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ζ-RELATED PROTEIN KINASE C IN NUCLEI OF NERVE CELLS

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To determine whether or not PKC is present in the nuclei of nerve tissue we made use of biochemical and immunocytochemical techniques. A 219-fold purification of rabbit brain nuclear protein kinase C was achieved by sequential steps of Triton X-100 extraction of isolated nuclei, DEAE-cellulose, Butyl-toyopearl and hydroxylapatite chromatography. The major peak of protein kinase C activity was eluted from the hydroxylapatite column at the KPO_4 concentration of 0.3 M. Both Ca^{2+} and Ptd Ser were required for stimulation of the enzyme. Immunoblot analysis revealed that the kinase fraction was immunoreactive with a polyclonal antibody, PC-ζ, that had been raised against a peptide synthesized according to the deduced sequence of rat ζ protein kinase C. Light-microscopy revealed strong immunoreactivity in the nuclei of Purkinje cells in cerebellum and pyramidal cells in the rat cerebral cortex. These observations suggest that a ζ-related protein kinase C is present in the nuclei of nerve cells.

Protein kinase C represents one of the major systems of protein phosphorylation which is apparently involved in cell growth, differentiation and neoplastic transformation (1-3). A number of nuclear proteins including histones, lamin B, matrix proteins, and DNA topoisomerase II are phosphorylated by protein kinase C (4-9). Moreover phorbol esters affect the transcription of many genes, including c-fos, plasminogen activator, ornithine decarboxylase, and interferon, however, the physiological nuclear substrates have yet to be identified (10-13). Initially it was documented that protein kinase C is either absent or is poorly represented in the nucleus (14), and the TPA-induced phosphorylation of intranuclear proteins such as histones and gene expressions may result from the action of a secondary kinase. More recently, Huang and colleagues presented biological and immunological evidence of the existence of protein kinase C in the rat liver nuclei (15, 16). We report here the isolation of protein kinase C isozymes from nuclei of the rabbit brain. Immunological studies done using protein

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kinase C isozyme-specific antibodies suggest the possibility that ζ related protein kinase C is specifically expressed in the nuclei of nerve cells.

MATERIALS AND METHODS

Preparation of antibodies. A synthetic peptide based on the deduced sequence of rat ζ protein kinase C (17 amino acids of carboxyl end) (17), coupled with hemocyanin was injected subcutaneously into New Zealand White rabbits together with Freund's complete adjuvant. After two subsequent immunizations at two week intervals, the rabbits were bled and polyclonal antibodies were obtained using an affinity chromatographic technique on an antigen-coupled CNBr-activated Sepharose 4B.

Preparation of rabbit brain nuclei. Rabbit brains weighing 37 g were removed and cut into small pieces in ice-cold buffer containing 1.3 M sucrose, 1.0 mM $MgCl_2$, 1 mM DTT, 0.001% leupeptin, 0.001% pepstatin, 10 mM potassium phosphate buffer, pH 6.8. The small pieces were homogenized in 200 ml of the same buffer in a Potter-Elvehjem homogenizer with six strokes at 1000 rpm. The procedure for isolation of nuclei was as described by Masmoudi et al. (16). The homogenate was filtered and centrifuged at 1000 x g for 15 min (after carefully removing the supernatant), the pellet was resuspended in 2.4 M sucrose, 1.0 mM $MgCl_2$, 1 mM DTT, 0.001% leupeptin, 0.001% pepstatin, 10 mM potassium phosphate buffer, pH 6.8. The suspension was centrifuged at 100,000x g for 1 hr. The floating lipids and other cellular components were removed. The pellet was washed and re-centrifuged with the same medium at 100,000 x g for 1 hr. The nuclear pellet was suspended in a buffer containing 0.25 M sucrose, 0.5 mM $MgCl_2$, 1 mM DTT, 0.001% leupeptin, 0.01% pepstatin, 20 mM Tris-HCl, pH 7.5, and centrifuged at 1000 x g for 10 min. The washing was repeated twice to obtain a white nuclear pellet. The pellet was then suspended in 60 ml of buffer (A) (10% glycerol, 1 mM DTT, 0.001% leupeptin, 0.001% pepstatin, 2 mM EDTA, 20 mM Tris-HCl, pH 7.5) containing 1% Triton X-100. The suspension was sonicated six times, 10s each, with a 1-min interval period in between two sonications, incubated for 30 min on ice, and centrifuged at 100,000 x g for 30 min. The resulting supernatant was termed "nuclei fraction".

