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Purification of a Heat-Stable Activator for Phospholipase C-γ1 from Bovine Brain Cytosol and its Identification as Microtubule-Associated Protein Tau

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During the purification of phospholipase C (PLC)-γ1 which was over-expressed in HeLa cells transfected with a recombinant vaccinia virus carrying a complete cDNA sequence for PLC-γ1[1], we noticed the presence of a heat-stable activator in crude cytosolic extract of HeLa cells which markedly stimulated phosphatidylinositol (PI) hydrolysis, when reconstituted with purified PLC-γ1. Moreover, this putative factor was also found to be present in bovine brain cytosol. Subsequently, based on its ability to stimulate PI hydrolysis of PLC-γ1 as an assay, this activation factor was purified to homogeneity from bovine brain cytosol. It was purified by heat treatment, trichloroacetic acid precipitation, and successive chromatographic steps on DEAE-5PW, phenyl-5PW, and heparin-5PW HPLC columns. The purified protein, as seen on SDS-PAGE, consisted of 4 or 5 closely spaced bands of apparent molecular weight between 48 and 62 kDa. However, on gel filtration chromatography on TSK-G3000SW HPLC, the estimated molecular weight was revealed to be approximately 350 kDa.

The purified activator was identified as a microtubule-associated protein tau by electroelution from an SDS-polyacrylamide gel, partial peptide mapping by Staphylococcal V_8 protease, and amino acid sequencing of cyanogen bromide cleaved peptides. The identity of the activator was re-confirmed by an immunoblotting using a specific anti-tau monoclonal antibody. In addition, reconstituting PLC-γ1 with tau protein purified by an alternative method described elsewhere[2] resulted in the same magnitude of activation.

Tau protein mediated activation of PLC isozymes was calcium dependent. Isozyme specific activation of PLCs in 0.1% deoxycholate substrate appeared to be preferential toward PI hydrolysis. The magnitude of activation of PLC isozymes in PI substrate were PLC-γ_1 > PLC-γ_2 > PLC-δ_1 > PLC-β_1 in decreasing order. Approximately 200 nM concentration of tau-protein produced 15.5-, 5.4-, 4.2-, and 1.6-fold increase in activity of these enzymes, respectively, in the presence of 1 mM free calcium ion.

Key Words: Tau protein, Activator, Phospholipase C-γ1

INTRODUCTION

It is well recognized that a variety of hormones, neurotransmitters, growth factors, ligands as well as antigens, through a receptor mediated process, produce rapid hydrolysis of phosphatidyl inositol 4,5-biphosphate in order to generate two principal second messengers; namely, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG)[3~7]. IP3 binds to a specific receptor and causes a rapid rise in local calcium concentration by mobilizing calcium from intracellular storage sites such as endoplasmic reticulum[3~5]. DAG, in turn, activates protein kinase C which causes phosphorylation of numerous cellular components essential for exihibiting mitogenic activity, growth, and differentiation[8,9]. PLC plays a central role in this signal transduction pathway by catalyzing

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the hydrolysis of phosphatidylinositol and it is one of the most commonly used transmembrane signaling mechanisms in a wide range of tissues and cells[10].

Evidences suggest that there are at least two major pathways known, by which activation of PLC can be achieved. One pathway of activation is through receptors which has intrinsic tyrosine-specific protein kinase activity as seen in epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) receptors[11,12]. The ligand binding to these receptors causes autophosphorylation of specific tyrosine residues of their cytoplasmic tyrosine kinase domain, which in turn phosphorylates specific tyrosine residues on PLC-γ_1 and -γ_2 isozymes, causing them to be activated[6,7,13] and to be translocated to the cytoskeletal compartment of the cell[14~16]. Alternative pathway known is activation through receptors which has seven transmembrane spanning helices. Stimulation of these receptors may cause conformational changes that are conveyed through the membrane to the intra-cytoplasmic loops of the receptor, eliciting the activation of guanine-nucleotide binding protein (G-protein), there by making them GTP bound. GTP bound α subunit of G-protein q family[17~21] or more recently, βγ subunit of the G-protein has been shown to activate β family of phospholipase C[22~25].

During the purification of PLC-γ_1 from HeLa cells transfected with recombinant vaccinia virus carrying full cDNA for PLC-γ_1, we had observed that, in regards to the relative amount of PLC-γ_1 present, PLC activity in crude extracts was consistently higher compared to that of the purified enzyme. We have also made the same observation in bovine brain cytosol and here we report purification and identification of a constitutively active, tyrosine kinase independent activator of PLC-γ from bovine brain, identified as a microtubule-associated protein tau.

