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Type I Protein Kinase C Isozyme in the Visual-Information-Processing Pathway of Monkey Brain

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Previously using PKC isozyme-specific antibodies for immunoblot analysis, we demonstrated the heterogeneous distribution of PKC isozymes in various regions of monkey and rat brains and that type I PKC was most abundant in cerebellum, hippocampus, amygdala, and cerebral cortex (Huang et al.: *J Biol Chem* 262:15714-15720, 1987). Using these antibodies, we have also demonstrated that type I, II, and III PKC are products of PKC genes γ, β, and α, respectively (Huang et al.: *Biochem Biophys Res Commun* 149:946–952, 1987). By immunocytochemical analysis, type I PKC-specific antibody showed strong reactivity in various types of neuron in hippocampal formation, amygdala, cerebellum, and neocortex. In hippocampal formation, granule cells of dentate gyrus and pyramidal cells of hippocampus were heavily stained. By immunoblot analysis, relative levels of PKC isozymes in several areas of monkey cerebral cortex involved in the visual information processing and storage were determined. Both type II and III PKCs appeared to be evenly distributed and at moderate levels, type I PKC formed a gradient of increasing concentration rostral along the cerebral cortex of occipital to temporal and then to the limbic areas. Neurobehavioral studies have demonstrated that the neocortical and limbic areas of the anterior and medial temporal regions participate more directly than the striate, prestriate, and posterior temporal regions in the storage of visual representations and that both hippocampus and amygdala are important in the memory formation. As type I PKC is present at high levels in hippocampus, amygdala, and anterior temporal lobe, we predict that the type I protein kinase C may participate in the plastic changes important for mnemonic function.

Abbreviations used: CNS, central nervous system; DG, diacylglycerol; LTP, long-term potentiation; PBS, phosphate-buffered saline; PKC, protein kinase C; PS, phosphatidylserine.

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Protein kinase C (PKC), a Ca^{2+}/phospholipid/diacylglycerol (DG)-dependent serine/threonine protein kinase, has been implicated in the regulation of many cellular processes [1,2]. This enzyme is widely distributed in a variety of tissues and is most concentrated in brain [3,4]. In the central nervous system (CNS) activation of PKC through receptor-mediated mechanism has been related to enhancement of neurotransmitter release [5,6], regulation of ion channels [7,8], and modification of neuronal plasticity [9,10]. The role of PKC in these diverse neural functions is, however, still unknown. Investigation of the function of this enzyme is especially complicated by the identification of multiple PKC genes [11–19] and isozymes [20]. There are at least seven cloned cDNAs [19] and three isozymes [20,23] have been identified. The three PKC isozymes, types I, II, and III, have been shown to exhibit similar immunoreactivities [21] and chromatographic behavior [22] to products of γ, βI and βII[1], and α genes, respectively. However, PKCs encoded by the newly discovered δ, ϵ, and ζ genes have not been identified. Among the various PKCs and their genes identified so far, message of the γ gene, or the type I PKC, was found to be present mainly in the brain [19,21,24] and was highly enriched in cerebellum, hippocampus, amygdala, and cerebral cortex [23], whereas the other species were found in CNS as well as peripheral tissues [19,21,24].

Previously, the localization of PKC in rat brain has been investigated by immunocytochemical analysis with polyclonal [25] and monoclonal [26,27] antibodies against PKC and their mRNA by in situ hybridization histochemistry with probes for α, βI, and βII cDNA [28]. However, in those studies, the polyclonal antibodies employed could not differentiate isozymes [25], and the isozyme specificities of the monoclonal antibodies were not determined [26,27]. Using well-characterized isozyme-specific antibodies, we have immunocytochemically localized PKC isozymes in various brain regions and neurons [21,29]. In this study we employed the type I PKC-specific antibodies to determine the cellular localization of this enzyme in rat hippocampal formation and to determine the content of this enzyme in the areas that make up the visual-information processing pathway of the monkey brain. Our results demonstrated that the type I PKC is highly enriched in the hippocampal neurons and in the cortical regions of monkey brain that are important for the storage of visual information. These results suggest that the type I PKC may participate in the processes involved in the mnemonic function.