Enzyme assay and determinations. Protein kinase C activity was assayed in a reaction mixture conatining in a final volume 0.1 ml, 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 μg/ml Pst Ser, 0.1 mM Ca^{2+} or 0.2 mM EGTA, 0.2 mg/ml H1 Histone and 10 μM [γ-^{32}P]ATP (1.0 x 10^6 cpm). The incubation was carried out at 30°C for 5 min and was terminated by the addition of ice-cold 20% trichloroacetic acid followed by 100 μg of bovine serum albumin as a carrier protein. The mixture was centrifuged at 3000 rpm for 15 min and the pellet was washed in ice-cold 5% trichloroacetic acid solution. The final pellet was dissolved in 0.5 ml of 1N NaOH and the radioactivity measured in a liquid scintillation counter.

Immunoblot of protein kinase C. Immunoblotting was carried out as described by Towbin et al. (18). The nuclei fraction and the partially purified protein kinase C from a hydroxylapatite column were separated by 10%-SDS-polyacrylamide gel electrophoresis followed by electrophoretic transfer to a nitrocellulose membrane. After blocking with 5% skim milk/PBS, the membrane was incubated with a polyclonal antibody to ζ subspecies of protein kinase C for 1 hr, and with horseradish peroxidase-conjugated anti rabbit IgG (DAKO) for 1 hr. Following each incubation, the membrane was washed extensively with PBS containing 0.05% Tween 20 or PBS. The immunoreactive band was visualized by making use of a solution of DAB (50 mg DAB, 0.03% H_2O_2 in 100 ml PBS).

Immunohistochemical procedures. Male adult rat was anesthesized with an iv injection of pentobarbiturate (Nembutal) at 30 mg/kg. The brain was removed after 10 min perfusion with phosphate-buffered paraformaldehyde, trimmed of adventitia, and cut into small pieces. The tissue was immersed in the same fixatives for 3 hr. and washed with PBS containing 0.1 M Lysine and 10%, 15%, or 20% Sucrose for 3 hr., respectively. The tissue frozen in liquid nitrogen was sectioned at 6 μm, using cryostat, and placed on slide glass. The sections were exposed to 10% normal horse serum in PBS for 1 hr and then incubated with a purified antibody, PC-ζ (20 μg/ml) for 2 hr. at room temperature. The immunostaining procedures were carried out as described by Sternberger et al. (19), using a DAKO universal PAP kit. Staining specificity was assayed by replacing antibodies with the IgG fraction derived from nonimmune rabbit serum.

