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Protein kinase C in rat brain synaptosomes

βII-subspecies as a major isoform associated with membrane-skeleton elements*

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A small fraction (approximately 5%) of protein kinase C (PKC) in the adult rat brain synaptosomes is tightly associated with Triton X-100-insoluble components (most likely membrane-skeleton elements), and is solubilized only after denaturation with sodium dodecyl sulfate. The kinase domain of this PKC can be released as a soluble form after limited proteolysis with calpain, whereas the regulatory domain which binds phorbol ester remains insoluble. The PKC in this fraction was identified as the βII-subspecies or its related molecule. Presumably, this enzyme subspecies is responsible for the phosphorylation of a major PKC substrate protein, growth-associated protein-43, which is located in nerve endings as well as in growth cones in association with the membrane-skeleton elements.

Protein kinase C; Synaptosome; Membrane-skeleton; Growth-associated protein-43; Rat brain

1. INTRODUCTION

It has been postulated that protein kinase C (PKC) in synapses plays roles in transmitter release, ion channel modulation, long-term potentiation, and neuronal development and regeneration (for reviews see [1–5]). A previous report form this laboratory [6] described a quantitative analysis of the PKC subspecies in the synaptosomal soluble fractions. Although relative ratios of the α-, βI-, βII- and γ-subspecies in the synaptosomes greatly vary with the brain tissue areas, a small fraction of PKC is always associated tightly with insoluble components which are not extractable by any detergent or by high salt. The nature of these detergent-insoluble components is presently unclear. The assumption has been made, however, that the components represent some membrane-skeleton elements [7]. These elements are known to be tightly associated with a major PKC-specific substrate, growth-associated protein-43 (GAP-43, F1, B-50, neuromodulin) (for reviews see [4,5,8]), which is localized specifically in the presynaptic region and growth cones. The present studies will identify the PKC in this detergent-insoluble fraction as the βII-subspecies. This finding is consistent with a recent report that GAP-43 is phosphorylated rapidly by the β-subspecies [9].

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Abbreviations: PKC, protein kinase C; GAP-43, growth-associated protein-43.

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2. MATERIALS AND METHODS

2.1. *Materials*

PKC subspecies, α-, βI-, βII- and γ-subspecies, were prepared as described [6,10]. Calpain II was purified from rat kidney [11].

2.2. *Antibodies and immunoblot analysis*

A polyclonal antibody specific to the α-subspecies, CKpV5α-a, was raised against a synthetic oligopeptide corresponding to a part of the carboxyl-terminal variable region V_5 of α-PKC (residues 662–672, QFVHPILQSAV). The specificity of this antibody was described [12]. A polyclonal antibody specific to the βI-subspecies, CKpV5βI-a, was prepared with a synthetic oligopeptide corresponding to a part of the variable region V_5 of βI-PKC (residues 661–671, SYTNPEFVINV). A polyclonal antibody specific to the βII-subspecies, CKpV5βII-a, was prepared with a synthetic oligopeptide corresponding to a part of the variable region V_5 of βII-PKC (residues 660–673, SFVNSEFLKPEVKS). The detailed procedure and specificity of these antibodies were described [13]. Fig. 1 shows the specificity of CKpV5βI-a and CKpV5βII-a employed in the present studies, which could distinguish the βI- and βII-subspecies. A polyclonal antibody specific to the γ-subspecies, CKpV3γ-a, was prepared with a synthetic oligopeptide corresponding to a part of the variable region V_3 of γ-PKC (residues 315–328, SPIPSPSPSPTDSK) as described [14]. A monoclonal antibody recognizing all of the α-, βI-, βII- and γ-subspecies, CKmC1β-a, was prepared with a synthetic oligopeptide corresponding to a part of the conserved region C_1 of βI/βII-PKC (residues 20–39, FARKGALRQKNVHEVKNHKF). The specificity of this antibody was described [10].

Immunoblot analysis with the PKC subspecies-specific antibodies was performed under the conditions specified [10,12–14].

