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ISOLATION AND CHARACTERIZATION OF PROTEIN KINASE C FROM RAT BRAIN SYNAPTOSOME CYTOSKELETON

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INDEXING WORDS

protein kinase C; synaptosome; cytoskeleton

SYNOPSIS

Synaptosomes prepared from brain tissues are known to retain morphorogical and functional characteristics of the nerve ending. Little information is available, however, as to the biochemical events underlying synaptogenesis and transmitter release. Increasing body of evidence suggests that protein kinase C (PKC) plays crucially important roles through phosphorylation of membrane proteins such as GAP-43 (for 43-kDa growth-associated protein) and 87-kDa MARCKS (for myristoylated, alanine-rich C kinase substrate) in many aspects of the neuronal function. Among them, arrangement of membrane cytoskeletal protein is proposed to be one of the primary sites of PKC action. The present study is an attempt to isolate and characterize PKC associated with synaptosomal membrane cytoskeleton. Rat brain synaptosomal Triton X-100 insoluble elements (cytoskeleton) contains specific

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Abbreviation used are: PKC, protein kinase C; PS, phosphatidylserine; DO, diolein; PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; 4αPDD, 4α-phorbol-12,13-didecanoate; MBP, myelin basic protein; EGTA, ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide; APMSF,(p-Amidinophenyl)metanesulfonyl fluoride hydrochloride

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[^{3}H]phorbol dibutyrate binding activity and 78-kDa protein which reacts with an antibody against βII-PKC subspecies. Although 78-kDa protein could not be solublized by the treatment with various ionic and non-ionic detergents and/or high concentrations of salts such as NaCl and LiBr, the fragment of 78-kDa protein was produced and solublized from cytoskeleton by limited proteolysis with calpain II, which cleaves PKC at one or two specific sites of the enzyme to produce catalytic and regulatory fragments. The solublized 46-kDa fragment was identical with the catalytic fragment of βII-PKC. The results indicate that this PKC subspecies is tightly associated with the cytoskeletal network of synaptic membranes.

INTRODUCTION

The receptor-mediated hydrolysis of polyphosphoinositides initiates signal transduction to regulate many neuronal functions such as modulation of membrane excitability and neurotransmitter release.[20] The primary products of this reaction, inositol 1,4,5-trisphosphate and 1,2-sn-diacylglycerol, act as the second messengers for Ca^{2+}-mobilization from its intracellular storage pool,[4] and the activation of PKC,[10] respectively. In addition, unsaturated fatty acids such as arachidonic acid are known to serve as PKC activator,[19] and Ca^{2+} may also come from an extracellular source by the activation of plasma membrane ion channels.[30] PKC is a large family of proteins with multiple subspecies that reveal subtly different enzymological characteristics and diverse distribution among mammalian tissues. At present, seven cDNA clones, α, βI, βII, γ, δ, ε, and ζ have been isolated from rat brain cDNA library.[21] PKC from cytosol and detergent-soluble fractions of rat brain is normally resolved into three subfractions, type I, II, and III, upon hydroxyapatite column chromatography, which correspond to the enzymes encoded by γ-, β (βI plus βII)-, and α-cDNA clone, respectively.[28] Immunocytochemical studies with subspecies-specific antibodies indicate that these PKC subspecies are differently located in particular neuronal cells, and at limited intracellular locations.[21] In brain tissue areas such as the hippocampus and cerebellum, it is proposed that PKC is persistently translocated to the membrane after prolonged electric stimulation and plays a role in the long-term potentiation of synaptic transmission.[1] Two specific substrates for PKC, GAP-43 (F1, B-50) and 87-kDa MARCKS, are known to be intercalated into the membrane.[18, 27] The soluble and detergent-extractable PKC subspecies in rat brain synaptosomes and their corelation to neurotransmitter release have been analyzed biochemically.[22, 31] In addition to these soluble and Triton-extractable subspecies, the existance of PKC molecules, which is tightly associated with and not extractable from the membranes, has been suggested.[33] This paper will describe the extraction, identification, and characterization of the PKC

subspecies associated with Triton X-100 insoluble cytoskeletal elements of rat brain synaptosomes.

EXPERIMENTAL PROCEDURES

Materials

PS and DO were obtained from Serdary Research Laboratories. [γ-^{32}P]ATP (3 Ci/mmol) and [^{3}H]PDBu (17.5 Ci/mmol) were obtained from New England Nuclear. PMA, PDBu, and 4αPDD were the products of LC service. H1 histone (calf thymus) and MBP (bovine brain) were prepared by the methods of Oliver et al.[23] and Oshiro and Eylar,[25] respectively. Rat brain PKC and rat kidney calpain II were purified by the methods of Sekiguchi et al.[28] and Yoshimura et al.,[35] respectively. The catalytic fragment of PKC was prepared on gel filtration after digestion of PKC by calpain II as described.[14] The peptides employed in the present studies were synthesized using an Applied Biosystems peptide synthesizer model 430A: Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu (MBP_{4-14}) [34] and Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val (PKC_{19-31}).[6] Superose 12HR 10/30 column and TSK-gel ODS-120T reverse-phase column (4.6 x 250 mm) were obtained from Pharmacia-LKB Biotechnology and Tosoh, respectively.

