Purification and Characterization of Protein Kinase C ϵ from Rabbit Brain†

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ABSTRACT: Protein kinase C ϵ was chromatographically purified from rabbit brain to electrophoretic homogeneity. We identified the enzyme as the ϵ species of novel-type protein kinase C (nPKCϵ), originally discovered and defined by cDNA cloning [Ohno, S., et al. (1988) *Cell 53*, 731-741], on the basis of the following observations: (i) the enzyme reacts specifically with an antipeptidic antiserum to nPKCϵ but not with antisera to any of the other molecular species of PKC thus far known; (ii) it exhibits enzymatic behavior essentially identical to that of a recombinant nPKCϵ purified from transfected COS cells [Konno, Y., et al. (1989) *J. Biochem. 106*, 673-678] and distinct from that of conventional PKC (α, βI/II, and γ) in its dependence on magnesium concentration and cofactors such as phospholipids, calcium, and phorbol ester; and (iii) it has an apparent molecular weight of 95.7K ± 0.4K on SDS-PAGE, significantly greater than the other conventional and novel PKCs thus far identified. Notably, calcium exhibits a complex effect, both positive and negative, on the kinase activity of ϵ depending on the kind of substrate and the coexisting phospholipid, calling for a modification of the current notion that ϵ is a kinase unresponsive to calcium. The amount of ϵ species in the brain was estimated to be comparable to that of each conventional species, indicating that ϵ stands as one of the major PKC family members in brain. Furthermore, the enzyme shows a broader substrate spectrum than conventional PKC when examined with endogenous substrates, implying that it may cover a wider or different range of physiological functions. As is the case with the conventional PKC species, nPKCϵ is also proteolytically converted to an active form with no cofactor requirement by the calcium-dependent proteases μ- and *m*-calpain. This first demonstration that an nPKC member is a calpain substrate predicts that the whole family of PKC, the conventional and the novel, may be under proteolytic regulation by calpain, and thus by calcium, in cells.

The implication that protein kinase C (PKC)[1] family members play pivotal roles in cellular signal transduction, coupled with receptor-mediated lipid metabolism, has attracted tremendous interest in wide areas of life science (Nishizuka, 1984, 1986, 1989). Hata et al. (1989) and Muramatsu et al. (1989) presented direct evidence that these kinases participate in transcriptional activation through a TPA-responsive element. The family is now known to consist of at least eight molecular species (α-η), on the basis of the results of cDNA cloning (Ohno et al., 1987, 1988a,b; Ono et al., 1988; Osada et al., 1990). Some of these species exhibit distinct tissue-specific expression (Huang et al., 1987; Ohno et al., 1987; Wada et al., 1989), implying that they play differential physiological roles, but our present knowledge of their enzymatic properties is not yet sufficient to enable a discussion of their individual functions.

The PKC family can be classified into two categories, the conventional and the novel, on the basis of primary domain structures (Ohno et al., 1988b; Ono et al., 1988; Osada et al., 1990). The major difference between the two groups is the presence or absence of the putative calcium binding domain designated as C2. The conventional PKC (cPKC) group [α, β (βI/II), γ] contains the C2 domain and is activated in the presence of calcium, phospholipids, and diacylglycerol/phorbol ester; the novel PKC (nPKC) group (δ, ϵ, ζ, η) lacks the C2 domain and thus is expected to have activity independent of [Ca^{2+}] as has been shown for a recombinant nPKCϵ under certain conditions (Ohno, 1988b). However, information on the enzymatic properties of nPKC is quite limited since assignment of the cDNA clones to the corresponding natural enzyme molecules is not yet achieved as they were originally discovered as byproducts along with cDNA cloning of cPKC, which, in contrast, had been enzymatically well characterized. Identification, isolation, and characterization of these nPKC species from natural sources, therefore, are crucial to verify the discovery by cDNA cloning, to analyze possible post-translational modifications, and to speculate on the physiological behavior and functions of the enzymes. Enzymatic characterization of the kinase cofactor dependence and substrate specificity is of primary importance in comparing the two groups of PKC. Another structural characteristic important to examine is the susceptibility of nPKC to proteolytic activation by calpain (Kishimoto et al., 1983), a feature commonly shared by cPKC family members, which may provide a major pathway for PKC metabolism (Suzuki et al., 1987; Ito et al., 1989).

Among the novel PKC species, ϵ was the first to be expressed as a recombinant in mammalian cells and enzymatically characterized (Ohno et al., 1988b; Konno et al., 1989; Akita et al., 1990a), although it has not been successfully isolated from natural sources to date. The recombinant nPKCϵ ex-

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[1] Abbreviations: nPKC, novel protein kinase C; cPKC, conventional protein kinase C; TPA, 2-*O*-tetradecanoylphorbol 13-acetate; PS, phosphatidylserine; CL, cardiolipin; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride).

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pressed in COS cells, characterized with histone and myelin basic protein as substrates, exhibited distinct enzymatic properties as compared with cPKCα. ε differed from α in that (i) it did not require Ca^{2+} for activation, (ii) it was activated more by cardiolipin than by phosphatidylserine, (iii) it required lower Mg^{2+} concentrations for activity, and (iv) its molecular weight was about 90K, significantly greater than that of any other known PKC species. These observations provide clues for isolation from crude natural sources by making it possible to design PKC assays capable of discriminating the ε activity from cPKC activity. Since Northern blot analysis indicates that the brain, among the major mammalian organs, is abundant in ε mRNA (Ohno et al., 1988b), we set out to purify ε from brain taking advantage of our current knowledge of the enzyme together with an antiserum specific for ε as another probe.

Here, we provide the first report of the purification and characterization of nPKCε from a natural source, rabbit brain. The enzyme, which has been an exclusive possession of a limited number of biochemists with the cDNA and an expression system to date, is now rendered open to the public for examination and utilization by any enzymologist.

Experimental Procedures

Reagents. Bovine brain phosphatidyl-L-α-serine (PS) and heart cardiolipin (CL) were purchased from Avanti. 2-*O*-Tetradecanoylphorbol 13-acetate (TPA) and bovine serum albumin (BSA, essentially free of immunoglobulin and fatty acid) were from Sigma. [γ-^{32}P]ATP was a product of DuPont–New England Nuclear. Biotinylated lectins were from EY Labs. All the unspecified chemicals were of analytical grade, purchased either from Wako Pure Chemicals, Nacalai Tesque, or from Sigma. Synthetic peptides were produced with an Applied Biosystems Model 430A peptide synthesizer by the Fmoc/NMP method (Chang & Meienhofer, 1978) and purified by HPLC. Partially purified recombinant nPKCε was prepared from transfected COS cells as described (Konno et al., 1989). Other nPKC antigens (δ, ζ, η) were prepared in a similar manner (Osada et al., 1990). μ- and *m*-calpains were purified from rabbit muscle as described (Inomata et al., 1983).