MATERIALS AND METHODS

The following materials were obtained from the indicated sources: affinity-purified rabbit antigoat IgG, biotin-conjugated rabbit antigoat IgG, and Texas Red-conjugated avidin from Cooper Biochemical; normal goat and rabbit sera from Miles; crystallized bovine plasma albumin from Armour Pharmaceutical; and polyacrylamide gel electrophoresis reagents from Bio-Rad; [^{125} I]protein A from Dupont -New England Nuclear; and nitrocellulose membrane from Schleicher and Schuell.

[1]Type II PKC-specific antibody recognizes PKC in βII [21] as well as βI (unpublished data)-transfected COS cell extracts.

PKC-isozyme-specific antibodies were purified from the polyclonal antibodies against a mixture of rat brain PKC isozymes [4] by immunoabsorption according to the method of Olmsted [30]. PKC isozymes were purified to near homogeneity as previously described [20]. For immunofluorescent staining, frozen brain sections (12-μm thickness) used were warmed to room temperature and rehydrated thoroughly with phosphate-buffered saline (PBS) before staining. Staining was carried out by incubations with primary antibody at 4° for 40 h, biotin-conjugated rabbit antigoat IgG at room temperature for 1 h, and Texas Red-conjugated avidin (7 μg/ml, molar ratio of Texas Red/avidin = 3) at room temperature for 1 h. Bovine plasma albumin, 2 mg/ml in PBS, was used as diluent at all steps. Between incubations, sections were washed twice with PBS, each for at least 10 min. Four washes were done at the completion of the staining, and the sections were rinsed with deionized water, air dried, and mounted with buffered polyvinylalcohol for fluorescent microscopy and photography.

Immunoblotting was carried out by initial separation of proteins by SDS-PAGE and followed by electrophoretic transfer to nitrocellulose membrane. The membrane was incubated successively with 3% gelatin in TBS (20 mM Tris-Cl buffer, pH 7.5, containing 500 mM NaCl) for 30 min, with various isozyme specific antibodies in TBS containing 1% gelatin for 90 min, with affinity-purified rabbit antigoat IgG (2,000-fold dilution) for 1 h, and with [^{125}I]protein A (0.4 μg, 4–6 μCi/20 ml) for 1 h. Following each incubation the membrane was extensively washed with TBS containing 0.05% Tween 20. The immunoreactive bands were visualized by autoradiography. Levels of PKC isozymes in the cerebral cortex of monkey brain were determined by immunoblotting with PKC-isozyme-specific antibodies [23,24]. Quantification of specific antigen was based on densitometric measurement of the[^{125}I]protein A-labeled band following autoradiography with known amounts of purified PKC isozymes as standards.

Fresh brain tissue was obtained from two male *Macaca fascicularis* monkeys. Each animal was deeply anesthetized with intrahepatic nembutal and the brain quickly removed and chilled in ice-cold saline prior to dissection. Cortical and subcortical tissues were dissected and immediately frozen in dry ice.

RESULTS

Characterization of Isozyme-Specific Antibodies

PKC isozyme-specific antibodies were prepared by immunoabsorption of a preparation of goat anti-rat brain PKC antibodies which cross reacted with all three isozymes [4]. Each of the purified antibodies was specific for the isozyme tested as described previously [23] by immunoblot analysis (Fig. 1A) and it gave proportional signals as quantified by[^{125}I]protein A binding with increasing amounts [20–200 ng] of specific PKC antigen applied [24]. Thus these antibodies are appropriate for immunocytochemical studies and for quantification of PKC levels in tissues. Routinely, at least three amounts of the purified rat brain PKC isozymes were included in each immunoblot analysis as standards to quantify the level of the isozyme in the tissue homogenates (Fig. 1B). Though reactive with proteolytic fragments of PKC (data not shown), these isozyme-specific antibodies detected predominantly the native PKC (82 kD) in the monkey and rat tissues [23,24] (also see Fig. 4). On immunoblot

