purified protein kinase C available, the following absorption protocol was used. Sections were incubated in CK 1.4 diluted 1:200. Adjacent sections were incubated in CK 1.4 diluted to the same concentration containing either 0.1, 0.01, or 0.001 μM protein kinase C. This absorption series produced decreased staining relative to the unabsorbed sections; however, some very light staining persisted at 0.1 μM.

In a second series of absorption controls, sections were stained with higher dilutions of CK 1.4 (up to 1:5000); adjacent sections were stained with the same dilution of CK 1.4 containing 0.1 μM protein kinase C. Immunoactivity was still observed at this dilution of antibody, particularly in the thalamus, but the sections immunostained with antibody-absorbed protein kinase C were negative. Incubation with cAMP-dependent kinase had no effect on the staining.

Many of the characteristics of the staining produced by CK 1.4 were also seen in immunofluorescent preparations with more than 20 additional anti-protein kinase C monoclonal antibodies. Fig. 3 shows the results obtained with two of these antibodies CK 1.3 and CK 1.12. In all areas examined, antibody CK 1.3 stained astrocytes and related glial elements. Astrocytes were stained throughout the cerebral cortex (Fig. 3A), but the density of astrocyte staining was much less than in the hippocampus. Fig. 3C illustrates dense, intense astrocyte labeling in the dentate gyrus. Like CK 1.4, CK 1.3 stained Bergman glial fibers in the molecular layer of the cerebellum; the cell bodies of these radial glial cells, however, were not stained (Fig. 3E and F). The white matter of the cerebellum contained large numbers of stained astrocyte cell bodies (Fig. 3E). Finally, Fig. 3H shows the dense staining of tanyctyes of the basal hypothalamus (comparable to that seen with CK 1.4 in Fig. 2E).

In contrast, CK 1.12 exclusively stained neuronal structures; staining was most dense in dendrites of cerebral cortical (Fig. 3B) and hippocampal pyramidal cells (Fig. 3D). There was also intense staining of the dendrites of cerebellar Purkinje cells; the cell bodies were only lightly labeled (Fig. 3G). There was relatively uniform staining of the thalamic
protein kinase stains predominantly the "V" astrocytes. Antibody CK and 105 (1.4). Immunoreactive protein this monoclonal antibodies and irreversibly the kinase protein not but a cells (5, 20, structures. It is kinase of protein and the kinase protein of kinase C-bearing cells in phorbol ester binding studies of rat brain (10).

The biochemical information about CK 1.3 and CK 1.12 is more limited. Both were positive in ELISAs using purified protein kinase C. In preliminary experiments, CK 1.12 inhibited protein kinase C activity (data not shown). That it stained Purkinje cells, as did another anti-protein kinase C monoclonal antibody (1), is consistent with it recognizing protein kinase C. The neuronal staining was also comparable to that reported using polyclonal sera (12, 13) and phorbol esters (10).

It is now clear that protein kinase C is a family of closely related, but distinct enzymes (22, 23). Five regions within the coding sequences for protein kinase C isoenzymes were found to be different (22, 24-26) and are likely to result in immunological distinction. Since the purification method for the enzyme (27) that was used in our study (14) would not separate these isoenzymes (23, 28), it is possible that we have obtained monoclonal antibodies that bind to these variable regions and thus distinguish between the various protein kinase C isoenzymes. The CK 1.4 epitope may be located in variable region V3 of one or two of the isoenzymes. This variable region was found to be close to the ATP binding site (22), which agrees with the kinase inhibitory activity of CK 1.4. Furthermore, it has been suggested that V3 contains the site attacked by proteolytic enzymes to produce the catalytic domain (22, 26, 28). This would be consistent with the observed loss of the CK 1.4 inhibition.

In support of the interpretation that the monoclonal antibodies distinguished between the various isoenzymes is the fact that the combined staining patterns of CK 1.3, CK 1.4, and CK 1.12 are similar, although not identical, to the pattern obtained with phorbol esters (10). For example, the staining patterns in the cortex of CK 1.3 and CK 1.12 but not of CK 1.4 (Figs. 1 and 3 A and B) were comparable to the phorbol ester staining pattern of CK 1.3. Antisera reacting with CK 1.3 and CK 1.12 did not stain the medial hypothalamus or the substantia nigra (10).

In this paper we describe the use of the anti-protein kinase C monoclonal antibodies CK 1.4, CK 1.3, and CK 1.12 to localize immunoreactive protein kinase C in the rat brain. At least three major staining patterns were revealed: exclusively neuronal (CK 1.12), exclusively astroglial (CK 1.3), and both neuronal and astroglial (CK 1.4). CK 1.4, CK 1.3, and CK 1.12 appear to be specific for protein kinase C. CK 1.4 inhibited the phospholipid and calcium-dependent activity but did not affect the activity of the irreversibly activated catalytic fragment of protein kinase C. CK 1.4 also did not affect the activity of other protein kinases, and the CK 1.4 staining was reduced or abolished when the antibody was preabsorbed with protein kinase C, but not with cAMP-dependent kinase. Several other anti-protein kinase C monoclonal antibodies gave similar or identical staining patterns. In agreement with several reports that a large quantity of protein kinase C is present in astroglia cells (5, 20, 21), CK 1.4 and CK 1.3 antibodies heavily stained astroglia structures (Figs. 2 C, D, and E; 3 A, C, E, F, and H).

It is of interest that astroglial cells were not identified as protein kinase C-bearing cells in phorbol ester binding studies of rat brain (10).

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ester binding pattern (10). The resolution of light microscopic autoradiographic techniques makes it difficult to establish whether phorbol esters bind astrocytes. Similarly, in the hippocampus, the staining pattern of CK 1.12, but not CK 1.4, agreed with the phorbol ester binding pattern (10); very high levels of phorbol ester binding (10) and CK 1.12 binding (Fig. 3D) were found on dendrites of pyramidal cells in stratum radiatum in the hippocampus.

Based on mRNA hybridization studies, Ohno et al. (26) determined that two of the three isoenzymes are more abundant in brain; the third is found predominantly in liver, muscle, and kidney. The different patterns of staining that we observed in the rat brain suggest that there is a cell-specific expression of particular protein kinase C isoenzymes within the central nervous system as well. Such differential expression of protein kinase C isoenzymes may indicate distinct functions. Two of the isoenzymes (identified by CK 1.12 and CK 1.3) are likely to be involved in specific functions common to neuronal cells and astroglial cells, respectively. In contrast, protein kinase C immunoreactivity as detected exclusively by CK 1.4 is likely to be associated with a much more regionally restricted function in the brain. Protein kinase C identified with CK 1.4 was most abundant in specific sensory relay nuclei (e.g., lateral geniculate) and in some of the nonspecific nuclei. This suggests that protein kinase C is likely to contribute to the information processing that occurs when signals are transmitted from thalamus to cortex. It was of particular interest that three major thalamic nuclei that do not project to cortex, specifically, the ventrolateral geniculate, the zona incerta, and the reticularis thalami, were not stained.

In conclusion, we have isolated monoclonal antibodies to protein kinase C and used these antibodies to determine the distribution of protein kinase C in the rat brain. We found different pattern of staining with different antibodies. One antibody stained neuronal structures exclusively (CK 1.12), another stained astroglial structures (CK 1.3), and a third (CK 1.4) stained astroglial and neuronal cell body structures, the latter mainly in thalamic nuclei. Our data suggest that immunologically distinct protein kinase C isoenzymes are expressed selectively in different cell types within distinct brain regions.

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