MATERIALS AND METHODS

PLC activity and PLC activation factor assay

Materials: [^{3}H] PI and [^{3}H] PIP_2 were obtained from Du Pont-New England Nuclear, PI from Sigma, PIP_2 from Boehringer Mannheim.

PLC activity assay in PI (or PIP_2) substrate: Unless otherwise specified, PLC activity assay performed during the course of the activator purification was done using [^{3}H] PI as the substrate in 1 mM (or 10^{-6} M) free calcium concentration. PI- (or PIP2) hydrolyzing activity was assayed in either a 200 μl (or 100 μl) reaction mixture containing 150 μM PI (or 30 μM PIP_2), 20,000 cpm ^{3}H PI (or 30,000 cpm [^{3}H] PIP2), 50 mM Hepes (pH7.0), 3 mM $CaCl_2$, 2 mM EGTA, 0.1% sodium deoxycholate and an enzyme source. Incubations were performed at 37℃ for 5 minutes (information in parentheses indicate the conditions of PLC activity assay for PIP2 substrate).

In the assay described above, the substrate mixture with other lipids dissolved in chloroform solution, was dried under a stream of nitrogen gas, resuspended in 50 mM Hepes (pH 7.0) and 0.1% deoxycholate by sonication and used for the assay. After the incubation the reaction was terminated by adding 1 ml of chloroform : methanol : HCl (100 : 100 : 0.6, v/v/v) followed by an additional 0.3 ml of 1 M HCl containing 5 mM EGTA. Following termination, after a brief centrifugation, 0.5 ml of upper aqueous phase of chloroform-methanol extraction was collected and counted for ^{3}H radioactivity by liquid scintillation counter spectroscopy for PLC activity.

PLC activator activity: The activity of the activator protein was measured by its ability to stimulate PI hydrolysis of PLC. Therefore the activator activity was assayed in an essentially same manner as the PLC activity. However, along with a known purified enzyme source, either the column fractions or purified activator protein (0.5 to 1.5 μg protein) was added to the assay. The reaction mixture was then treated in the same way as PLC assay described above and ^{3}H radioactivity counted. The resulting counts from the samples containing activator were compared to the control which contained only a PLC enzyme source. The amount of PLC enzyme in the assays was carefully adjusted to maintain unstimulated basal count in the range between 500 and 1,200 cpm in an attempt to include the reaction in the linear portion of the curve as much as possible.

Purification of PLC isozymes: PLC isozymes used in this experiment was purified from the HeLa cells which had been transfected with recombinant vaccinia virus containing entire coding sequence of each of the isozymes used in the experiment (PLC-β_1, PLC-γ_1, PLC-γ_2, and PLC-δ_1), as described elsewhere[1].

Activation factor in HeLa cell and bovine brain cytosol: HeLa cell and bovine brain cytosolic extracts (about 0.4

~4.0 ug protein) were mixed with purified PLC-γ_1 in order to prove the presence of an activation factor. The cytosolic fractions containing about 400 μg protein was subjected to various treatments such as heating at 95℃ for 5 min, filtration through Centricon-30 microconcentrator (Amicon, Beverly, MA), nuclease and protease digestion including DNase (0.02 μg/ul), RNAse (0.02 μg/μl), trypsin (0.4 μg/μl), and chymotrypsin (0.2 μg/μl) (Worthington Biochemical Corp., Freehold, NJ). The resulting samples after the treatments were again reconstituted with purified PLC-γ_1 in activator assay in order to determine the nature of the activator.

Purification procedures : All manipulation were performed at 4~6℃ in a cold room or on ice unless otherwise indicated. The activator activity was monitored by measuring [^{3}H] PI hydrolysis throughout the purification procedures. This was done by mixing purified PLC-γ_1 (about 10~50 ng) and column fractions (0.5 to 3.0 μl) as described above.

(i) Preparation of bovine brain cytosolic extracts : Fifteen bovine brains (4.5 kg), freshly acquired from a local slaughter house were mixed with 10 liters of homogenization buffer containing 10 mM Tris (pH 7.4), 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol (DTT), 2 μg/ml leupeptin, 2 μg/ml aprotinin in a Waring blender. The homogenate was then centrifuged at 1,000×g for 10 minutes. The resulting supernatant was centrifuged further at 13,000×g for 1 hour. The soluble fraction (supernatant) was then brought to 60% saturation with ammonium sulfate. After 30 min, it was precipitated by centrifugation for 30 min at 13,000×g. The resulting precipitate (about 600 ml) was saved and frozen at −70℃ for purification of the activator protein.