Enzyme assay

PKC activity was routinely assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into the synthetic peptide, MBP_{4-14}.[34] The reaction mixture (50 µl) contained 20 mM Tris/HCl at pH 7.5, 5 mM magnesium acetate, 10 µM [γ-^{32}P]ATP (3-6 x 10^5 cpm), 25 µM MBP_{4-14}, 10 µg/ml PS, 1 µg/ml DO, 0.66 mM $CaCl_2$, and enzyme preparation to be assayed. After incubation for 6 min at 30°C, a 30-µl aliquot of the mixture was spotted onto a square (2 x 2 cm) of phosphocellulose paper (Whatman P-81), and then the paper was washed with 75 mM phosphoric acid 4 times each time for 5 min. The radioactivity trapped on the paper was quantitated by scintillation spectroscopy. The catalytic fragment of PKC was assayed under the same condition except that 0.5 mM EGTA was added to the reaction mixture instead of $CaCl_2$, PS, and DO. One unit of the enzyme was defined as that amount of enzyme which incorporated 1 nmol of phosphate from ATP to MBP_{4-14} per min.

Subcellular fractionation of rat brain

Male Sprague-Dawley rats were decapitated and brains were quickly removed. All subsequent operations were carried out at 0-4°C. The fractionation procedure of

cerebrum was essentially same as described [15] except that a Tris-buffered sucrose solution (TBSS, 20 mM Tris/HCl at pH 7.5, 0.32 M sucrose, 2 mM EDTA, 2 mM EGTA, 1 mM leupeptin, 0.4 mM APMSF) was employed. The metal chelators were indispensable to prevent the association of cytosol PKC with membrane, and also to block the proteolysis of enzyme by calpain during the subcellular fractionation procedures.[7] Starting from 10 g of the wet tissues, nuclear (P_1, 507 mg of protein), crude mitochondrial (P_2, 184 mg of protein), microsomal (P_3, 79 mg of protein), and soluble (413 mg of protein) fractions were obtained. Then, Fraction P_2 was fractionated further by the method of Kreuger et al.[15] which was a modification of the procedure originally described by Gurd et al.[5] Fraction P_2 from 10 g of rat brain (184 mg of protein) was suspended in 10 ml of TBSS. This was then diluted with 30 ml of 19% (W/V) Ficoll (Pharmacia) dissolved in TBSS to give a final Ficoll concentration of about 14%. A discontinuous density gradient was formed by overlaying 7 ml of this suspension, first with 3.5 ml of 7.5% (W/V) Ficoll in TBSS, and then with 1.6 ml of TBSS alone in a swinging bucket (Beckman model SW40Ti). Six buckets were centrifuged for 120 min at 90,000 x g. The materials at the 0 to 7.5% Ficoll interface (Fraction P_2A), that at the 7.5 to 14% Ficoll interface (Fraction P_2B), and the pellet (Fraction P_2C) were harvested with a Pasteur pipette. Each of these fractions was diluted with 100 ml of TBSS and centrifuged for 10 min at 20,000 x g. Then, pellets were separately suspended in TBSS. Fraction P_2A (25 mg of protein, mainly myelin and membranes), Fraction P_2B (135 mg of protein highly enriched in synaptosomes with a slight contamination of myelin, mitochondria, and unidentified particulate materials), and Fraction P_2C (23 mg of protein, mainly mitochondria with a few synaptosomes) were obtained.

Preparation of Triton X-100 insoluble elements

Triton X-100 (2% (V/V) at final concentration) and leupeptin (0.1 mM at final concentration) were added to each subcellular fraction. The solution was sonicated 4 times each time for 15 sec, allowed on ice for 30 min, and then centrifuged for 30 min at 100,000 x g. The pellet was suspended with 10 volumes of Buffer A (50 mM HEPES at pH 7.5, 0.5 mM EGTA, 0.5mM EDTA, 0.1 M NaCl) containing 1% (V/V) Triton X-100, and sonicated 4 times each time for 15 sec followed by centrifugation for 30 min at 100,000 x g. This procedure was repeated two more times. The resultant pellet was washed once with Buffer A, and the pellet was finally suspended in Buffer A. These preparations were stored at -80°C until use.