RESULTS

The major protein kinase C isozyme was purified by a procedure including ion-exchange, hydrophorbic, and hydroxylapatite column chromatography. The nuclei fraction (60 ml, 33.7 mg of protein) was applied to a DEAE-cellulose column (ø2 x 17 cm) preequilibrated with buffer A. The column was washed with 500 ml of the same solution. The enzyme was eluted from the column by application of a 400 ml linear concentration gradient of NaCl (0.0-0.5 M) in buffer A at a flow rate of 30 ml/hr. Fractions of 4 ml were collected and each was assayed for protein kinase, in the absence or presence of Ca^{2+}·Ptd Ser. The elution profile obtained was essentially the same as that for the whole rat brain (14). The DEAE-eluted protein kinase C fraction (110 ml, 6.6 mg of protein) was dialyzed against buffer A containing 1 M NaCl and the dialyzed fraction was applied to a Butyl-Toyopearl 650 M column (ø5 x 0.8) previously equilibrated with buffer A containing 1 M NaCl. The column was washed with 200 ml of the same buffer and the enzyme was eluted with buffer A containing 0.1 M NaCl at a flow rate of 180 ml/h. Seventy ml of active fraction (1.5 mg of protein) was pooled as kinase fraction. Butyl-Toyopearl elutes containing protein kinase C activity were then dialized against 0.02 M KPO_4 buffer and loaded onto a hydroxylapatite column (ø0.8 x 35 cm). Protein kinase C was eluted using 0.02 - 0.6 M KPO_4 gradient from the hydroxylapatite column at a flow rate of 30 ml/h after 30 ml wash with buffer A. Approximately 65 percent of protein kinase C activity eluted at 300 mM KPO_4 (Fig. 1). Other minor peaks of protein kinase C eluted KPO_4 concentrations at 80 and 140 mM. Pooling of the major peak fractions yielded a protein kinase C preparation, that has specific activity of 71 nmolP/min/mg protein. These results of 3 steps of column chromatography are summarized in Table 1. The major 80 kDa band in Fig. 1 correlated with the protein kinase C activity. Immunoblot analysis of the kinase fractions obtained through hydroxylapatite column chromatography with polyclonal antibodies that had been raised against a synthesized peptide of a deduced sequence, 476-492 of rat brain protein kinase C ζ revealed an immunoreactive band with a

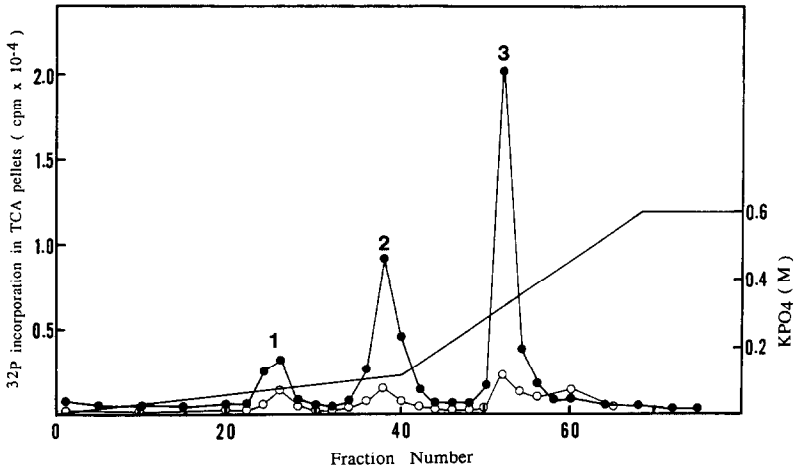

Fig. 1. Elution profile of protein kinase C from a hydroxylapatite column.
Protein kinase C fraction eluted from a Butyl Toyopearl column was dialyzed against 0.02 M KPO_4 buffer, pH 7.5, containing 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, and 10% glycerol before application to a hydroxylapatite column (ø0.8 x 35 cm). The enzyme was eluted with a KPO_4 gradient (0.02-0.3 M) (-) delivered by a gradient GP-250 PLUS and P-500 pumps (Pharmacia) at a flow rate of 30 ml/h. Fractions of 1 ml were collected and 10 µl of the fraction was used for the measurements of protein kinase activity with Ca^{2+}·Ptd Ser (●) or with EGTA (○). The three peaks of activity were designated peak 1, 2, and 3.

molecular mass of 80,000 kDa (Fig. 2). Other minor peaks eluted at 80 or 140 mM KPO_4 were not immunoreactive with this antibody.