(ii) Purification of the activator protein by its physical property : The frozen ammonium sulfate precipitate of the bovine brain cytosol was thawed and diluted by adding two-volumes of distilled water. Taking the advantage of its heat stability, suspension was then heated at 95℃ for 5 min and was centrifuged at 1,000×g for 15 min. The resulting supernatant was filtered through Whatman #1 filter paper. The filtrate was then brought to 2% trichloroacetic acid and centrifuged at 17,000×g for 10 min. The supernatant was discarded and the resulting pellet was scraped thoroughly from the centrifuge tubes. Immediately, the sample was resuspended by adding a small volume of 1 M Tris (pH 8.4) buffer and dialyzed extensively against the dialysis buffer (50 mM Tris, 1 mM EGTA, and 0.1 mM DTT). After dialysis, any insoluble materials were removed by centrifugation at 75,000 rpm for 10 min. Approximately 40 ml of the sample containing 420 mg protein with conductivity equivalent to that of 60 mM NaCl was obtained and subjected to subsequent chromatographic steps.

(iii) Ion exchange HPLC on preparative DEAE-5PW column : The sample from previous step was divided into two equal parts and applied twice onto a preparative TSKgel DEAE-5PW HPLC column (21.5×150 mm, TosoHaas, Montgomeryville, PA) which had been equilibrated with 50 mM Tris (pH7.4), 1 mM EGTA, 0.1 mM DTT. The protein was then eluted at a flow rate of 5 ml/min by successive application of the gradients starting with equilibration buffer for 5 min, and an increasing linear NaCl gradient of 0 to 0.3 M for the next 35 min, followed by a second increment of NaCl gradient from 0.3 M to 1 M in 5 min. The column was then washed with the buffer containing 1 M NaCl. 5 ml fractions were collected and assayed for the presence of the activator, and peak fractions (26 and 27) from two consecutive runs were pooled and saved for the next step.

(iv) Reverse phase HPLC on TSKgel phenyl-5PW column : Solid KCl was added to the pooled fractions (40 mg protein) from the previous step to give the final salt conentration of 3 M, and any insoluble material was removed by centrifugation. The clear supernatant was then injected into an analytical TSKgel phenyl-5PW HPLC column (7.5×75 mm, TosoHaas) which had been equilibrated with 20 mM Hepes (pH 7.0), 3 M NaCl, 1 mM EGTA, and 0.1 mM DTT. The proteins were eluted at a flow rate of 1 ml/min by applying equilibration buffer for 5min, a decreasing linear NaCl gradient of 3 M to 1.2 M in 10 min, followed by an another linear decrease from 1.2 M to 0 M in next 25 min. The column was then washed with NaCl free buffer. 1 ml fractions were collected and assayed for the activator activity, and peak fractions (26 and 27) were pooled. The pooled sample was then washed with 20 mM Hepes (pH 7.0), 1 mM EGTA, 0.1 mM DTT in a Centriprep-30 (Amicon) to lower the salt concentration below 0.1 M. The part of the sample (6 μg/lane) was mixed with 5× Laemmli's sample buffer, heated for 5 min, and subjected to an analysis on 8% SDS-polyacrylamide gels.

(v) HPLC on TSKgel heparin-5PW column : The desalted fractions pooled from the previous phenyl column (4

mg protein) was then applied to a TSKgel heparin-5PW column (7.5×75 mm, TosoHaas) that had been equilibrated with 20 mM Hepes (pH 7.0), 1 mM EGTA, 0.1 mM DTT. Proteins were eluted at a flow rate of 1.0 ml/min by a gradient consisting of equilibration buffer for 15 min, linear increase in NaCl concentration from 0 to 0.64 M during 40 min, and final rapid increase in gradient form 0.64 to 1.0 M for 10 min. Then, the column was washed with 1 M NaCl 1 ml fractions were collected and were assayed for the activator activity. Peak fractions (32 and 33) were pooled and concentrated. The samples were then aliquoted, part of the sample was analyzed on 8% SDS-polyacrylamide gel (4 μg/lane), and the remainder stored at −70℃ for future use.