Analysis of PKC subspecies

The PKC subspecies, α, β(βI plus βII), and γ, in the soluble fraction, were separated and quantitated upon hydroxyapatite column chromatography as described previously.[29] The ratio of βI- and βII-subspecies was determined by immunological procedures.[29]

Phosphopeptide analysis of MBP

The ^{32}P-labeled MBP (5 μg) was dissolved in 0.05 ml of 0.1 M ammonium bicarbonate at pH 8.0, and digested with trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (0.1 μg) for 2.5 h at 37°C. Fresh trypsin (0.1 μg) was again added, and the mixture was incubated for additional 2.5 h. After lyophilization, the resulting tryptic phosphopeptides were taken up with 0.05 ml of 0.1% (V/V) trifluoroacetic acid and subjected to HPLC equipped with a TSK-gel ODS-120T reverse-phase column (5 μm silica, 120Å pore, 4.6 x 250 mm; Tosoh) equilibrated with 0.1% trifluoroacetic acid. After the column was washed for 21 min with the same solution at a flow rate of 1 ml/min, elution was performed using an 80-min linear gradient from 0.1% trifluoroacetic acid to 20% (V/V) acetonitrile in 0.1% trifluoroacetic acid at a constant flow rate of 1 ml/min. Fractions of 1 ml each were collected and the radioactivity was determined.

Preparation of antibodies

A monoclonal antibody designated CKmC1β-a, was raised against the synthetic peptide, FARKGALRQKNVHEVKNHKF, which corresponds to the amino acid residues from 20 to 39 deduced from the β-cDNA clone of the rat brain PKC.[28] This sequence is almost common among the equivalent regions of the predicted amino acid sequences encoded by α-, βI-, βII-, and γ-cDNA clones.[8] The polyclonal antibodies, designated CKpV5βI-a, CKpV5βII-a, CKpV3γ-a, and CKpV5α-a were prepared against oligopeptides, SYTNPEFVINV (βI-sequence, amino acid residue 661-671), SFVNSEFLKPEVKS (βII-sequence, amino acid residue 660-673), SPIPSPSPSPTDSK (γ-sequence, amino acid residue 322-335), QFVHPILQSAV (α-sequence, amino acid residue 662-672), respectively, as described.[12] These polyclonal antibodies were specific to βI-, βII-, γ-, and α-PKC subspecies, respectively.

Immunoblot analysis

Samples were electrophoresed on a SDS-10% (W/V) polyacrylamide gel by the method of Laemmli,[16] and transfered to a polyvinylidene difluoride membrane (Millipore). The membrane was incubated overnight at room temperature with 20 mM Tris/HCl at pH 7.5 containing 150 mM NaCl (TBS) supplemented with 3% (W/V)

gelatin and 0.02% (W/V) NaN_3. Then, the membranes were exposed to the respective antibodies (1 μg/ml) in TBS containing 1% gelatin for 60 min at room temperature. Immunoreactive bands were visualized using an anti-mouse or anti-rabbit IgG antiserum and avidin-biotinylated horseradish peroxidase complex (Vectastain, Vector Laboratories), employing diaminobenzidine and H_2O_2 for color reaction according to the manufacture's procedure. The visualized bands were scanned using a dual wave length TLC scanner (Shimazu) and the relative intensities were quantitated as integrated areas of the optical density.

Assay of [^{3}H]PDBu binding

The binding assay was done as described previously [32] with a slight modification. The standard mixture (0.1 ml) contained 20 mM Tris/HCl at pH 7.5, 100 mM KCl, 0.15 mM $CaCl_2$, 0.05 mM EGTA, 100 μg/ml PS, 10 nM [^{3}H]PDBu (8-10 x 10^3 cpm), 0.5% (V/V) DMSO, and 24 μg of synaptosomal Triton X-100 insoluble elements or 0.04 μg of purified PKC. After incubation for 20 min at 30°C, the reaction was stopped by the addition of 2 ml of ice-cold 0.5% (V/V) DMSO. Then, the solution was immediately poured onto a glass-fiber filter (Whatman GF/A) under a high vacuum produced by a suction apparatus. The filter was washed three times, each time with 3 ml of ice-cold 0.5% (v/v) DMSO. After drying, the bound radioactivity was determined in a toluene-based liquid scintillation fluid. Non-specific binding was measured in the presence of 50 μM non-radioactive PDBu. Specific binding represents the difference between total and non-specific binding.

Protein phosphorylation in synaptosomal Triton X-100 insoluble elements

Protein phosphorylation in synaptosomal Triton X-100 insoluble elements was carried out at 30°C in the reaction mixture (50 μl) containing 20 mM Tris/HCl at pH 7.5, 5 mM magnesium acetate, 10 mM 2-mercaptoethanol, 0.01% (V/V) Triton X-100, 10 μM [γ-^{32}P]ATP (3 x 10^5 cpm), 10 μg/ml PS, 1 μg/ml DO, 0.2 mM $CaCl_2$, and the synaptosomal Triton X-100 insoluble elements. Where indicated, purified PKC was added to the mixture. The phosphorylation reaction was terminated by the addition of SDS-containing sample solution according to Laemmli.[16] Then, a 25-μl aliquot of the solution was electrophoresed. The dried gel was exposed on a Fuji RX film sheet for 12 h in the presence of a intensifying screen at -80°C.