Antisera. Anti-PKCα, anti-βI, anti-βII, and anti-ε antisera have been previously described (Ohno et al., 1988b; Akita et al., 1990a). Rabbit antisera specific to γ, δ, ζ, and η were produced by using synthetic peptides corresponding to the C-terminal sequences of the enzymes as follows. Synthetic peptides with a cysteine residue added onto the N-termini (γ, CFVHPDARSPSSPVPVPVM, δ, CTAFKGFSFVNPKYEQFLE; ζ, CFEGGEYINPLLLSAEESV, η, CINQDEFRNFSYVSPELQL) were covalently conjugated to amino groups of keyhole limpet hemocyanin (KLH, Calbiochem) via a heterobifunctional cross-linker, *N*-hydroxysuccinimidyl *m*-maleimidobenzoyl ester (Pierce) by the method of Lerner et al. (1981). The ratio of peptides of KLH was (0.5–1):1 (w/w). Rabbits were immunized with the peptide–KLH conjugates (0.25–1 mg/rabbit) 4 or 5 times at intervals of 2–3 weeks; the first and the second injections of antigens included Freund's adjuvant, complete and incomplete (Wako), respectively. Antisera were obtained 1 week after the final booster shot.

Buffers. The following buffers were used for purification of PKC: homogenization buffer, 20 mM Tris/HCl, 10 mM EGTA, 2 mM EDTA, 5 mM β-mercaptoethanol, 250 mM sucrose, 0.1 mg/mL leupeptin, 1 mM phenylmethanesulfonyl fluoride, and 0.5 mM diisopropyl fluorophosphate (pH 7.5); buffer A, 20 mM Tris/HCl, 5 mM β-mercaptoethanol, 0.5 mM EGTA, and 0.5 mM EDTA (pH 7.5); buffer B, buffer A plus 1 M NaCl; buffer H-A, 20 mM KH_2PO_4/K_2HPO_4, 0.5 mM EGTA, 0.5 mM EDTA, and 10% (v/v) glycerol (pH 7.5); buffer H-B, 320 mM KH_2PO_4/K_2HPO_4, 0.5 mM EGTA, 0.5 mM EDTA, and 10% (v/v) glycerol (pH 7.5).

Protein Kinase Assays. Two different conditions were employed for assaying cPKC and nPKCε activities based on our previous results (Konno et al., 1989). The reaction mixture for cPKC assay (C assay) contained 20 mM Tris/HCl (pH 7.5), 5 mM $Mg(CH_3COO)_2$, 20 μM ATP, [γ-^{32}P]ATP (0.05 μCi), 0.5 mM Ca^{2+}, 50 μg/mL phosphatidylserine, 50 μg/mL synthetic peptide substrate, 0.25 mg/mL essentially fatty acid free bovine serum albumin, 0.1 mM leupeptin, and 5 μL of enzyme sample in a total volume of 50 μL.[2] The nPKCε assay (N assay) mixture was of identical composition except that PS and Ca^{2+} were replaced by cardiolipin (50 μg/mL) and 1 mM EGTA. The phosphate acceptor substrates used in the assays were a synthetic peptide with a sequence of myelin basic protein (QKRPSQRSKYL), termed MBP_{4-14} (Yasuda et al., 1990), and another peptide corresponding to the nPKCε pseudosubstrate sequence with a single alanine residue substituted by serine (PRKRQGSVRRRVHQVNG), termed ε-peptide (Ohno et al., 1988b; House & Kemp, 1987). MBP_{4-14} was used throughout unless otherwise mentioned. The reaction was started by the addition of the enzyme sample and terminated, after incubation at 30 °C for 10 min, by placing a 40-μL aliquot of the reaction mixture onto Whatmann DE81 paper (2 × 2 cm). The ion-exchange pepers were subsequently washed in 75 mM phosphoric acid 5 times in 20 min, and the remaining radioactivity on the papers was measured through Cerenkov radiation by an Aloka scintillation counter. Background levels in the absence of enzyme were less than 100 cpm and thus negligible. One unit of kinase activity was defined as the ability to transfer 1 nmol of phosphate from ATP to MBP_{4-14} per minute under the conditions described above.

Purification of nPKCε. All the chromatographic procedures described were carried out at 4 °C in columns connected to and controlled by a Pharmacia FPLC system. Whole brain (50 g) from Japanese white rabbits, stored frozen at −80 °C, was homogenized in 200 mL of homogenization buffer with a mortar and a pestle. This rather mild procedure reduced oxidative damage to PKC as compared to other methods using a sonicator or a blender-type homogenizer. The homogenate was ultracentrifuged at 130000*g* for 60 min to remove insoluble materials, and the supernatant, passed through glass wool, was chromatographed on a DEAE-Toyopearl 650S (Tosoh) column (1.6 × 40 cm) equilibrated with buffer A (Figure 1A). After the column was washed with buffer A, the bound protein was eluted by a linear gradient of NaCl concentration up to 200 mM (0.4 mL/min, 5 mL/fraction). Fractions 29–33, corresponding to the second peak of PKC activity, were collected and applied directly onto a threonine–Sepharose 4B column (1 × 10 cm, Figure 1B) prepared by the method of Kitano et al. (1986). Elution was also conducted by a linear NaCl gradient (0.2 mL/min, 5 mL/fraction). Fractions 10 and 11, corresponding to the first peak of PKC activity, were pooled, and its ionic strength was brought to approximately 0.8 M by the addition of 2 M NaCl and applied to a phenyl-Sepharose CL-4B column (Pharmacia, 0.5 × 5 cm, Figure 1C). This column, equilibrated with buffer B, bound the enzyme activity which was then eluted by a linear negative gradient of NaCl (0.2 mL/min, 1 mL/fraction). Fractions

[2] Phorbol ester was not included as it would lessen the difference in responsiveness in each assay between cPKC and nPKCε.

with the kinase activity were collected, diluted with 1 volume of buffer H-A, and chromatographed on a hydroxyapatite column (Koken, 0.78 × 10 cm, Figure 1D). A linear gradient of potassium phosphate created by buffers H-A and H-B (0.4 mL/min, 2 mL/fraction) eluted the kinase activity. Fractions 11 and 12 were collected as the final purified nPKCε sample and stored in the presence of 20% (v/v) glycerol and 0.1 mg/mL BSA at −80 °C. The activity remained stable under these conditions for more than 6 months.

Purification of Conventional PKC. Conventional PKC, a mixture of α, βI/II, and γ, was purified from the same source essentially by the method of Kitano et al. (1986) with minor modifications as follows. Fractions 21–25 from DEAE-Toyopearl chromatography (Figure 1A), corresponding to the first peak of PKC activity, were pooled and applied onto the threonine–Sepharose column. The cPKC activity was eluted at NaCl concentrations of 0.6–0.8 M and collected. The pooled fractions were applied directly onto a phenyl-Sepharose column equilibrated with buffer B, and the cPKC was eluted by a negative linear gradient of NaCl. The final sample was stored in 20% glycerol and 0.1 mg/mL BSA at −80 °C. The individual cPKC species [α, β (βI/II), γ] were obtained by further separation of the cPKC sample on a hydroxyapatite column (Huang et al., 1986a).