The purified enzyme requires phosphatidylserine and calcium for histone H1 phosphorylation, but no further activation was observed by adding TPA (Table 2). A light-microscopic study of the rat brain was performed (Fig. 3A). In the neocortex, nuclei of pyramidal cells showed strong immunoreactivity and neuronal somata and dendrites were weakly stained. In the cerebellum (Fig. 3B), Purkinje cells and granular cell layers

Table 1. Steps of partial purification

Fraction	Volume ml	Total protein mg	Activity pmol P/min	Specific activity n mol P/min/mg	Purification -fold	Yield %
Nuclei fraction	60	33.7	10960	0.33	1	-
DEAE-eluted	110	6.6	28526	4.32	13.3	100
Butyl Toyopearl-eluted	70	1.51	24266	24.18	73.27	85
Hydroxylapatite-eluted (peak 3 fraction)	5	0.066	4710	71.36	219.4	16

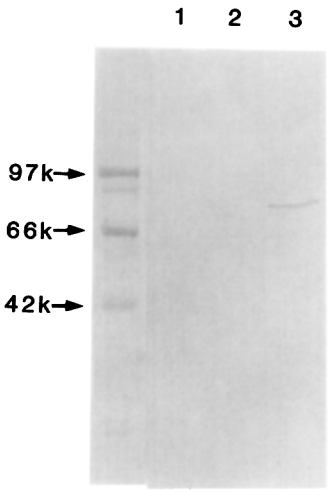

Fig. 2. Immunoblot analysis of peaks 1, 2, and 3 eluted from a hydroxylapatite column by anti-ζ polyclonal antibody, PC-ζ.
Fractions of No. 26 (lane 1), 38 (lane 2), and 52 (lane 3) (10 μl each) of Fig. 1 were separated by SDS-PAGE (10% gel) and electrophoretically transferred to a nitrocellulose membrane. The 80 kDa immunoreactive band was detected by DAB after incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG.

were strongly stained. Most of the Purkinje cells showed intense immunostaining in the nuclei and weak immunostaining in the cytosol. The nuclei of some granular cells also showed immunoreactivity.

DISCUSSION

Protein kinase C was originally purified from rat brain "cytosol" as a monomeric protein with an apparent molecular mass of 80 kDa (14).

Table 2. Stimulation of purified protein kinase C activity from the nuclei

Condition	Protein kinase activity (pmol P/min)
Ca^{2+} + Ptd Ser + TPA	5.00 (100%)
Ca^{2+} + Ptd Ser	4.33 (87%)
EGTA + Ptd Ser + TPA	2.76 (55%)
EGTA + Ptd Ser	1.55 (31%)
Ca^{2+}	0.63 (13%)
EGTA	0.59 (12%)

Purified protein kinase C from the nuclei of rabbit nerve cells was used to measure protein kinase activity in a basic mixture (25 mM Tris-HCl, pH 7.5 / 5 mM $MgCl_2$ / 10 μM [γ-^{32}P]ATP / 0.2 mg/ml of H1 Histone) plus the indicated compounds in the following concentrations: Ca^{2+} (0.1 mM), Ptd Ser (50 μg/ml), TPA (100 mM), and EGTA (2 mM).