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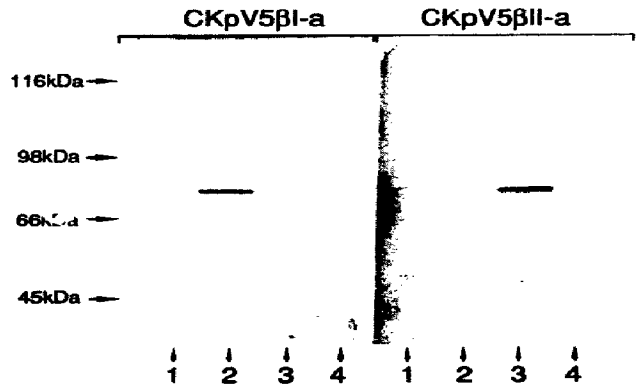

Fig. 1. Specificity of polyclonal antibodies against the βI- and βII-subspecies of PKC. Specificities of CKpV5βI-a and CKpV5βII-a was analyzed by immunoblot procedures using the standard preparations of the α-, βI-, βII- and γ-subspecies which were prepared as described [6,10]. The molecular sizes in kDa of the standards are indicated by arrows. Lane 1, α-subspecies; lane 2, βI-subspecies; lane 3, βII-subspecies; and lane 4, γ-subspecies.

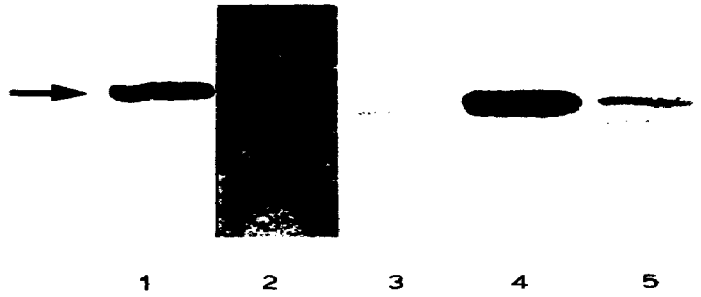

Fig. 2. Immunoblot analysis of the synaptosomal Triton X-100 insoluble components. The synaptosomal Triton X-100-insoluble fraction (51 μg protein) and purified PKC (0.24 μg protein) were subjected to SDS/PAGE and followed by immunoblot analysis. Lane 1, rat brain purified PKC (mixture); lanes 2–5, synaptosomal Triton X-100 insoluble elements. The first antibodies used were CKmClβ-a for lane 1, CKpV3γ-a for lane 2, CKpV5βI-a for lane 3, CKpV5βII-a for lane 4, and CKpV5α-a for lane 5, respectively. The gel was calibrated with the following standards: myosin, 205 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 29 kDa. The position of the purified PKC (82 kDa) is indicated by an arrow.

2.3. *Preparation of synaptosomal insoluble components*

The cerebral tissues from adult male Sprague–Dawley rats were fractionated into the subfractions (P_2A, myelin; P_2B, synaptosomal; P_2C, mitochondrial; and P_3, microsomal fractions) by the procedure described by Kreuger et al. [15] except that a Tris-buffered sucrose solution (20 mM Tris-HCl, pH 7.5, 0.32 M sucrose, 2 mM EDTA, 2 mM EGTA, 1 mM leupeptin and 0.4 mM (*p*-amidinophenyl)-metanesulfonyl fluoride hydrochloride) was employed. The P_2B synaptosomal fraction was sonicated 4 times, each time for 15 s in the presence of 2% (v/v) Triton X-100 and 0.1 mM leupeptin, stirred for 30 min at 0°C, and centrifuged for 30 min at 100 000 × *g*. The pellet was suspended with 10 vols of buffer A (50 mM HEPES at pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, and 0.1 M NaCl), containing 1% (v/v) Triton X-100. This extraction procedure was repeated 2 more times. The resultant pellet was washed once with buffer A, and the final pellet was suspended in buffer A.