Phosphorylation of Electroblotted Protein. Membrane and cytosol fractions of brain were electrophoresed and electroblotted to examine phosphorylation by the purified kinase samples. Rabbit brain homogenate (supernatant after centrifugation at 7000*g* for 15 min) was ultracentrifuged at 120000*g* for 1 h, and the cytosol fraction (supernatant) and membrane fraction (precipitate) were obtained. Aliquots of each (10–20 μg) were subjected to SDS–PAGE on a 4–20% gradient gel and electroblotted onto poly(vinylidene difluoride) (PVDF) membranes (Atto) (Akita et al., 1990a), which then were blocked with 1% BSA in 20 mM Tris/HCl and 150 mM NaCl (pH 7.5) for more than 1 day. The blocked membranes were treated with purified nPKCε and cPKC (0.5–1 unit/mL) under conditions for N assay and C assay, respectively, with the addition of 50 nM TPA at 30 °C for 15 min. The membranes were subsequently washed 10 times with phosphate-buffered saline (pH 7.2) containing 0.5% Triton X-100 and 1 mM ATP over a day and subjected to autoradiography.

Proteolysis of nPKCε by Calpain. The purified nPKCε (2 units/mL, final) was treated with varying amounts of μ- or *m*-calpain in 25 mM Tris, 5 mM β-mercaptoethanol, 1 mg/mL BSA, and Ca^{2+} (0.4 mM for μ-calpain and 1 mM for m-calpain) (pH 7.5) in a volume of 40 μL at 30 °C for 30 min. Proteolysis was stopped by the addition of 10 μL of 1 mM leupeptin and 10 mM EGTA, and 10-μL aliquots were assayed for kinase activity with and without 50 μg/mL cardiolipin. The remaining aliquots were mixed with SDS–PAGE solubilization buffer and subjected to Western blot analysis.

Other Procedures. Protein assay was conducted by the method of Bradford (1976). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemlli, 1970) and Western blotting (Konno et al., 1989; Akita et al., 1990a) were performed as described before. Two sets of molecular weight markers were used for molecular weight calibration in SDS–PAGE; one was a Pharmacia set of alkaline phosphatase (94K), BSA (67K), ovalbumin (43K), carbonic anhydrase (30K), soybean trypsin inhibitor (20.1K), and α-lactalbumin (14.4K), and the other was a Bio-Rad set of dye-conjugated markers of phosphorylase *b*, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. The molecular weights of the latter were recalibrated by the former. Immunostaining procedures employed different second antibodies depending on the animal origin of the primary antisera: alkaline phosphatase conjugated anti-rabbit IgG (Tago) for rabbit antisera and biotinylated anti-guinea pig IgG together with ABC (avidin–biotin conjugate) alkaline phosphatase kit (Vector) for guinea pig antisera. An alkaline phosphatase substrate kit, Vector Red (Vector), was used to visualize the antigens.

Results and Discussion

Purification of Brain nPKCε. The strategy we took to identify nPKCε during purification employed a set of enzymatic and immunochemical means: PKC assays capable of discriminating ε from cPKC and immunoblot assays using a specific antiserum for ε. PKC assays under two different conditions, C and N assays, enabled us to distinguish ε from α, β, and γ, as the C assay is more sensitive to cPKC activity and the N assay to nPKCε (Konno et al., 1989). Furthermore, the phosphate acceptor peptide used in the assay, MBP_{4-14}, has been shown to be a specific substrate for PKC but not for other kinases such as cyclic AMP dependent protein kinase, casein kinases I and II, Ca^{2+}/calmodulin-dependent kinase, or phosphorylase kinase (Yasuda et al., 1990), and thus is expected to detect PKC and PKC-related activities selectively.

In the first chromatographic step of the brain cytosolic fraction on DEAE-Toyopearl (Figure 1A), three separate peaks of protein kinase C activity were observed: the first at 80–100 mM NaCl, the second at 110–140 mM, and the third at 150–180 mM. The C and N assays showed rather parallel profiles, indicating that ε was not separated from cPKC by this step. We had initially expected the different peaks to correspond to different molecular species of PKC, but each peak was actually a mixture containing at least all of the conventional species of PKC (α, βI/II, γ) as examined by specific antisera, with peak 1 containing the largest amount (Figure 2A and data not shown). Each conventional species was presumably separated on the basis of its individual level of posttranslational modification. The ε antigen, as probed by Western blotting, appeared mainly in the second peak and had a molecular weight of 96K (Figure 2A). The first peak also contained a smaller but appreciable amount of 94K ε antigen, but its quantity varied depending on the experiment, indicating that this antigen may have been produced during storage or homogenization of the starting materials and that it is a rather minor component in brain. The amount of another ε antigen in peak 3 was negligibly small as subsequently separated by threonine–Sepharose (see below). Only peak 2 fractions reproducibly contained the majority of the ε antigen/kinase, consistent with the previous observation by Ono et al. (1988) that a recombinant ε elutes at higher salt concentrations in a DEAE column than cPKC, and thus were subjected to subsequent purification steps.

On threonine–Sepharose chromatography (Figure 1B), two distinct peaks of kinase activity appeared which had different profiles depending on the assay conditions. The first peak (320–480 mM NaCl) was much more responsive to the N assay and the second (640–800 mM) to the C assay, indicating that the former contained the ε activity while the latter corresponded to cPKC. The Western blot analysis of the threonine–Sepharose fractions (Figure 2B) showed that the ε antigen of 96K coincided exactly to the first peak and the α antigen of 82K to the second peak and thus confirmed the identity of the separated kinases. The 70–80K ε antigen in fractions 12–14 did not parallel any kinase activity and is as yet unidentified. The other cPKC antigens (βI/II and γ) coeluted with α in parallel and were thus separated from ε as

Table I: Purification of nPKCε[a]

purification step	total protein (mg)	total act. (units)	sp[b] act. (units/mg)	purification	recovery[c] (%)
cytosol	1450	2204	1.52	1	100
DEAE-Toyopearl	87.0	501.1	5.76	3.8	85.5
Thr–Sepharose	11.2	156.8	14.0	9.2	35.6
phenyl-Sepharose	0.73	68.5	93.9	61.8	15.6
hydroxyapatite	0.017[d]	42.0	2430	1600	9.6

[a] Protein kinase C ε antigen/kinase was purified from rabbit brain as described under Experimental Procedures and under Results. [b] Assays were conducted under conditions for "N assay" as described under Experimental Procedures. [c] Since the cytosol and the DEAE peak 2 included other kinase activities subsequently separated from nPKCε, the ratios of ε activity to total kinase activity were estimated on the basis of the chromatography elution profiles (Figure 1) as 20% for the cytosol and 75% for DEAE peak 2 and. These values were used to calculate the recovery values. [d] The protein concentration was measured by densitometric quantification of the sample separated by SDS–PAGE and stained with CBB. Varying amounts of phosphorylase b were used for calibration.