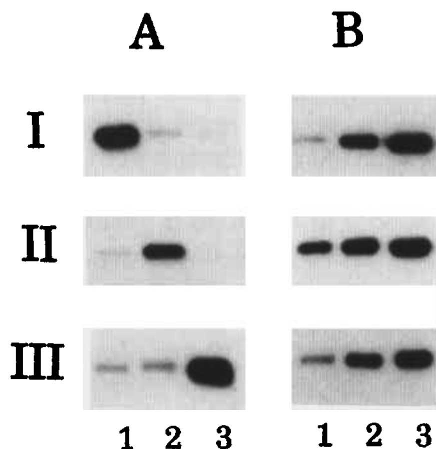

Fig. 1. Characterization of PKC isozyme-specific antibodies and quantification of isozyme levels by immunoblotting. **A:** purified rat brain PKC isozymes, 100 ng each of type I (lane 1), type II (lane 2), and type III (lane 3) were immunoblotted with type I (I), type II (II), or type III (III) isozyme-specific antibodies after SDS-PAGE and electrophoretic transfer to nitrocellulose membrane. **B:** PKC isozymes, 20 ng (lane 1), 40 ng (lane 2), and 80 ng (lane 3), of type I (I), type II (II), or type III (III) were immunoblotted with their corresponding antibodies. Routinely, these purified PKC isozymes were immunoblotted simultaneously with tissue samples; the immunoreactive signals were determined by densitometric tracing of the autoradiograms by using a scanner equipped with an integrator; thus, the level of PKC isozyme in tissue can be determined.

by these antibodies the type I PKC was found mainly in the brain [24] and had a highest level in cerebellum, hippocampus, amygdala, and neocortex [23].

Immunocytochemical Localization of the Type I PKC in Rat Brain

Indirect immunofluorescent staining of coronal sections through the level of rostral hippocampus revealed a high level of type I PKC in hippocampal formation and neocortex (Fig. 2). We have also detected high level of this kinase in various amygdaloid nuclei; it was less prominent in tail of caudate, thalamus, and hypothalamus and least in internal capsule, corpus callosum, and other fiber-tract-enriched areas (data not shown). Detailed staining patterns of these areas as well as those of other isozyme-specific antibodies will be compared elsewhere [29]. The distribution of immunoreactivities of the type I PKC in hippocampus and dentate gyrus resembled that of a Nissl-stained section, indicating an association of the enzyme with neuronal somata.

The immunoreactivities of the type I PKC in the hippocampal formation were present largely in hippocampal pyramidal cells and granule cells of the dentate gyrus (Fig 3A) and much less in cells of the strata oriens, radiatum, and lacunosum moleculare, and the molecular layer of dentate gyrus. Staining was visible throughout somata of dentate gyrus granule cells (Fig. 3B) and hippocampal pyramidal cells (Fig. 3C) and in the apical dendrites of the pyramidal cells (Fig. 3C). These results suggest that type I PKC is probably responsible for the signal transduction within the cell bodies and at the postsynaptic loci of the hippocampal pyramidal cells.

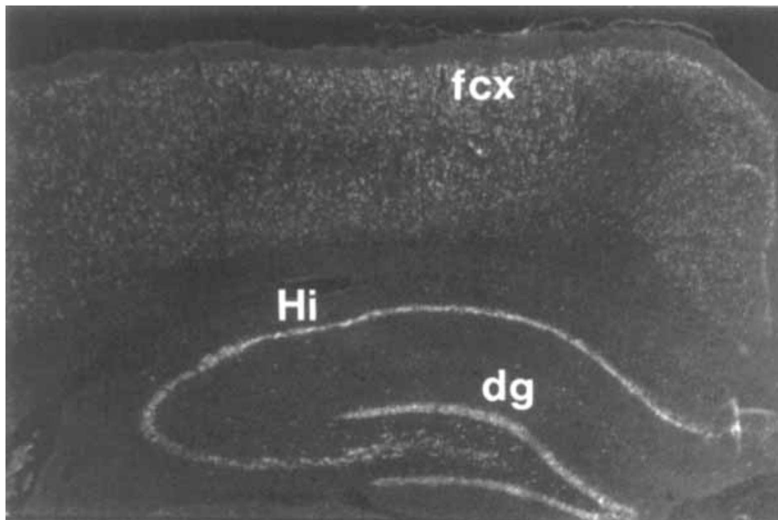

Fig. 2. Immunocytochemical localization of type I PKC in coronal section of rat brain at the level of rostral hippocampal formation. Immunofluorescent staining with type I PKC-specific antibody showed prominent reactivity at hippocampus (Hi), dentate gyrus (dg), and frontal cortex (fcx). The immunoreactivities are mostly associated with neurons and not the nerve fibers.