Characterization and identification of the activator protein

(i) HPLC gel filtration chromatography : 120 μg of purifed activator protein was applied to a TSKgel G3,000-SW column (7.5×600 mm) that had been equilibrated with a solution containing 20 mM sodium phosphate (pH 7.0) and 100 mM NaCl. The proteins were eluted at a flow rate of 0.5 ml/min with the same buffer. Absorbance at 280 nm was monitored. The activity assay and the molecular weight estimation was made in reference to the molecular weight standard mixture (Bio-Rad, Hercules, CA).

(ii) Electroelution of the activator protein from SDS-PAGE gels : Purified proteins from heparin column (about 700 μg) was loaded onto a preparative SDS-polyacrylamide gels (8%, 3 mm thickness, single well comb). After the electrophoresis using constant current of 30 mA, the gels were stained lightly with Coomassie brilliant blue and each visualized protein bands were cut with razor blades. The proteins were subsequently electroeluted according to the protocols and apparatus (C.B.S. Scientific Co., Del Mar, CA) described by Hunkapillar et al[27]. Following the electroelution, Coomassie brilliant blue was extracted using isobutanol, and ice cold acetone precipitation was done to remove SDS[28]. Then, the electroeluted proteins were again reconstituted in an assay for the activator actvity to determine which protein band was responsible for the activation.

(iii) Partial peptide mapping of the activator : In situ peptide mapping of the activator protein in SDS-PAGE was done according to the methods described by Cleveland et al[29] with a slight modification. Briefly, the electroeluted proteins (5 μg each) were mixed with 5× sample buffer and 0.75 μg of Staphylococcus V_8 protease (Takara biochemical Inc., Berkeley, CA) was added. The samples were then left to stand at 37℃ for 30 min, loaded onto 10% SDS-polyacrylamide gel, and run at the constant current of 20 mA. After electrophoresis, the pattern of cleavage was visualized with silver stain and compared.

(iv) Cyanogen bromide cleavage peptide and amino acid sequencing : Purified activator protein from heparin column (20 μg) and electroeluted protein bands (10 μg each) were subjected to chemical cleavage by 100 mM CNBr in the presence of 70% formic acid for 16 hours. The reaction was quenched by adding excess methionine crystals to the reaction mixture. Following the cleavage, the samples were vaccuum dried and resuspended in 50 mM Tris (pH 8.4) solution. After the addition of 5× sample buffer and heating, the samples were visualized on 16% SDS-polyacrylamide gel with silver stain to examine their cleavage pattern. Purified activator from heparin column (150 μg) was also cleaved with CNBr and resulting peptides were either blotted onto PVDF membrane (Millipore, Bedford, MA) as described[29] or isolated on an C_{18} reverse phase HPLC column (4.6×250 mm, Vydac, Hesperia, CA). In reverse phase HPLC, the column was first equilibrated with 0.05% trifluoroacetic acid in water and the peptides were eluted at a flow rate of 1.0 ml/min by application of the equilibration buffer for 20 min, followed by a linear gradient from 0 to 50% (v/v) of acetonitrile in 0.05% trifluoroacetic acid for 50 min and a second linear increase of elution buffer from 50 to 100% in 10 min. The elution of the peptides was monitored by absorbance at 215 nm and peak fractions were collected manually. Four peptide bands cut-out from blotted PVDF membrane and two peaks isolated from C_{18} HPLC column were subjected to the amino acid analysis on an Applied Biosystems Model 477A Sequenator and the resulting amino acid sequence was searched from Swiss-Pro data files.

Confirmation of the activator as tau protein

(i) Immunoblotting of the activator : Both the purified activator and electroeluted eluted protein bands were blotted with a commercially available specific anti-tau monoclonal antibody (ICN Immunobiological, Lisle, IL). Purified activator and electroeluted bands were treated with 5× sample buffer, resolved by 8% SDS-PAGE, and transferred to the

nitrocellulose membrane. Nitrocellulose membrane was then incubated with specific anti-tau monoclonal antibody and visualized with alkaline phosphatase conjugated secondary antibody.

(ii) Purification of tau-protein using CM-Sephadex chromatography and perchloric acid precipitation : Another tau purification was done from fresh bovine brain by an alternative method described elsewhere[2] with a slight modification. The purification involved 45% ammonium sulfate fractionation, CM-Sephadex 50 (Pharmacia, Piscataway, NJ) column chromatography, and 2.5% perchloric acid precipitation. Tau protein purified in this way was dialyzed extensively to change the buffer to 50 mM Hepes (pH 7.4) to be used in PLC assay and was reconstituted with purified PLC-γ_1 for the activator activity.