Table II: Immunoreactivities of PKC Isozymes to Isozyme-Specific Antisera[a]

antibody	antigen α	β	γ	ε[b]	ε[c]	δ	ζ	η
anti-α	++	–	–	–	–	nd	nd	nd[d]
anti-βII	–	++	–	–	–	nd	nd	nd
anti-γ	–	–	++	–	–	nd	nd	nd
anti-ε	–	–	–	++	++	–	–	±
anti-δ	–	nd	nd	–	–	++	nd	–
anti-ζ	+	+	nd	±	±	–	++	–
anti-η	–	nd	nd	±	●	–	nd	++
molecular weight (deduced from cDNA)	77K	77K	78K	84K	84K	78K	68K	78K
molecular weight (SDS–PAGE)	82K	82K	82K	96K	90K–94k	82K	76K	82K

[a] Each PKC isozyme antigen was examined by Western blotting as described under Experimental Procedures. The apparent molecular weight of each isozyme was calculated from the molecular weight markers. "++" indicates strongly positive, "±" very weakly positive, and "–" negative. [b] Purified nPKCε antigen/kinase from rabbit brain. [c] Recombinant nPKCε from transfected COS cells. [d] Not determined.

well (data not shown). The threonine–Sepharose column was a key step to the successful separation of ε from cPKC.

The ε activity was further purified by subsequent column chromatographies on phenyl-Sepharose and hydroxyapatite (Figure 1C), both of which have been used routinely for the purification of the conventional PKC species (Kitano et al., 1986; Huang et al., 1986a). The ability of ε to bind to these columns seems to reflect a structural similarity among the isozymes. The ε antigen with a molecular weight of 96K always coeluted with the activity. In the hydroxyapatite column, the ε activity/antigen appeared at an elution volume close to that of βI/II, in accordance with the former observation with a recombinant ε (Konno et al., 1989). In SDS–PAGE, the final ε sample appeared as a single band of M_r 95.7K ± 0.4K (mean of four measurements ± standard deviation) as stained by Coomassie Brilliant Blue; the purified cPKC had a molecular weight of 82K (Figure 3). Table I summarizes the purification of nPKCε. The final samples of purified nPKCε and cPKC showed similar specific activities, indicating that their catalytic capacity to transfer phosphate groups from ATP to a substrate are about the same.[3] The amount of nPKCε in brain can also be calculated from the activity and the recovery and is estimated to be about one-fifth that of cPKC. Since cPKC is a mixture of four isozymes, the ε species exists in brain at a level comparable to each of the cPKC species. This is in good agreement with the ε mRNA content shown by Northern blot analysis, demonstrating that ε is the major nPKC isozyme present in brain (Ohno et al., 1988b; Wada et al., 1989).

Immunoreactivity of nPKCε with Antisera Specific to Various PKC Species. Table II summarizes the immunoreactivities of nPKCε and the other isozymes to the isozyme-specific antisera as analyzed by Western blotting. Brain ε and recombinant ε showed identical immunoreactivities in that they reacted strongly with anti-ε, very weakly with anti-ζ and anti-η, and not at all with the other sera. They also possessed similar relative molecular weights more than 90K, significantly greater than the other species. The results support the molecular identity of brain ε with recombinant ε.

The only ε-like nPKC isozyme, η, which we described recently as sharing the largest homology with ε (Osada et al., 1990), had a clearly different immunoreactivity and an apparent molecular weight. The possible ε-related isozyme predicted by Schaap et al. (1990) in conjugation with alternative splicing is probably the η species as deduced from the mRNA sizes (Ohno et al., 1988b; Osada et al., 1990); hence, there seems to be no other ε-like isozyme that could be mistaken for nPKCε in the brain. Together with the fact that the brain and recombinant ε samples showed similar chromatographic behaviors, these results confirm that the purified brain nPKCε antigen/kinase is identical to the nPKCε originally discovered and defined by cDNA cloning (Ohno et al., 1988b).

Incidentally, lectin staining experiments using biotinylated concanavalin A, *Ricinus communis* agglutinin, and wheat germ agglutinin (data not shown) indicated that the brain nPKCε is probably not glycosylated.

Effect of Phospholipids, Calcium, and TPA on nPKCε Activity. A comparison was made among brain nPKCε, cPKC, and recombinant ε samples with respect to the dependence of their kinase activities on cofactors known to activate cPKC using two different substrate peptides, ε-peptide and MBP_{4-14} (Figure 4). Surprisingly, the ways in which the kinase activities were affected by these cofactors greatly differed depending on the substrate used for the assay.

Figure 4A shows the results with the ε-peptide. The kinase activity of cPKC was activated in the presence of Ca^{2+} and phosphatidylserine (PS), and this activation was enhanced by TPA. Cardiolipin (CL) also activated cPKC but was less effective than PS. The brain and recombinant ε, in contrast, showed clearly different patterns of cofactor dependence in that calcium exerted a negative effect and that CL was a

[3] The purified cPKC sample exhibited a specific activity of 2520 units/mg and a total activity of 38.8 units with 9.0% recovery.