Distribution of PKC Isozymes in Cerebral Cortex of Monkey Brain Involved in the Visual Information Processing

PKC has been implicated in the control of neurotransmitter release and neuroplasticity [9]. Regional distribution of PKC isozymes along the occipitotemporal cortex of visual-information-processing pathway in monkey brain was analyzed by immunoblotting with PKC isozyme-specific antibodies (Fig. 4). When carefully quantitated against purified rat brain PKC isozymes, the type I PKC was found to have increasing concentration rostral along the cerebral cortex of occipital and temporal lobes and peaked at perirhinal and entorhinal areas (Fig. 5). Unlike the type I enzyme, the levels of both type II and III PKC did not show any gradient in the cortical areas of the visual-information-processing pathway. Levels of type I PKC present in the cortical regions of perirhinal (station 5), entorhinal (station 6), and temporal pole (station 7) were almost ten times more than those in the striate and prestriate areas (stations 1 and 2).

Immunoblot analyses for PKC isozymes in other areas of cerebral cortex were also carried out (Fig. 6). While these PKC isozymes were also abundantly present in various cortices, they did not indicate any special pattern of distribution, except that the primary somatosensory cortex (Fig. 6, lane 6) also showed the same low level of type I PKC as that found in the primary visual cortex (lanes 1 and 2 in Fig. 4). Preliminary analysis suggests that a gradient of increasing concentrations of type I PKC exists along the somatosensory information processing pathway (Fig. 6D, stations 6,7, and 10). There was a low level of this enzyme in the primary somatosensory cortex (Fig. 6A, lane 6), a higher level in the second somatosensory area (Fig. 6A, lane 7), and a highest level in the posterior insula (Fig. 6A, lane 10), which like the anterior temporal lobe projects directly to the limbic system.

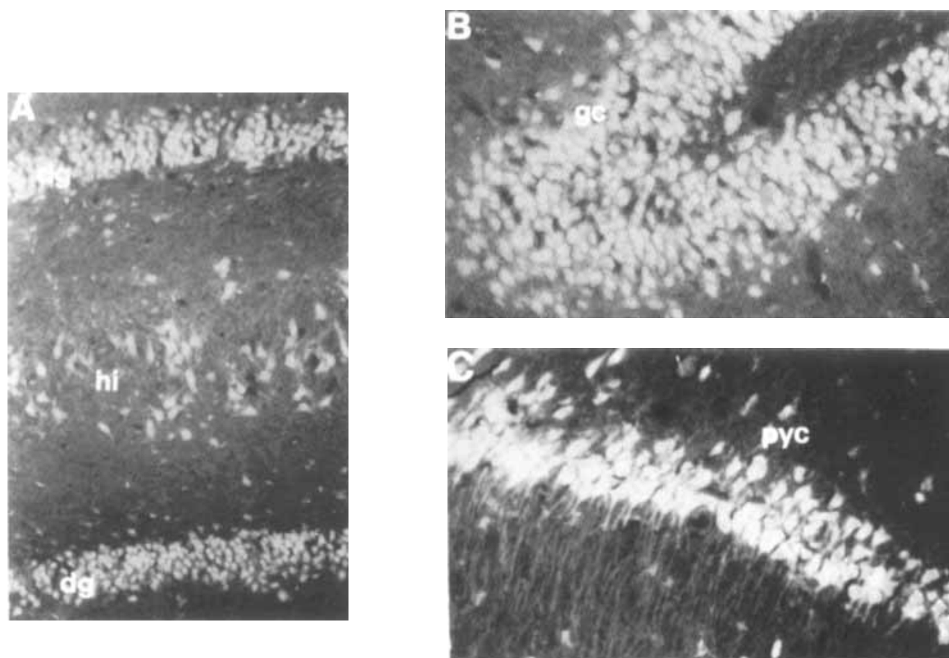

Fig. 3. Cellular localization of type I PKC in rat hippocampus and dentate gyrus. At higher magnification, immunofluorescent staining with type I-specific antibody showed strong reactivities with granule cells of dentate gyrus (dg) and pyramidal cells of hilus (hi) **(panel A)**. The cell bodies of granule cells (gc) in dentate gyrus **(panel B)** and both the cell bodies and apical dendrites of pyramidal cells (pyc) of hippocampal CAl region **(panel C)** were stained. Cells in other layers, such as strata oriens, radiatum, lacunosum, and moleculare, were only occasionally stained.