2.4. *Enzyme assay*

Protein kinase activity was assayed by measuring the incorporation of $^{32}P_i$ into a synthetic PKC-specific oligopeptide substrate, MBP_{4-14}, under the conditions specified [16].

2.5. *Protein phosphorylation*

The synaptosomal Triton X-100 insoluble components were incubated with [γ-^{32}P]ATP in the presence of $CaCl_2$, phosphatidylserine and diolein. Where indicated, purified PKC was added to the incubation mixture. The phosphorylation reaction was terminated by the addition of SDS-containing electrophoresis sample buffer, and aliquots of each sample solution were subjected to SDS/PAGE as described [6]. The gel was dried and exposed to an X-ray film.

2.6. *Other methods*

Phorbol ester-binding was measured by using [^{3}H]phorbol 12,13-dibutylate as described [17]. Protein was determined using bovine serum albumin as a standard [18].

3. RESULTS

3.1. *Immunoblot analysis of synaptosomal insoluble components*

Figure 2 shows immunoblot analysis of the Triton X-100 insoluble components using antibodies specific to the PKC subspecies. The insoluble components contained an immunoreactive band with an apparent molecular weight of 78 kDa, which is slightly smaller than that of the PKC subspecies in the soluble fraction (80–82 kDa) [10]. The band was recognized by the antibody specific to βII-PKC (lane 4) and reacted faintly with the antibody against α-PKC (lane 5), but the antibodies against the γ- and βI-subspecies did not recognize the 78 kDa protein. This 78 kDa protein was not solubilized by various ionic and non-ionic detergents such as deoxycholate and Triton X-100 or by salts such as KCl and LiBr even at high concentrations, indicating that this protein is associated tightly with structural elements. Semi-quantitative immunoblot analysis of the soluble and insoluble fractions by densitometry suggested that about 10% of the βII-subspecies in synaptosomes is associated with these insoluble components. It was previously found that the βII-subspecies is a major PKC isoform in the rat cerebral synaptosomes, and represents nearly one half of the total PKC activity [6]. Thus, approximately 5% of the PKC in synaptosomes is tightly associated with the Triton X-100-insoluble components.

3.2. *Calpain digestion of synaptosomal insoluble components*

A Ca^{2+}-dependent neutral protease, calpain, is known to cleave PKC to produce 2 fragments: catalytic and regulatory fragments. The synaptosomal insoluble components were incubated with calpain II, and the resulting soluble and insoluble fractions were subjected to immunoblot analysis. As shown in Fig. 3, after the calpain treatment, a protein band with an approximate molecular weight of 46 kDa, which corresponds to the