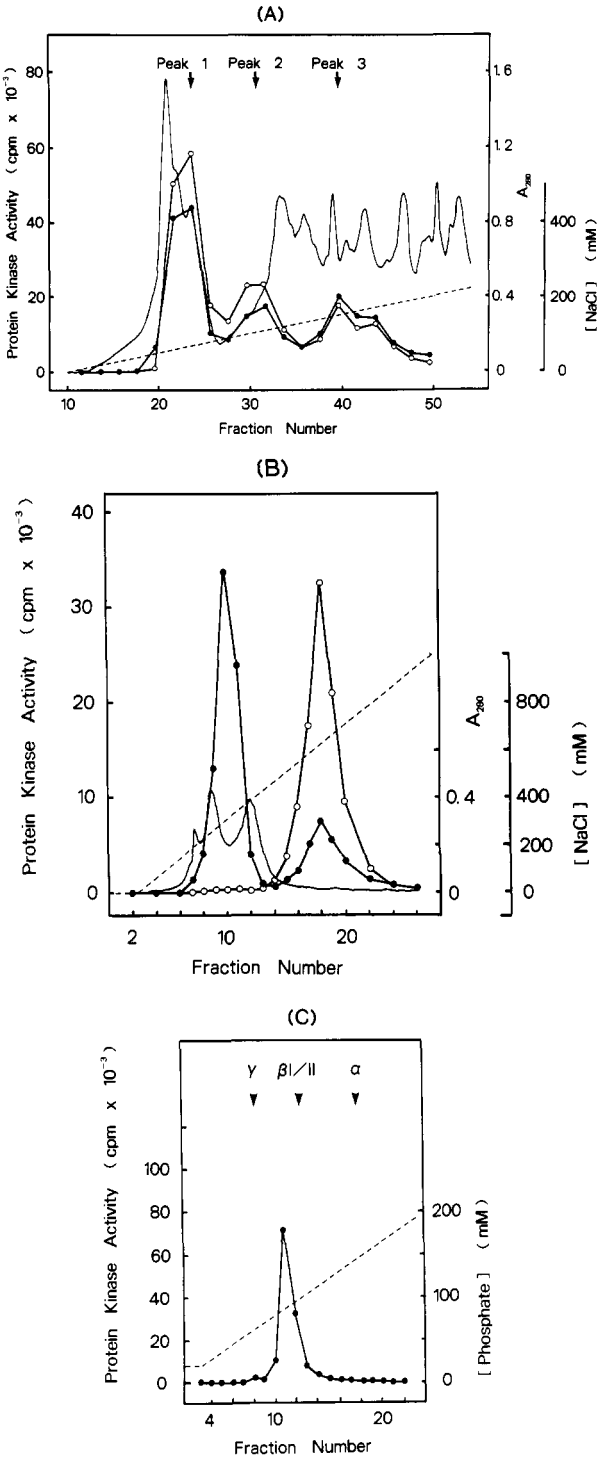

FIGURE 1: Purification of nPKCε from rabbit brain by chromatography on (A) DEAE-Toyopearl, (B) threonine–Sepharose, and (C) hydroxyapatite. Open circles represent kinase activity assayed under "C assay" conditions and closed circles that under "N assay" conditions (see Experimental Procedures). The dashed lines indicate the concentrations of NaCl (A, B) or phosphate (C) used to elute the adsorbed protein. The curved lines show the absorbance at 280 nm. Kinase assays were conducted with samples diluted 10-fold in (A) and (B). Elution volumes of cPKC α, β, and γ are shown by arrows in (C).

stronger activator than PS. Phorbol ester together with phospholipid also stimulated the ε activity, consistent with the previous observation that ε, too, is a phorbol ester receptor (Akita et al., 1990a). These results are quite similar to those obtained previously for recombinant ε when histone was used as a substrate (Konno et al., 1989). The results are also essentially similar to those reported by Schaap and Parker (1990) using recombinant ε expressed in insect cells except that they showed that PS was a stronger activator than CL. The reason for the difference is not clear, but it may have been caused by the different qualities of the commercially available phospholipids used in the experiments.

The negative effect of Ca^{2+} on the ε activity, however, was reversed when the other peptide substrate, MBP_{4-14}, was used as a phosphate acceptor (Figure 4B). In this case, Ca^{2+} enhanced the activity of both brain and recombinant ε in the presence of PS. Interestingly, this positive effect of Ca^{2+} was lost when PS was replaced by CL. CL was also unique in that it mimicked the enhancing effect of TPA on the ε activity when MBP_{4-14} was used as the substrate. The choice of substrates in combination with phospholipid, therefore, alters the way in which the enzyme responds to Ca^{2+}.[4] Incidentally, the ε antigen/kinase fractions in the threonine–Sepharose column (Figure 1B) showed different cofactor dependence in that it had little activity in the presence of Ca^{2+} and PS. This is probably because the crude sample contained factor(s) affecting the kinase activity under certain conditions like the PKC inhibitors previously described (Toker et al., 1990: Pearson et al., 1990).

The way in which the cPKC kinase activity was affected by these cofactors was similar in tendency regardless of the kind of substrate used, but the extent of TPA effect was clearly different with different substrates (Figure 4). TPA together with phospholipid in the absence of Ca^{2+} caused more activation with ε-substrate than with MBP_{4-14}. The interdependent manner in which the cofactors and substrates affect the kinase activities of both nPKCε and cPKC observed here suggests a complex nature for the activation mechanism of the enzymes.

These observations, therefore, cast doubt on the previous claims (Ohno et al., 1988b; Konno et al., 1989; Schaap & Parker, 1990) that nPKCε is a kinase unresponsive to Ca^{2+} and that the C2 domain of cPKC is responsible for Ca^{2+}-dependent activation, although the C2 domain undoubtedly plays an important role in the enzyme activation mechanism by distinguishing the ways in which the two PKC groups respond to Ca^{2+} (Ohno et al., 1988b; Ono et al., 1989). We assume that the mechanism for the effect of Ca^{2+} on PKC activity, whether that of cPKC or of nPKCε, is more complicated than the simple binding of Ca^{2+} to a single domain in the enzyme and needs be examined in terms of the complex interaction among the enzyme, substrates, phospholipids, and phorbol ester (or diacylglycerol). We would expect to gain more insight into the activation mechanism by examining genetically engineered PKC molecules such as chimeric derivatives composed of alternative cPKC and nPKC domains. The fact that the cofactor effect varies according to the kind of substrate implies that different intracellular signals such as lipid metabolites and Ca^{2+} may cause the kinase to act on different substrates depending on the physiological microenvironment. Furthermore, the activating effect of cardiolipin on ε activity in the absence of Ca^{2+} and phorbol ester implies that ε may also be activated solely by other cardiolipin like lipids possessing a cluster of phosphate residues and that the cellular activation mechanism of ε may not necessarily depend on the production of diacylglycerol by phospholipase C. Identification and characterization of the physiological substrates of ε, therefore, are critical not only to further investigate the function of nPKCε but also to verify the significance of the complex enzyme activation mechanism discussed here.

[4] A better "C assay", therefore, would have used ε-peptide instead of MBP_{4-14}, as the difference between cPKC and nPKCε would then be even larger in the presence of PS and Ca^{2+}.