Protein determination

Protein was determined by the method of Lowry et al.[17] with bovine serum albumin as a standard protein. Samples were first precipitated in 10% (w/v) trichloroacetic acid to remove the interfering materials, dissolved in 0.1 M NaOH, and subjected to protein determination.

RESULTS AND DISCUSSION

Figure 1 shows a typical elution profile of the PKC subspecies present in the soluble fraction of synaptosomes. The relative activities of α-, βI-, βII-, and γ-subspecies are quantitatively shown in Table 1. Although most of PKC activity was recovered in the soluble fraction, a small quantity of PKC remained thightly associated with Triton X-100 insoluble material. Figure 2 shows an immunoblot analysis of the Triton X-100 insoluble element. This element contained 78-kDa protein which was reactive with CKpV5βII-a (lane 4), and slightly with CKpV5α-a (lane 5). The antibodies against the γ- and βI- subspecies did not recognize this 78-kDa protein. This protein was not solublized by various non-ionic and ionic detergents, 0.4-1.0 M KCl, nor by 1.25 M LiBr, indicating that this protein is probably associated tightly with structural elements. Densitometric scanning of the

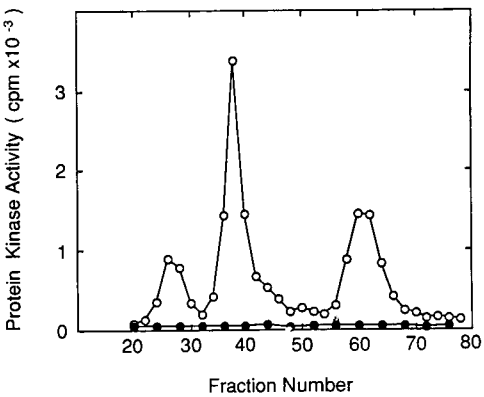

Fig. 1. PKC subspecies from rat brain synaptosomal Triton X-100 soluble fraction. The synaptosomal fraction was prepared from 0.5 g of rat brain as described under "EXPERIMENTAL PROCEDURES". Then, Triton X-100 (2% (v/v) at final concentration) was added to the synaptosomal fraction. The solution was sonicated 4 times each time for 15 sec, allowed on ice for 30 min, and then centrifuged for 30 min at 100,000 x g at 4°C. The supernatant was subjected to DEAE-cellulose column chromatography followed by the separation of PKC subspecies on a hydroxyapatite column as described under "EXPERIMENTAL PROCEDURES". A 10-μl aliquot of each fraction was assayed for PKC in the presence of either PS, DO, and $CaCl_2$ (○) or in the presence of 0.5 mM EGTA (●) under the standard condition.

Table 1. Realative ratios of PKC subspecies from rat brain synaptosomal Triton X-100 soluble fraction.

Subspecies	α	βI	βII	γ
Relative activities	34.1%	5.2%	46.5%	14.2%

Quantitations of α-, β(βI and βII)-, and γ-PKC subspecies were performed by measuring the areas from protein kinase activities of Fig. 1. Since βI- and βII-PKC were not able to be separated chromatographically, the relative ratio of βI- and βII-PKC was tentatively determined by immunoblot analysis of the fraction as described.[29]

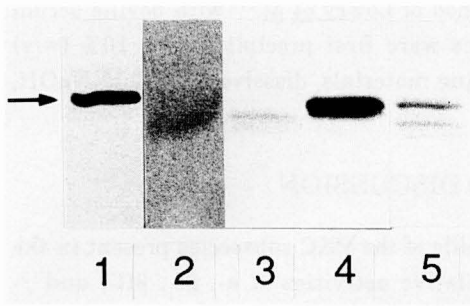

Fig. 2. Immunoblot analysis of purified PKC and synaptosomal Triton X-100 insoluble elements. Purified PKC (0.24 μg) and synaptosomal Triton X-100 insoluble elements (51 μg) were subjected to polyacrylamide gel electrophoresis followed by immunoblot analysis as described under "EXPERIMENTAL PROCEDURES". Lane 1, rat brain purified PKC (mixture); lanes 2-5, synaptosomal Triton X-100 insoluble elements. CKmC1β-a (lane 1), CKpV3γ-a (lane 2), CKpV5βI-a (lane 3), CKpV5βII-a (lane 4), and CKpV5α-a (lane 5) were used as the first antibody. The gel was calibrated with the following standards: myosin, 205 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa. A Position of purified PKC (82 kDa) is indicated by the arrow.