DISCUSSION

The type I PKC (γ PKC) has been detected primarily in the central nervous system and is especially highly enriched in cerebellum, hippocampus, and amygdala [23], the brain regions important for learning and memory [31]. In an attempt to determine a possible role of this kinase in mnemonic function we have determined the content of this enzyme in cerebral cortex of monkey brain along the visual-information-processing pathway. Quantification of the type I PKC by immunoblot appears to be adequate because the antibody is specific and the immunoreactive signal of PKC determined by[^{125} I]protein A is proportional to the amount of antigen present (Fig. 1) [also see ref. 23,24). By using this analysis we found that the levels of PKC isozymes in the cortical areas of the visual information processing pathway are distributed in a unique fashion. The type II and III PKC were evenly distributed along this pathway, whereas the type I enzyme formed a gradient of increasing concentration from the occipital to the temporal lobe areas. Neurobehavioral studies suggest that the latter areas, including perirhinal and entorhinal cortices and temporal pole, are more important for storing visual information than the former [31]. The type I PKC in these latter areas is at least ten times more than that of striate and prestriate cortices of the occipital region. It is tempting to speculate that this isozyme of PKC may be involved in the functioning of memory. In a separate study, we found that during brain development type I PKC was expressed at the stage of rapid synaptogenesis; in