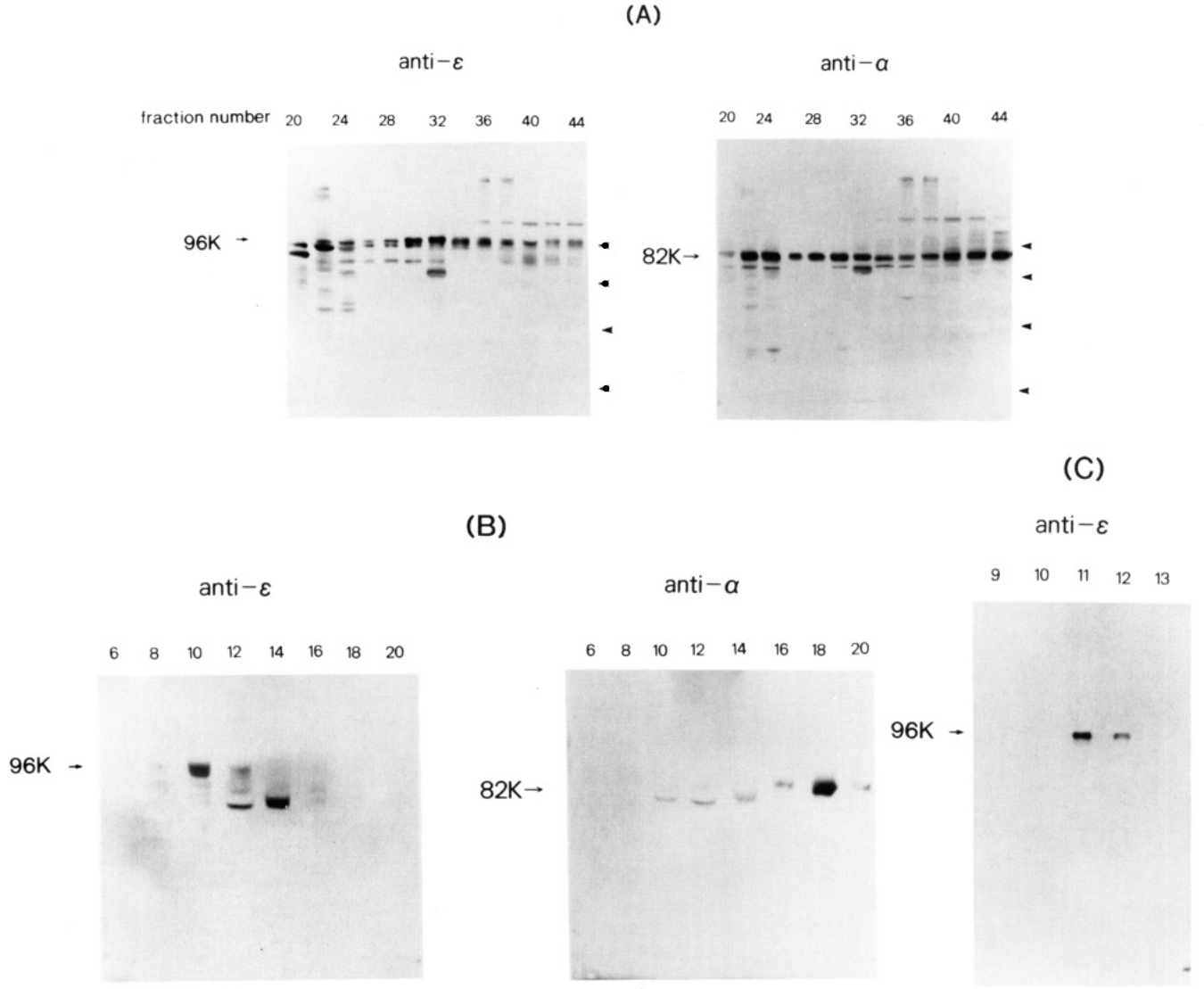

FIGURE 2: Immunoblot analysis of fractions from (A) DEAE-Toyopearl (Figure 1A), (B) threonine–Sepharose (Figure 1B), and (C) hydroxyapatite (Figure 1C) using anti-ε and anti-α antisera. Western blotting was performed as described under Experimental Procedures. The arrows indicate the apparent molecular weights derived from the molecular weight markers. The arrowheads on the right of the picture indicate molecular weight markers as in Figure 3.

In any case, both brain and recombinant ε's showed essentially identical profiles of dependence on cofactors except that the former had a greater basal level of kinase activity in the absence of cofactors than the latter. The possible presence of the kinase catalytic fragment produced by calpain or a calpain-like protease in the ε sample is unlikely because we were unable to detect such a fragment even after a week of storage at 4 °C by immunoblot analysis with a sensitivity sufficient to detect less than 10% conversion of 96K antigen to the catalytic fragment. The elevated basal level of kinase may be caused by autophosphorylation of the purified ε antigen as described below. The minor 94K ε antigen isolated from the peak 1 fractions of the DEAE column (Figure 1A), which was less autophosphorylated (see below), showed similar cofactor dependence, but its basal kinase activity was about half of that of the 96K ε (data not shown).

The effect of Mg^{2+} concentration on the kinase activity was also examined because a recombinant ε had been shown to require lower concentrations of Mg^{2+} than α (Konno et al., 1989). The Mg^{2+} concentrations required for half-maximal kinase activity were about 0.1–0.2 mM for nPKCε and about 0.8–1.0 mM for cPKC with MBP_{4-14} in the presence of 0.2 mM Ca^{2+}, 10 μg/mL PS, and 150 nM TPA. These results are similar to those obtained with cPKCα and a recombinant nPKCε.

Autophosphorylation Activity of Brain nPKCε. The purified brain 96K nPKCε lacked autophosphorylation activity (Figure 5), whereas both the purified cPKC and the 94K nPKCε from peak 1 of the DEAE column (Figure 1A) exhibited cofactor-dependent activity of autophosphorylation. The 94K ε antigen in peak 1 had also been purified in a similar manner.[5] This indicates that the 96K ε had already been fully autophosphorylated and that a large part of nPKCε in rabbit brain exists in such a form. It is not clear yet how many of the possible multiple autophosphorylation sites in the 94K ε molecule remain unphosphorylated. These differential autophosphorylation levels in nPKCε may be the cause for the subtle difference in electrophoretic mobility among the 94K and 96K ε samples from brain and the recombinant ε (Figure 2A, Table II), as previously shown in the case of cPKC (Borner et al., 1989; Huang et al., 1986b; Woodgett et al., 1987). Like the 94K and 96K nPKCε, the cPKC species that eluted at

[5] The chromatographic profiles of the 94K ε antigen/kinase after the DEAE column were identical to those of the 96K ε as shown in Figure 1B,C.

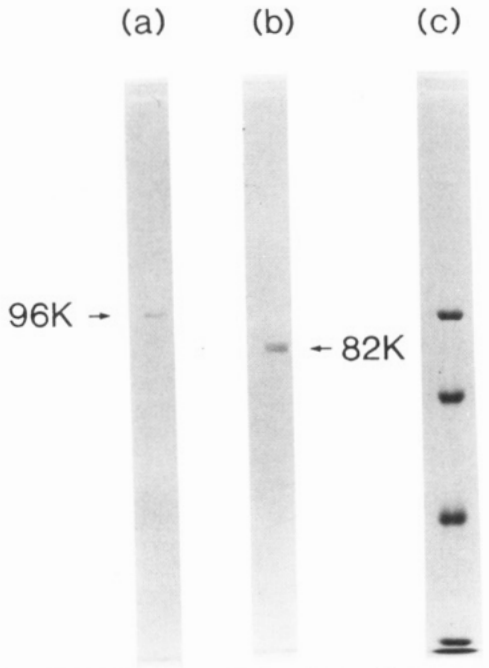

FIGURE 3: SDS–PAGE of purified nPKCε and cPKC. Purified samples of nPKCε (a) and cPKC (b) were resolved by SDS–PAGE and stained with Coomassie Brilliant Blue. Lane c shows molecular weight markers: from the top, 94K (phosphorylase *b*), 67K (BSA), 43K (ovalbumin), 30K (carbonic anhydrase), and 20K (soybean trypsin inhibitor).