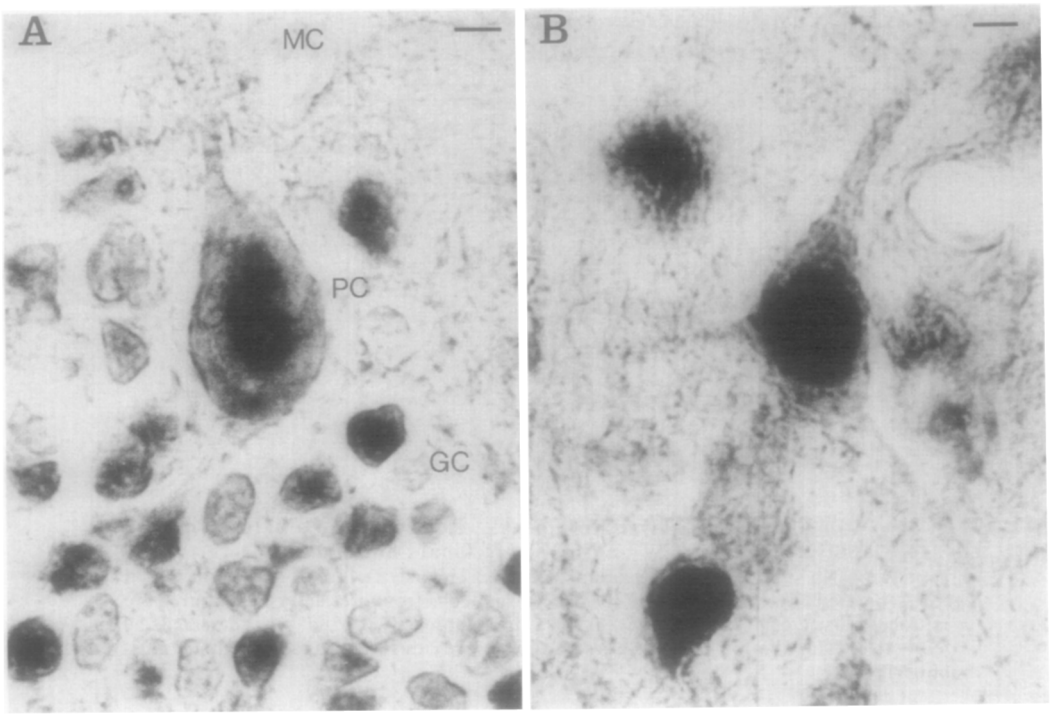

Fig. 3. Light micrographs (A) of a coronal section of the rat cerebellar cortex.
Premotor neocortex and (B) sagittal section rat cerebellar hemispherere. Immunoreactivity was relatively strong in the nuclei of pyramidal cells (A). Purkinje cells (PC) and granular cells (GC) (B). MC, molecular cells. Bar = 5 μm.

Molecular cloning analyses from several laboratories revealed shown that protein kinase C is a family of four subspecies having α, β1, β2, and γ sequences (20-24). Independent of the molecular cloning, protein kinase C was separated into three distinct types, types I, II, and III, on a hydroxylapatite column (25, 26). Using immunological and biochemical procedures, the α, β, and γ were shown to correspond to types III, II and I, respectively (27-29). Recently, additional cDNA clones, δ, ε, and ζ have subsequently been isolated and termed "nPKC' (30, 31). Although the existence of a 90 kDa protein molecule encoded by ε was suggested (31), protein molecules corresponding to δ or ζ have not been identified, except in expression experiments.

In our histochemical experiments using an anti ζ antibody, tissues such as Purkinje cells in the cerebellum and pyramidal cells in the cerebral cortex were clearly visible with a dense staining. These immunocytochemical related results showed a close agreement with our biochemical analysis, since the major activity peak of nuclear protein kinase C eluted from the hydroxylapatite column was immunostained. Western blots showed that our anti ζ antibody detected a 80 kDa protein in the rabbit brain nuclei. We

previously prepared monoclonal antibodies specific for the α, β, and γ isozymes and demonstrated differential expression of isozymes in rabbit cerebellum and other tissues (27). The immunocytochemical data also revealed that α, β, and γ isozymes are mainly localized in neuronal somata and dendrites of specific cells. Thus, ζ isozyme may play a specific role in nuclei.

Ono et al. reported that the ζ subspecies expressed in COS cells by transfection with cDNA showed phospholipid dependent activity but that the activity was not affected by the presence of Ca^{2+}, diolein, or TPA (17). The anti-ζ reactive enzyme purified from rabbit brain nuclei required both phospholipid and Ca^{2+}, but was independent of the presence of TPA. The molecular mass of the nuclear enzyme was 80 kDa, whereas the deduced molecular mass of ζ isozyme was 64 kDa. In rat liver nuclei, Masmoudi et al. (16) noted the presence of both 80 and 66 kDa protein kinase C. The 80 kDa protein kinase C of rat liver nuclei requires Ca^{2+} for activation of the enzyme, although the isozyme was not identified. Two types of protein kinase C isozymes, 64 kDa ζ and 80 kDa ζ-related enzyme may exist in rat liver nuclei. The exact role of nuclear protein kinase C in gene expression has yet to be determined. Precise knowledge of the location of protein kinase C-related enzymes at the site of the nucleus may provide a clue to the complex pathway of signal transduction involved in the control of growth.

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