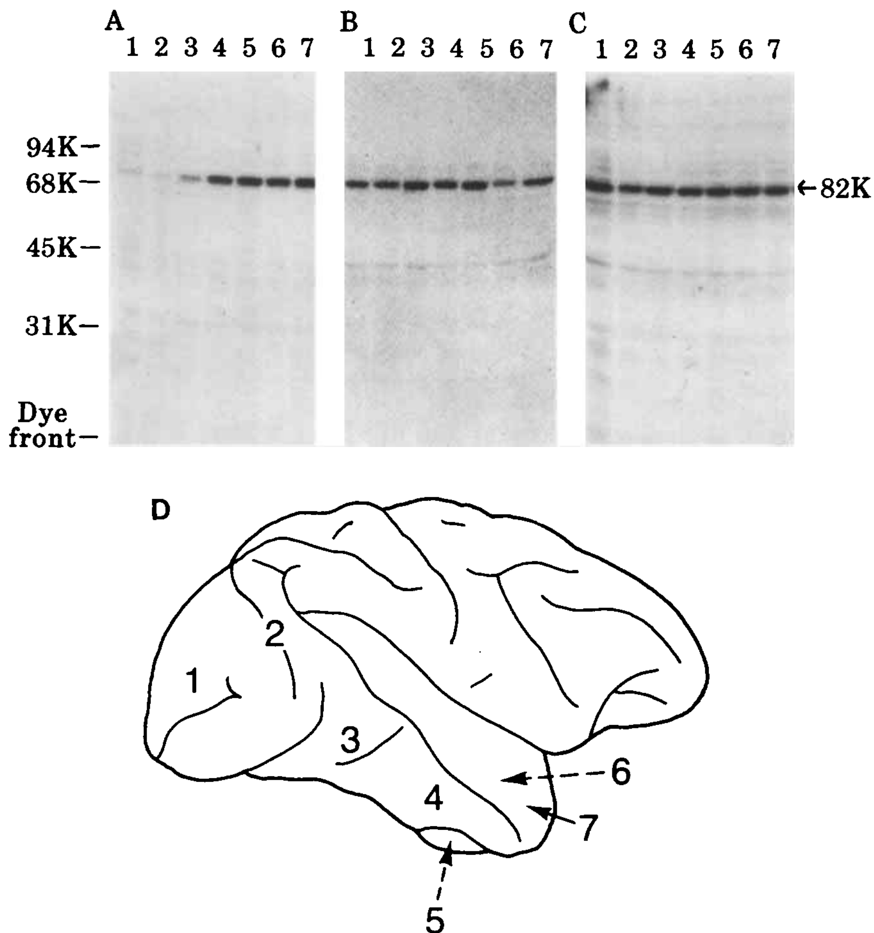

Fig. 4. Immunoblot analysis of PKC isozymes in cortex along the occipitotemporal visual-processing pathway of the monkey brain. Cortical regions of the visual processing pathway from two monkeys (male *Macaca fascicularis*) were dissected and freezed immediately in dry ice. **A–C:** Tissues dissected from striate (lane 1), prestriate (lane 2), posterior temporal (lane 3), anterior temporal (lane 4), perirhinal (lane 5), and entorhinal (lane 6) cortices and temporal pole (lane 7) were homogenized in 20 mM Tris-Cl buffer, pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA, 1 mM DTT, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of leupeptin, chymostatin, and pepstatin A. Samples in triplicate (50 μg protein) were analyzed by immunoblotting with type I (A), II (B), and III (C) PKC-specific antibodies. **D:** dissecting diagram. The numbers indicate the area of cortex analyzed in A–C: 1, striate; 2, prestriate; 3, posterior temporal; 4, anterior temporal; 5, perirhinal; 6, entorhinal cortices; and 7, temporal pole.

adult rat brain, this isozyme was mostly associated with the particulate fractions of the brain [24]. It seems likely that the expansion of neural network during learning and early brain development results in an elevation of this enzyme.

The formation and storage of visual information do not depend on the operation of the visual system alone. Brain regions such as amygdala and hippocampus are of crucial importance for memory function [31]. These two regions have also been shown to contain a high level of type I PKC [23]. In rat hippocampal formation, immunocytochemical analysis revealed the abundant presence of this enzyme in the

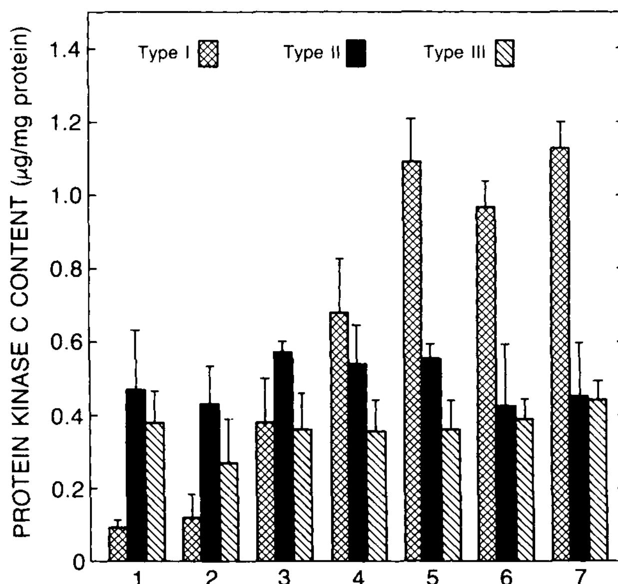

Fig. 5. Relative contents of type I, II, and III PKC in monkey cerebral cortex of visual-information-processing pathway. Quantification of PKC isozymes was carried out by densitometric tracing and integrating of the autoradiograms similar to those shown in Figure 4. Routinely, three increasing amounts of purified rat brain isozymes I, II, and III were also included for quantification. **1,** striate; **2,** prestriate; **3,** posterior temporal; **4,** anterior temporal; **5,** perirhinal; **6,** entorhinal; **7,** temporal pole. Data were calculated as means $\pm$ SE from four measurements.

cell bodies of hippocampal pyramidal cells and the granule cells of dentate gyrus. Prominent staining of the apical dendrites of the former cells was also visible under light microscopy. The presence of type I PKC in the dendrites of the cerebellar Purkinje cells has also been reported [21]. The dendritic localization of this kinase in hippocampal pyramidal cells suggests a postsynaptic role in information processing and/or storage. The hippocampal long-term potentiation (LTP), which was thought to be triggered by postsynaptic entry of Ca^{2+} , has been recognized as an experimental model for memory storage. The postsynaptic Ca^{2+} signal may strengthen a synapse through Ca^{2+} -dependent protein kinases, which phosphorylate target proteins to regulate neural functions. Both PKC and Ca^{2+} /calmodulin-dependent protein kinase II are likely candidates to involve in LTP processes. Based on the evidence that the phorbol-ester-mediated responses mimic LTP and occlude further potentiation by synaptic input [5], the involvement of PKC in this process is strongly suggested. Recently, Hu et al., [32] reported that injection of PKC, likely a mixture of all three isozymes, in CA1 pyramidal neurons in hippocampal slices elicited long-lasting enhancement of synaptic transmission. The changes induced by PKC injection were only seen when the cells were activated synaptically, suggesting a dendritic location for the effect of PKC.