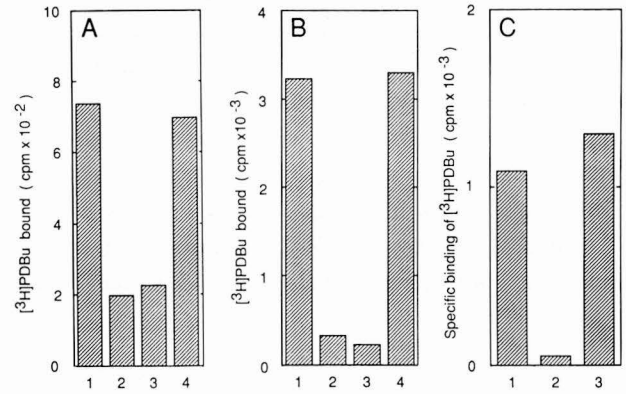

Fig. 3. [^{3}H]PDBu-bindings to synaptosomal Triton X-100 insoluble elements and purified PKC. A and B, [^{3}H]PDBu-binding to 24 μg of synaptosomal Triton X-100 insoluble elements and 0.04 μg of purified PKC (mixture), respectively. [^{3}H]PDBu-binding was measured as described under "EXPERIMENTAL PROCEDURES". Where indicated, 50 μM of non-radioactive PDBu, PMA or 4αPDD was added to the mixture. Lane 1, in the absence of non-radioactive material; lanes 2, 3, and 4, in the presence of 50 μM non-radioactive PDBu, PMA, and 4αPDD, respectively. C, specific [^{3}H]PDBu-bindings to synaptosomal Triton X-100 insoluble elements and those treated with calpain II. synaptosomal Triton X-100 insoluble elements (72 μg) were at first incubated with calpain II (0.9 μg) at 20°C for 30 min in the reaction mixture (50 μl) containing 20 mM Tris/HCl at pH 7.5, 5 mM 2-mercaptoethanol, 1% (v/v) Tween 20, 0.7 mM $CaCl_2$, 10 μg/ml PS, 1 μg/ml DO, and 0.5 μM H1 histone. After the incubation, the reaction was stopped by the addition of EGTA (4 mM at final concentration). Then, the mixture was centrifuged at 100,000 x g for 30 min at 4°C. The pellet was suspended with 50 μl of Buffer A. Then, a 20-μl aliquot of each fraction was subjected to the assay of specific [^{3}H]PDBu-binding as described above. Lane 1, synaptosomal Triton X-100 insoluble elements (24 μg); lanes 2 and 3, the supernatant and the pellet after the treatment of synaptosomal Triton X-100 insoluble elements with calpain II, respectively.

immunoblot analysis with the antibody against βII-PKC indicated that the 78-kDa protein was approximately 9 % of total synaptosomal soluble and insoluble βII-PKC. It has been shown that PKC shows a specific [^{3}H]PDBu-binding activity, and this binding site is located in the regulatory domain.[24] Figure 3 shows the specific binding of [^{3}H]PDBu to purified PKC and the synaptosomal Triton X-100 insoluble elements. Purified PKC and the Triton X-100 insoluble elements showed specific [^{3}H]PDBu-binding of 490 and 0.22 pmol/mg protein, respectively. Non-radioactive tumor promoting phorbol esters, such as PDBu and PMA, inhibited the specific

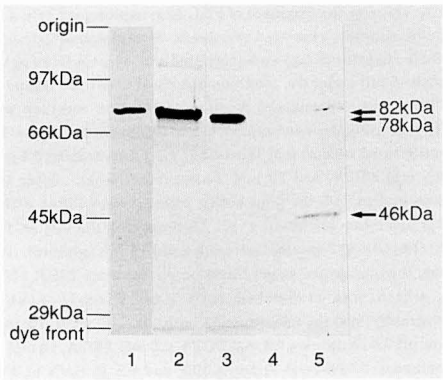

Fig. 4. Immunoblot analysis of synaptosomal Triton X-100 insoluble elements after calpain II-treatment. Synaptosomal Triton X-100 insoluble elements (900 μg) were incubated with calpain II (9 μg) at 20°C for 30 min on a large scale (0.5 ml) under the condition described under the legend to Fig. 3. After the centrifugation, the pellet was suspended in 0.5 ml of Buffer A. Aliquots (20 μl each) of the resultant supernatant and pellet were subjected to immunoblot analysis using antibodies CKmC1β-a (lane 1) and CKpV5βII-a (lanes 2-5) as described under the legend to Fig. 2. Lane 1, purified rat brain PKC (mixture); lane 2, synaptosomes (116 μg of protein); lane 3, synaptosomal Triton X-100 insoluble elements (51 μg); lanes 4 and 5, the pellet and supernatant after treatment of synaptosomal Triton X-100 insoluble elements with calpain II, respectively. The positions of 82 kDa, 78 kDa, and 46 kDa are indicated by the arrows.