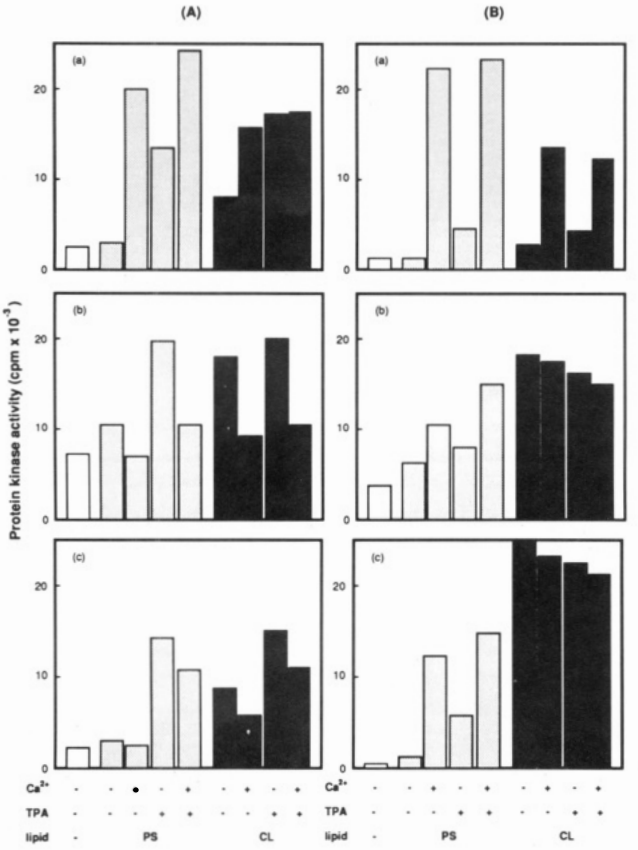

FIGURE 4: Effect of phospholipids, Ca^{2+}, and phorbol ester on kinase activity of (a) brain cPKC, (b) brain nPKCε antigen/kinase, and (c) recombinant nPKCε. The kinase activity was assayed with 0.1–0.2 unit/mL enzyme sample with either peptide ε [left panel (A)] or MBP_{4-14} [right panel (B)] as the substrate as described under Experimental Procedures. The concentrations of the cofactors were 0.4 mM for Ca^{2+}, 10 μg/mL for PS and CL, or 80 nM for TPA. Each value is the average of two measurements, each of which differed by less than 10% from the average.

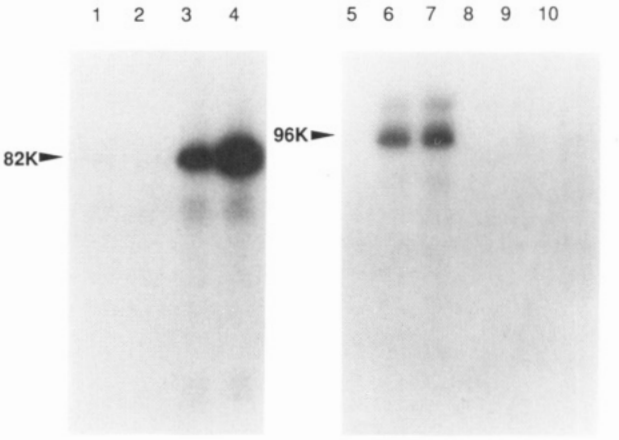

FIGURE 5: Autophosphorylation activity of purified cPKC and nPKCε. The purified cPKC (7.6 units/mL, lanes 1–4), the 94K nPKCε (1.3 units/mL, lanes 5–7), and the 96K nPKCε (1.3 units/mL, lanes 8–10) were incubated in the presence of 1 μM [γ-^{32}P]ATP, 0.1 mg/mL leupeptin, 2 mM vanadate, and 20 mM Tris/HCl (pH 7.5) with or without cofactors at 30 °C for 10 min and subjected to SDS–PAGE and autoradiography. The reaction mixtures contained the following cofactors: no cofactors (lanes 1, 5, and 8); 20 μg/mL PS (lane 2); 20 μg/mL PS and 0.2 mM $CaCl_2$ (lane 3); 20 μg/mL PS, 0.2 mM $CaCl_2$, and 50 ng/mL TPA (lane 4); 20 μg/mL CL (lanes 6 and 9); 20 μg/mL CL and 50 ng/mL TPA (lanes 7 and 10). The cPKC and 94K nPKCε samples showed cofactor-dependent autophosphorylation activity, but the 96K nPKCε had no detectable activity.

different ionic strengths from the DEAE column (Figure 1A and 2A) may also have been separated on the basis of different autophosphorylation levels. However, more detailed examination of autophosphorylation and dephosphorylation of nPKCε is necessary to clarify the effect of autophosphorylation on the enzyme properties.

Data presented by Strulovici et al. (1991) show that murine brain ε antigen has an apparently larger molecular weight than the thymocyte antigen and thus imply that the nPKCε molecules may be processed differently in different tissues even within individual animal species. Interestingly, the 96K ε described here was more resistant to degradation than the 94K sample during storage, implying that autophosphorylation may contribute to enzyme stabilization.

Substrate Spectra of nPKCε and cPKC. The substrate specificity of nPKCε and cPKC was examined in a unique manner using the brain cytosol and membrane fractions processed as for Western blotting as substrates (see Experimental Procedures). The substrate-containing filter was treated with the kinases instead of antibodies under phosphorylating conditions. This allowed phosphorylation of all the endogenous substrates solely by these kinases without interference by other endogenous kinases or phosphatases.

The phosphorylation patterns presented in Figure 6 show that the two kinases have clearly different substrate spectra, although some proteins (40K, 100K, etc.) seem to have been commonly phosphorylated. Most noticeably, nPKCε phosphorylated a larger number of proteins with various electrophoretic mobilities than cPKC. The degree of phosphorylation of each common substrate was also different, e.g., when compared to the 40K band as a standard. The reason for the relatively low level of phosphorylation of high molecular weight proteins (M_r >100K) presumably is the decreased efficiency of electroblotting of the proteins as no detergent such as SDS was included for transfer.