Activation of PKC through membrane association and phosphorylation of one of its membrane-bound substrates, protein F1, were shown to be directly correlated to the long-term enhancement of synaptic activity [10]. Phosphorylation of monkey brain cortical protein F1 in vitro is significantly higher in the temporal regions of the occipitotemporal visual processing pathway than in occipital regions [33]. Increased

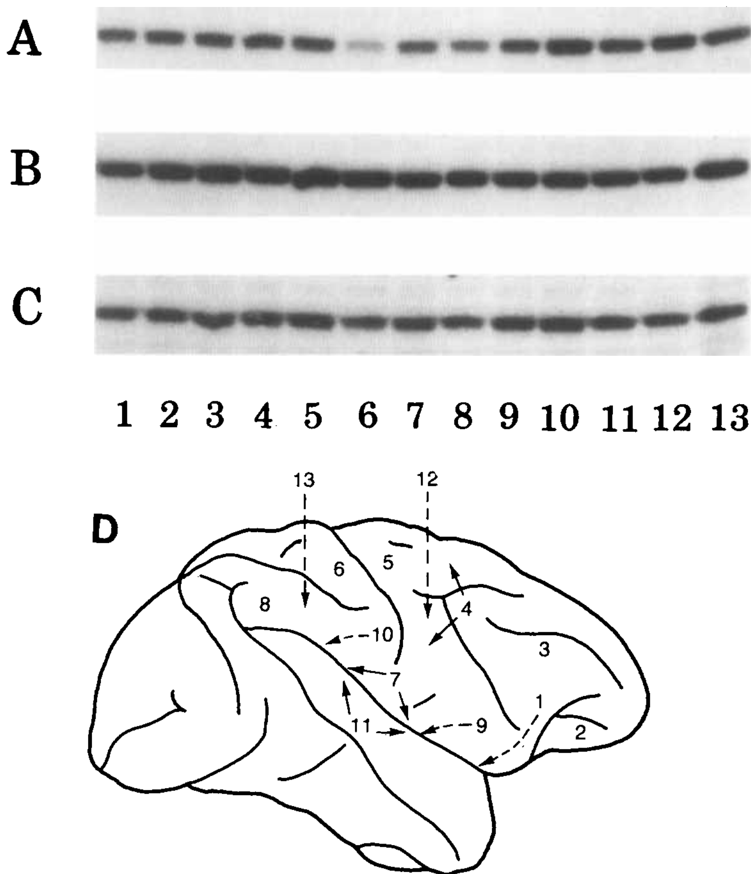

Fig. 6. Immunoblot analysis of PKC isozymes in monkey cerebral cortex. Other cortical regions not involved in the visual pathway described in Figure 4 were analyzed by immunoblot with isozyme-specific antibodies of type I (**A**), type II (**B**), or type III (**C**). The numbers indicate the area of cortex analyzed as shown in the dissection diagram, **D**: **1**, basal forebrain; **2**, orbital frontal; **3**, frontal; **4**, premotor; **5**, motor; **6**, primary somatosensory; **7**, somatosensory association; **8**, inferior parietal lobule; **9**, anterior insula; **10**, posterior insula; **11**, auditory; **12**, anterior cingulate; and **13**, posterior cingulate. Dotted lines indicate cortex in the medial surface. Preparation of tissue homogenate and immunoblot are as described in Figure 4.

phosphorylation of protein F1 accompanying LTP has been correlated with the change in electrophysiological response [34,35]. Increases in the level of type I PKC in the visual-information-processing pathway parallel the phosphorylation of protein F1, suggesting that this kinase may directly or indirectly influence the phosphorylation of protein F1. Protein F1 has been shown by immunocytochemical analysis to be present in the presynaptic terminals [36], whereas PKC is predominantly in the cell bodies and dendrites (Fig. 3) [21,29]. Whether protein F1 is phosphorylated in the cell bodies and then rapidly transported to the presynaptic terminals awaits to be determined. In this regard, protein F1 has been shown to be identical to a fast axonally transported growth-associated protein, GAP43, whose synthesis and transport are greatly increased during neural regeneration [37].

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