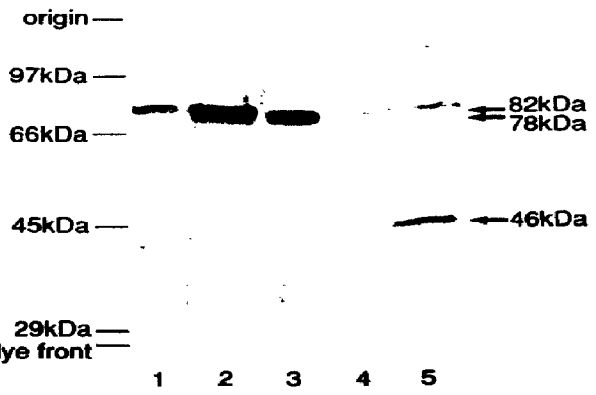

Fig. 3. Immunoblot analysis of the synaptosomal Triton X-100 insoluble elements after calpain treatment. The synaptosomal Triton X-100 insoluble fraction (900 μg protein) was incubated with calpain II (9 μg protein) at 20°C for 30 min in the reaction mixture (0.5 ml) containing 20 mM Tris-HCl at pH 7.5, 5 mM 2-mercaptoethanol, 1% (v/v) Tween 20, 0.7 mM $CaCl_2$, 10 μg/ml phosphatidylserine, 1 μg/ml diolein, and 0.5 μM H1 histone. After incubation, the reaction was stopped by the addition of EGTA (final concentration 4 mM), and the mixture was centrifuged at 100 000 × g for 30 min at 4°C. After 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. Lane 1, purified rat brain PKC (mixture); lane 2, whole synaptosomes (116 μg protein); lane 3, synaptosomal Triton X-100 insoluble components (51 μg protein); lanes 4 and 5, the pellet and supernatant fractions of the synaptosomal Triton X-100-insoluble components after the treatment with calpain II, respectively. The first antibodies used were CKmClβ-a for lane 1, and CKpV5βII-a for lanes 2–5. The positions of the 82 kDa, 78 kDa, and 46 kDa protein bands are indicated by arrows.

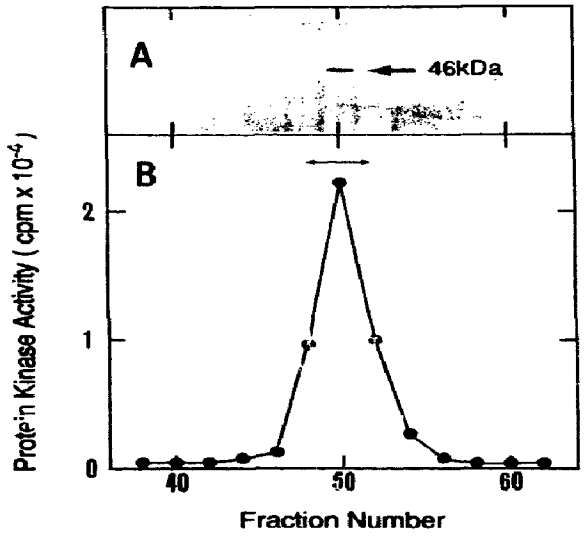

Fig. 4. Gel filtration analysis of protein kinase from synaptosomal Triton X-100-insoluble elements generated by calpain treatment. The synaptosomal Triton X-100-insoluble fraction (9 mg protein) was incubated with calpain II (90 μg protein) in a large scale reaction mixture (5 ml) as described in the legend to Fig. 2. The subsequent procedures were carried out at 0–4°C. The reaction mixture was centrifuged and the supernatant fraction was applied on a hydroxyapatite column (0.8 × 1 cm) 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 directly subjected to gel filtration on a Superose 12 HR10/30 column connected to a fast protein liquid chromatography system (Pharmacia-LKB) which was 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. Protein was eluted with the same solution at a flow rate of 0.3 ml/min, and fractions of 0.3 ml each were collected. (A) Immunoblot analysis. An aliquot of the peak fraction was subjected to immunoblot analysis using the polyclonal antibody CKpV5βII-a. The position of the 46 kDa protein reactive with the antibody is indicated by an arrow. (B) Protein kinase activity. An aliquot of each fraction was assayed for protein kinase activity using a synthetic peptide, MBP_{4-14}, as substrate. EGTA (0.5 mM) was added instead of $CaCl_2$, phosphatidylserine and diacylglycerol.

catalytic fragment of PKC [19], was found in the soluble (lane 5), but not in the insoluble (lane 4) fraction. A protein kinase reactive with a PKC-specific oligopeptide substrate, was recovered in the soluble fraction, which was independent on Ca^{2+}, phosphatidylserine and diolein. The soluble protein kinase thus generated from the insoluble components was subjected to gel filtration (Fig. 4). An immunoreactive 46 kDa protein band (Fig. 4A) appeared at the protein kinase peak (Fig. 4B). The pseudo-substrate peptide PKC_{19-31} [20], an inhibitory peptide specific to PKC subspecies, inhibited this protein kinase activity.