The results here indicate that ε probably has a broader substrate specificity in cells than cPKC, although the following precautions much be considered for interpretation of the data. (i) The substrates were denatured for SDS–PAGE, and their

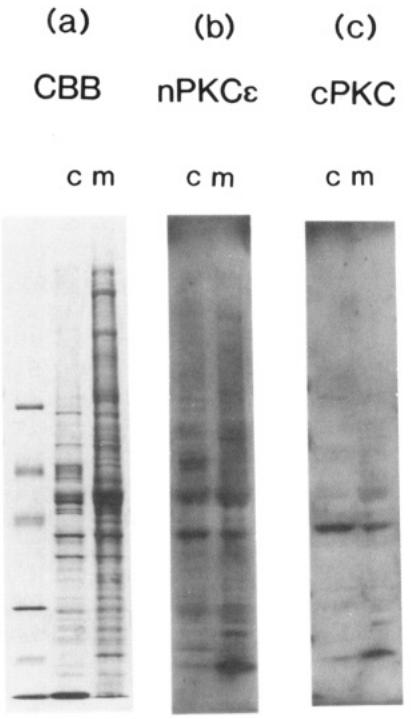

FIGURE 6: Phosphorylation of electroblotted cytosol and membrane proteins by purified nPKCε (b) and cPKC (c). Cytosol and membrane fractions (designated c and m, respectively) of brain homogenate were electroblotted onto PVDF membranes after SDS–PAGE and phosphorylated as described under Experimental Procedures. Panel a shows the protein stain patterns of the polyacrylamide gel with Coomassie Brilliant Blue. The first lane presents the molecular weight markers as in Figure 3. Panels b and c are autoradiograms of the phosphorylated proteins on the PVDF membranes. No phosphorylation was observed in the absence of the kinase sample under either of the phosphorylation conditions.

susceptibilities to phosphorylation may have been altered, although they were partly renatured on the PVDF membrane. (ii) The physiological intracellular environment surrounding the substrates was lost. (iii) The observed phosphorylation is a function of both the amount of substrates and their susceptibility to phosphorylation. The principal of the experiment, however, is essentially the same as of Western blotting where, in many cases, antibodies raised against "native" antigens are used to identify specific "denatured" proteins. The observation described here, therefore, should reflect the physiological enzyme–substrate interaction to a certain extent and reveals a new aspect of the substrate specificity of nPKCε, which has not been shown by the experiments using only synthetic peptides and commercially available proteins (Konno et al., 1989; Schaap & Parker, 1990).

Proteolytic Activation of nPKCε by Calpain. One of the general characteristics of cPKC is that limited proteolysis by calpain results in production of a catalytic fragment that does not require cofactors such as phospholipids, calcium, and phorbol ester for activity (Kishimoto et al., 1983). As examined with an antiserum specific to the C-terminal, when brain ε was treated with μ- and *m*-calpains, the 96K enzyme was proteolyzed and a 45K catalytic fragment was formed in a manner dependent on the amount of calpain (Figure 7A). The cardiolipin-independent kinase activity arose in accordance with the appearance of the catalytic fragment (Figure 7B). Thus, the ε species, too, can be a substrate for calpain and can be proteolytically activated in vitro. The results agree with the report that ε, like cPKC, is subject to down-regulation in cells such as stimulated GH_4C_1 cells (Akita et al., 1990b; Kiley et al., 1990) and murine thymocytes (Strulovici et al., 1991). The apparent molecular weight of 45K for the catalytic fragment indicates that the enzyme is cleaved in the D3 region (Kishimoto et al., 1989). The other nPKC species sharing common domain structure, therefore, are expected to undergo limited proteolysis as well at the hinge region between the catalytic and regulatory domains. As has been shown with cPKCγ, ε may be proteolyzed by μ-calpain at physiological Ca^{2+} concentrations under certain conditions, i.e., in the presence of polyphosphoinositide or other lipids (Saido et al., 1991). We predict that the whole PKC family, both novel and

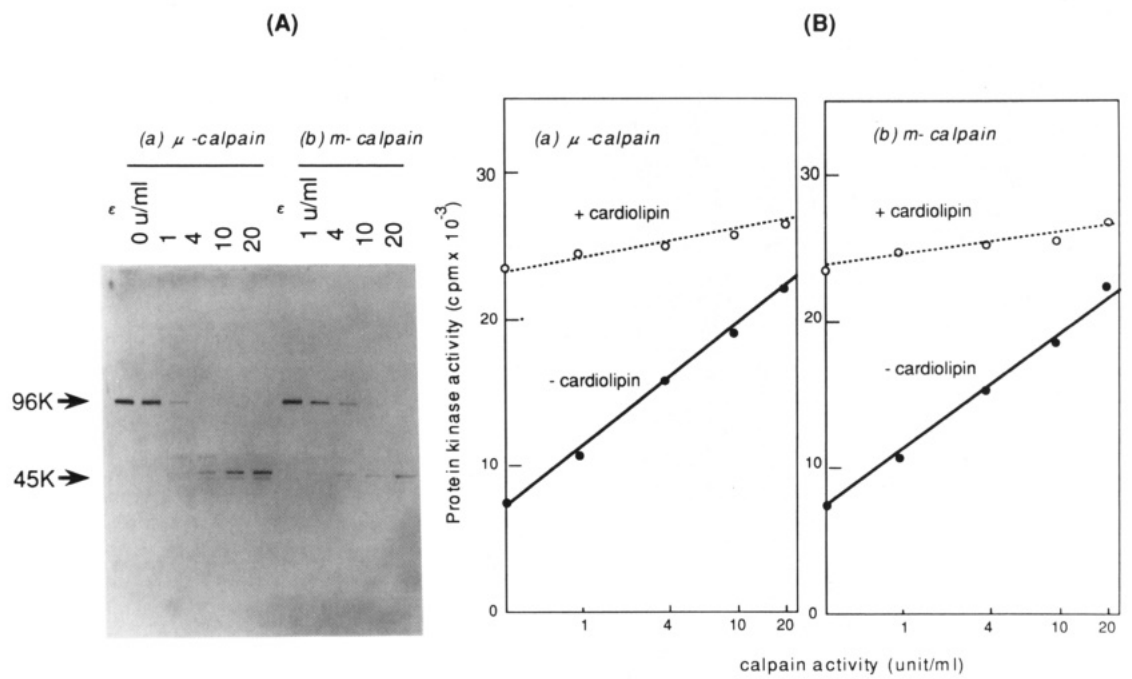

FIGURE 7: Proteolytic activation of nPKCε by μ- and *m*-calpain. (A) Western blot analysis using anti-nPKCε antiserum. Purified ε (lanes 1 and 7) was treated with μ-calpain (lanes 3–6) or *m*-calpain (lanes 8–11) at calpain concentrations of 1 unit/mL (lanes 3 and 8), 5 units/mL (lanes 4 and 9), 10 units/mL (lanes 5 and 10), or 20 units/mL (lanes 6 and 11). Lane 2 shows an ε sample treated with the proteolysis buffer in the absence of calpain. (B) Protein kinase activity of nPKCε treated with (a) μ-calpain and (b) *m*-calpain. Assays were conducted with MBP_{4-14} in the presence (open circles) or absence (closed circles) of 50 μg/mL cardiolipin.

conventional, is under proteolytic regulation by calpain, and thus by calcium, in cells.

Registry No. Ca, 7440-70-2; protein kinase, 9026-43-1; calpain, 78990-62-2.

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