Table II. Protein kinase activity released from the Triton X-100 insoluble elements by the treatment with calpain.

	without calpain	with calpain
phosphorylation of $MBP_{4\text{-}14}$ (cpm)	239	4,924

The Triton X-100 insoluble elements was incubated either with or without calpain II followed by centrifugation as described in the legend to Fig. 3. A 5-μl aliquot of the supernatant was assayed for the catalytic fragment of PKC with the PKC-specific substrate, $MBP_{4\text{-}14}$, under the standard condition.

binding of [^{3}H]PDBu, whereas non-tumor promoting phorbol ester, 4αPDD, was not inhibitory (Fig. 3A and B). Ca^{2+}-dependent neutral protease, calpain II, is known to cleave PKC in a limited mannar to produce two fragments, catalytic and regulatory fragments.[13, 14] The Triton-insoluble elements was incubated with calpain II, and then separated into soluble and insoluble fractions by centrifugation. Immunoblot analysis with polyclonal antibody CKpV5βII-a, which can recognize the catalytic fragment of βII-PKC, is shown in Fig. 4. A 46-kDa protein, whose molecular weight corresponds to the catalytic fragment of PKC,[14] was produced and recovered from the soluble fraction. Simultaneously, the enzymatic activity reactive with a synthetic oligopeptide substrate specific to PKC was released into the soluble fraction (Table II). After digestion with calpain II, the Triton X-100 insoluble elements still retained the [^{3}H]PDBu-binding activity (Fig. 3C). This suggests that the regulatory domain of PKC in the Triton X-100 insoluble elements was tightly bound to the cytoskeletal elements, whereas the catalytic fragment was converted to a soluble form after the limited proteolysis. The enzyme fraction obtained from the synaptosomal Triton X-100 insoluble elements by calpain was subjected to gel filtration on a Superose 12 HR

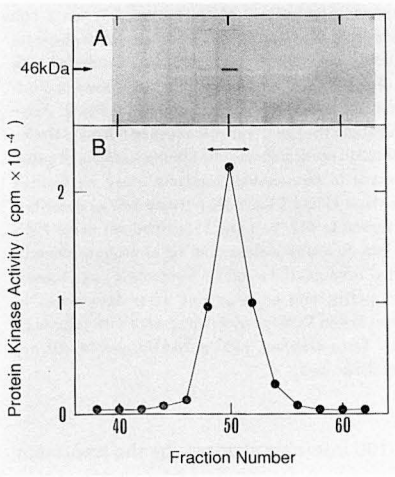

Fig. 5. Analysis of the fragment of PKC in synaptosomal Triton X-100 insoluble elements generated by calpain. Synaptosomal Triton X-100 insoluble elements (9 mg) were incubated with calpain II (90 μg) on a large scale (5 ml) under the condition described under the legend to Fig. 3. Then, the supernatant fraction of reaction mixture was prepared by centrifugation and applied to a hydroxyapatite column (0.8 x 1cm) equilibrated with 20 mM Tris/HCl at pH 7.5 containing 0.5 mM EGTA, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol. After the column was washed with the same buffer, proteins were eluted with 1 ml of 0.3 M potassium phosphate at pH 7.5 containing 0.5 mM EGTA, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, and 10% (v/v) glycerol. The eluate was, then, subjected to gel filtration on Superose 12HR 10/30 column, which was connected to a Fast Protein Liquid Chromatography system (Pharmacia) equilibrated with 20 mM Tris/HCl at pH 7.5 containing 0.5 mM EGTA, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, 0.02% (v/v) Triton X-100, and 0.5 M NaCl at 4°C. Elution of proteins was performed with the same solution at a flow rate of 0.3 ml/min and fractions of 0.3 ml each were collected. A, a 250-μl aliquot of each fraction was lyophilyzed and dissolved in 25 μl of H_2O, then the solution was subjected to immunoblot analysis using polyclonal antibody CKpV5βII-a, as described under the legend to Fig. 2. The 46-kDa protein reactive to the antibody was indicated by the arrow. B, a 10-μl aliquot of each fraction was assayed for the catalytic fragment of PKC with synthetic peptide, $MBP_{4\text{-}14}$, as substrate under the standard condition. The fractions containing kinase activity (48 to 52) were combined as indicated by the horizontal arrow in Panel B (cytoskeletal PKC).