PKC is well known as the receptive protein of tumour-promoting phorbol esters and the binding site is located in the regulatory domain [2]. The synaptosomal Triton X-100-insoluble components bind phorbol ester (0.22 pmol/mg protein). After digestion with calpain the insoluble fraction still retained 97% of the binding activity, whereas the soluble fraction could not bind phorbol ester. The results indicate that βII-PKC or its related molecule is tightly associated with the membrane-skeletal elements through its regulatory domain, and that the catalytic fragment is released as a soluble form after limited proteolysis by calpain.

3.3. *Phosphorylation of synaptosomal insoluble components*

The synaptosomal fraction (P_2B) contained a significant amount of Triton-insoluble PKC, whereas the myelin (P_2A) and microsomal fraction (P_3) contained only a small amount of PKC. Practically no PKC was detected in the mitochondrial fraction (P_2C). Incubation of the synaptosomal Triton X-100-insoluble components with radioactive ATP resulted in the phosphorylation of a protein with an approximate molecular weight of 45 kDa in the presence of Ca^{2+}, phosphatidylserine and diolein (Fig. 5A). This phosphorylation of the 45 kDa protein was more evident when a large excess of pure type II (β) PKC was added (Fig. 5B). The

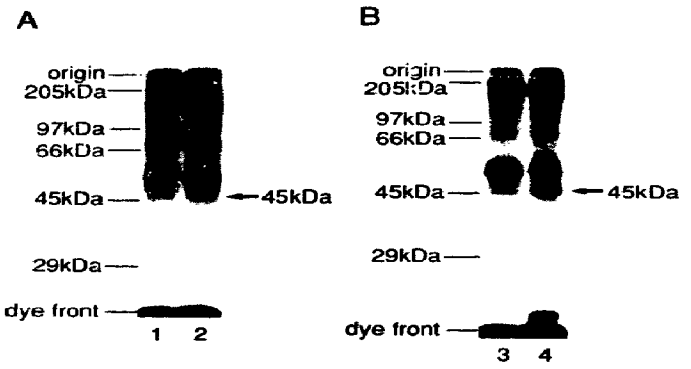

Fig. 5. Protein phosphorylation of synaptosomal Triton X-100-insoluble elements. The synaptosomal Triton X-100-insoluble elements (24 μg protein) were incubated at 30°C for 6 min in the reaction mixture (50 μl) containing 20 mM Tris-HCl at pH 7.5, 5 mM magnesium acetate, 10 mM 2-mercaptoethanol, 0.01 % (v/v) Triton X-100, 10 μM [γ-^{32}P]ATP (3×10^5 cpm), 10 μg/ml phosphatidylserine, 1 μg/ml diolein and 0.2 mM $CaCl_2$, in the presence or absence of purified type II(β) PKC (0.2 μg protein). In a control experiment, EGTA (0.7 mM) was added to the mixture instead of phosphatidylserine, diolein and $CaCl_2$. After the reaction was terminated, the samples were subjected to SDS/PAGE and then to autoradiography. A and B, without and with purified type II(β) PKC, respectively. Lanes 1 and 3, in the presence of EGTA; and lanes 2 and 4, in the presence of phosphatidylserine, diolein and $CaCl_2$. The position of 45 kDa protein is indicated by arrows.

results indicate that the 45 kDa protein, presumably GAP-43 is a major substrate protein.

4. DISCUSSION

Association of PKC with cytoskeletal elements has been suggested in cultured cells [21], rat brain postsynaptic densities [22], and chicken neuron and brain [7], as estimated by either phorbol ester-binding assay or immunoblot assay. In the present studies, the 78 kDa protein, which is recognized by the antibody against βII-PKC, was detected in Triton X-100-insoluble components of rat brain synaptosomes. This protein can be released as a soluble form only after calpain treatment, and the proteolytic fragment shows a molecular weight as well as enzymatic properties identical with those of the authentic catalytic fragment which is generated from the purified PKC. It is concluded that the βII-subspecies of PKC is tightly associated with the insoluble components, presumably the cytoskeletal network, and may have some role in the control of synaptic processes. Moss et al. [7] have reported that more than 50% of membrane-associated GAP-43, which is a major phosphoprotein in the synaptic plasma membrane and growth cone preparations [8], is resistant to non-ionic detergent extraction. GAP-43 is known to be a preferred target of PKC, and phosphorylation of Ser[41] of this protein by PKC decreases its ability to bind calmodulin [23]. It is plausible that the 45 kDa protein presented above is GAP-43, and is phosphorylated by βII-PKC in cytoskeletal elements.