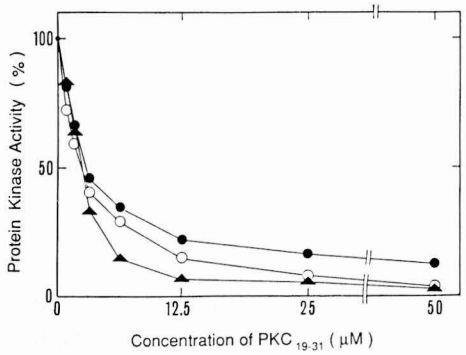

Fig. 6. The effect of synthetic peptide, $PKC_{19\text{-}31}$, upon protein kinase activities of PKC, the catalytic fragment of PKC, and the catalytic fragment of cytoskeletal PKC. Purified PKC (0.036 μg), the catalytic fragment of purified PKC (0.036 μg as PKC), and a 5-μl aliquot of the catalytic fragmemt of cytoskeletal PKC prepared in Fig. 5 were assayed under the respective standard condition except that various concentrations of $PKC_{19\text{-}31}$ were added to the mixture. (●), (○), and (▲), the catalytic fragmemt of cytoskeletal PKC, purified PKC, and the catalytic fragment of purified PKC, respectively.

10/30 column (Fig. 5). The elution position of 46-kDa protein was shown by the immunoblot analysis using polyclonal antibody, CKpV5βII-a (Fig. 5A). This antibody recognizes the C-terminal end of βII-PKC. Protein kinase activity toward PKC-specific peptide substrate was eluted at the exactly same fraction (Fig. 5B). Thus, this 46-kDa protein, which was recognized by the antibody against βII-PKC, was likely to possess the enzymatic activity. The fractions containing kinase activity were pooled and employed for the subsequent studies as the catalytic fragment of cytoskeletal PKC.

The peptide, $PKC_{19\text{-}31}$, which corresponds to the pseudosubstrate region of PKC, is known to inhibit PKC specifically at micromolar concentrations.[6] Figure 6

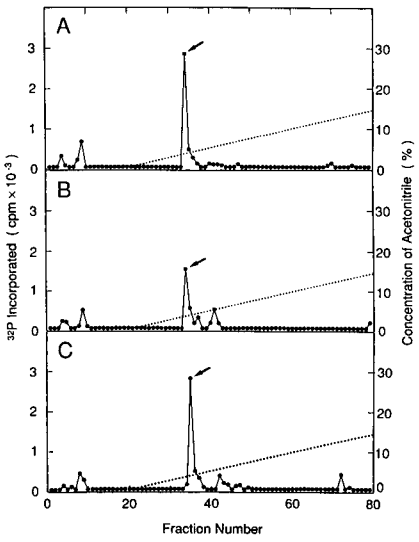

Fig. 7. Phosphopeptide analysis of MBP phosphorylated by either purified type II PKC, the catalytic fragment of purified type II PKC, or the catalytic fragment of cytoskeletal PKC. MBP (88 μg) was incubated with type II PKC (0.054 μg) in the reation mixture (0.22 ml) containing 20 mM Tris/HCl at pH 7.5, 0.1 mM [γ-^{32}P]ATP (4.1 x 10^6 cpm), 30 mM 2-mercaptoethanol, 5 mM magnesium acetate, 0.01% (v/v) Triton X-100, 10 μg/ml PS, 1 μg/ml DO, and 0.34 mM $CaCl_2$. MBP was also phosphorylated by either the catalytic fragment of type II PKC (0.054 μg as PKC) or that of cytoskeletal PKC (170 μl) under the same condition except that 0.66 mM EGTA was added to the reaction mixture instead of PS, DO, and $CaCl_2$. After the incubation at 30°C for 100 min, 0.48 mol, 0.67 mol, and 0.64 mol of ^{32}P were incorporated into 1 mol of MBP with type II PKC, the catalytic fragment of type II PKC, and the catalytic fragment of cytoskeletal PKC, respectively. The reaction was stopped by the addition of an ice-cold saturated ammonium sulfate solution (final 45% saturation). The precipitated ^{32}P-labeled MBP was collected by centrifugation for 15 min at 20,000 x g at 4°C and washed with ice-cold 45% saturated ammonium sulfate solution followed by centrifugation. Precipitated materials were dissolved in distilled water and dialyzed overnight at 4°C with Spectrapor 3 (Mr cut off: 3,500) membrane tube against a large volume of distilled water. Then, the ^{32}P-labeled MBP was lyophilized and phosphopeptide analysis was performed by the trypsin digestion followed by a reversed-phase HPLC under the condition described under "EXPERIMENTAL PROCEDURES." A, with purified type II PKC; B, with the catalytic fragment of purified type II PKC; C, with the catalytic fragment of cytoskeletal PKC, respectively. The Arrow indicates the position of the peptide, Arg-Pro-Ser(8)-Gln-Arg.

shows the effect of this synthetic peptide inhibitor, PKC_{19-31}, on PKC, the catalytic fragment of PKC, and the catalytic fragment of cytoskeletal PKC. Activities of these enzyme fractions were all inhibited by this peptide (2-10 μM). Purified PKC phosphorylates preferentially Ser-8 in MBP.[11] Phosphopeptide analysis of the MBP phosphorylated by purified type II PKC, the catalytic fragment of type II PKC, and the catalytic fragment of cytoskeletal PKC was performed to examine the substrate specificities of these enzymes (Fig. 7). With these enzymes, an identical phosphopeptide was obtained. This phosphopeptide has been identified as, Arg-Pro-Ser(8)-Gln-Arg.[11] The results indicate that Triton-insoluble elements of rat brain synaptosomes tightly bind most likely βII- or its related PKC subspecies, and that its catalytic fragment is released by the limited proteolysis with calpain.