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REFERENCES

[1] Gispen, W.H. (1988) Ann. N. Y. Acad. Sci. 525, 141–149.
[2] Shearman, M.S., Sekiguchi, K. and Nishizuka, Y. (1989) Pharmacol. Rev. 41, 211–237.
[3] Kennedy, M.B. (1989) Cell 59, 777–778.
[4] Linden, D.J. and Routtenberg, A. (1989) Brain Res. Rev. 14, 279–296.
[5] Coggins, P.J. and Zwiers, H. (1991) J. Neurochem. 56, 1095–1106.
[6] Shearman, M.S., Shinomura, T., Oda, T. and Nishizuka, Y. (1991) J. Neurochem. 56, 1255–1262.
[7] Moss, D.J., Fernyhough, P., Chapman, K., Baizer, L., Bray, D. and Allsopp, T. (1990) J. Neurochem. 54, 729–736.
[8] Skene, J.H.P. (1989) Annu. Rev. Neurosci. 12, 127–156.
[9] Sheu, F.-S., Marais, R.M., Parker, P.J., Bazan, N.G. and Routtenberg, A. (1990) Biochem. Biophys. Res. Commun. 171, 1236–1243.
[10] Sekiguchi, K., Tsukuda, M., Ase, K., Kikkawa, U. and Nishizuka, Y. (1988) J. Biochem. 103, 759–765.
[11] Yoshimura, N., Kikuchi, T., Sasaki, T., Kitahara, A., Hatanaka, M. and Murachi, T. (1983) J. Biol. Chem. 258, 8883–8889.
[12] Ito, A., Saito, N., Hirata, M., Kose, A., Tsujino, T., Yoshihara, C., Ogita, K., Kishimoto, A., Nishimoto, A., Nishizuka, Y. and Tanaka, C. (1990) Proc. Natl. Acad. Sci. USA 87, 3196–3199.
[13] Ase, K., Saito, N., Shearman, M.S., Kikkawa, U., Ono, Y., Igarashi, K., Tanaka, C. and Nishizuka, Y. (1988) J. Neurosci. 8, 2850–3856.
[14] Kishimoto, A., Saito, N. and Ogita, K. (1991) Methods Enzymol. 200, 447–454.
[15] Krueger, B.K., Forn, J. and Greengard, P. (1977) J. Biol. Chem. 252, 2764–2773.
[16] Yasuda, I., Kishimoto, A., Tanaka, S., Tominaga, M., Sakurai, A. and Nishizuka, Y. (1990) Biochem. Biophys. Res. Commun. 166, 1220–1227.
[17] Tanaka, Y., Miyake, R., Kikkawa, U. and Nishizuka, Y. (1986) J. Biochem. 99, 257–261.
[18] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[19] Kishimoto, A., Mikawa, K., Hashimoto, K., Yasuda, I., Tanaka, S., Tominaga, M., Kuroda, T. and Nishizuka, Y. (1989) J. Biol. Chem. 264, 4088–4092.
[20] House, C. and Kemp, B.E. (1987) Science 238, 1726–1728.
[21] Kiley, S.C. and Jaken, S. (1990) Mol. Endocrinol. 4, 59–68.
[22] Wolf, M., Burgess, S., Misra, U.K. and Sahyoun, N. (1986) Biochem. Biophys. Res. Commun. 140, 691–698.
[23] Apel, E.D., Byford, M.F., Au, D., Walsh, K.A. and Storm, D.R. (1990) Biochemistry 29, 2330–2335.