The synaptosome (P_2B) fraction contained a significant amount of Triton-insoluble PKC, whereas the myelin (P_2A) and microsome/plasma membrane fraction (P_3) contained nearly negligible activity. No enzymatic activity was associated with the mitochondria (P_2C) fraction. Although a considerable amount of enzyme was also released from P_1 fraction, its specific activity was relatively low. This may be due to the contamination of P_2 and P_3 fractions and cell debris to P_1 fraction.

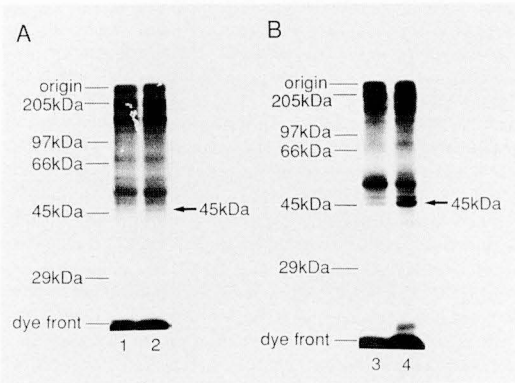

Fig. 8. PKC-mediated protein phosphorylation in synaptosomal Triton X-100 insoluble elements. Incubations were carried out at 30°C for 6 min in the reaction mixture (50 µl) containing 20 mM Tris/HCl at pH 7.5, 5 mM magnesium acetate, 10 mM 2-mercaptoethanol, 0.01% (v/v) Triton X-100, 10 µM [γ-^{32}P]ATP (3 x 10^5 cpm), 10 µg/ml PS, 1 µg/ml DO, 0.2 mM $CaCl_2$, and synaptosomal Triton X-100 insoluble elements (24 µg) alone (A) or synaptosomal Triton X-100 insoluble elements (24 µg) plus purified type II PKC (0.2 µg) (B). In control experiment, 0.7 mM EGTA was added to the mixture instead of PS, DO, and $CaCl_2$. The reaction was terminated by the addition of 25 µl of the sample solution according to Laemmli.[16] The samples were subjected to SDS-10% (w/v) polyacrylamide gel electrophoresis followed by autoradiography. Lanes 1 and 3, in the presence of EGTA; lanes 2 and 4, in the presence of PS, DO, and $CaCl_2$. Position of 45-kDa protein is indicated by the arrow.

Finally, the endogenous substrate of PKC in synaptosomal Triton X-100 insoluble elements was examined (Fig. 8). Phosphorylation of 45-kDa protein was enhanced slightly by the addition of Ca^{2+}, PS, and DO to the reaction mixture (Fig. 8A). When purified type II PKC was exogenously added to the mixture, phosphorylation of 45-kDa protein was markedly increased in the presence of Ca^{2+}, PS, and DO (Fig. 8B). The result indicates that 45-kDa protein in the cytoskeletal elements was a major substrate protein for PKC. Recently, Moss et al.[18] has reported that more than 50% of membrane-associated GAP-43 (F1, B-50, P57), which is a major phosphoprotein of synaptic plasma membrane and growth cone preparations,[26] is resistant to non-ionic detergent extraction. GAP-43 is known to be a preferable target of PKC,[2] and phosphorylation of Ser41 of GAP-43 by PKC decreases its activity to bind calmodulin.[3] Thus, it is plausible that this 45-kDa protein is GAP-43 and is phosphorylated by βII-PKC in cytoskeletal elements.

The presence of PKC in the cytoskeletal elements was previously suggested in cultured cells,[9] rat brain post synaptic densities,[33] and chicken neuron and brain.[18] These authors detected PKC using [^{3}H]PDBu-binding assay or the antibodies against PKC, but they could not extract nor assay the enzymatic activity of PKC. In the present studies, the 78-kDa protein, which was recognized by the antibody against βII-PKC, was detected in Triton X-100 insoluble elements of rat brain synaptosomes. The 46-kDa protein released from the cytoskeleton by calpain treatment showed an identical molecular weight and enzymatic activity with the authentic catalytic fragment of the purified PKC. It is concluded that the βII-subspecies of PKC is thightly associated with the cytoskeletal network, and may have some roles in the control of axonal growth and transmitter release in mature synapses.

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