Isozyme specificity and free calcium dependence of the PLC activation : Purified activator (tau protein) was reconstituted with various PLC isozymes (β1, γ1, γ2, and δ1) to test its isozyme specific effects in either PI and PIP2 substrate systems. Tau-protein (0.2 to 0.75 μg) was reconstituted with 10 to 75 ng of PLC isozymes depending on the enzymes and substrate systems used. The free calcium concentration was varied from 10^{-8} to 10^{-3} as described above[26].

RESULTS

Activation factor in HeLa cells and bovine brain cytosol: It was well recognized from our cummulative experience that, when purifying PLC-γ_1 from HeLa cells, crude cytosolic extract consistently showed 2 to 4 fold higher level of PI hydrolyzing activity compared to the purified PLC-γ_1. Moreover, in a serial dilution of the crude cytosolic extract,

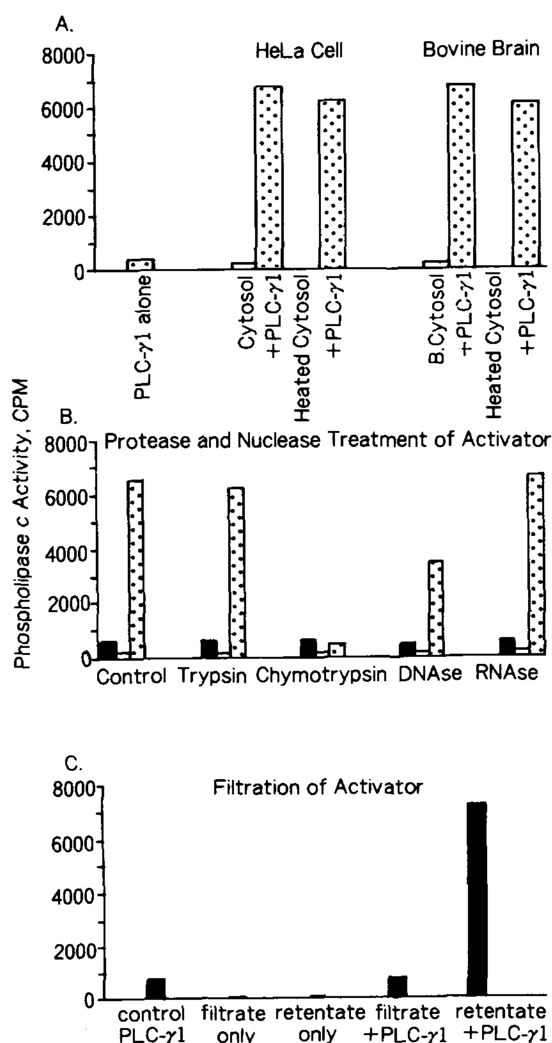


Fig. 1. Presence of activation factor in HeLa cells and bovine brain cytosol. Activator activity was tested in crude cytosolic extracts from HeLa cells (left) or bovine brain (right). Either fresh or heat treated for 5 min at 95℃, the cytosolic extracts containing 4 to 5 μg protein was mixed with purified PLC-γ1 (50 ng) in an PLC activity assay in PI substrate (A). *Filled bar*, PLC activity in cytosol or heated cytosol; and *hatched bar*, PLC-γ1 plus cytosol extracts. Heat treated cytosolic extract from bovine brain was treated with trypsin (0.4 μg/ul), Chymotrypsin (0.2 μg/μl), DNase (0.02 μg/μl), or RNase (0.02 μg/μl) at 37℃ for 1 hour, subsequently heated at 100℃ for 3 min to inactivate appropriate protease or nuclease, and finally reconstituted in an assay with PLC-γ1(B). *Filled bar*, control PLC-γ1 activity; *hatched bar*, PLC activity in treated cytosol; and *open bar*, PLC-γ1 plus treated cytosol extracts. Heat treated bovine brain cytosol was filtered in Centricon-30 microconcentrator at 1,500 x g for 20 min. The retentate by the filter (1.0 μg protein) or an equal volume of filtrate was reconstituted with PLC-γ1 for activation assay (C). In all three figures, PI substrate (150 μM) was used in a mixed micelle system with 0.1% deoxycholate for the PLC assay as described under "Experimental Procedures". The free Ca^{2+} concentration in the assay was 1 mM. The data shown are a mean of duplicate values and are a represenative of three similar experiments.

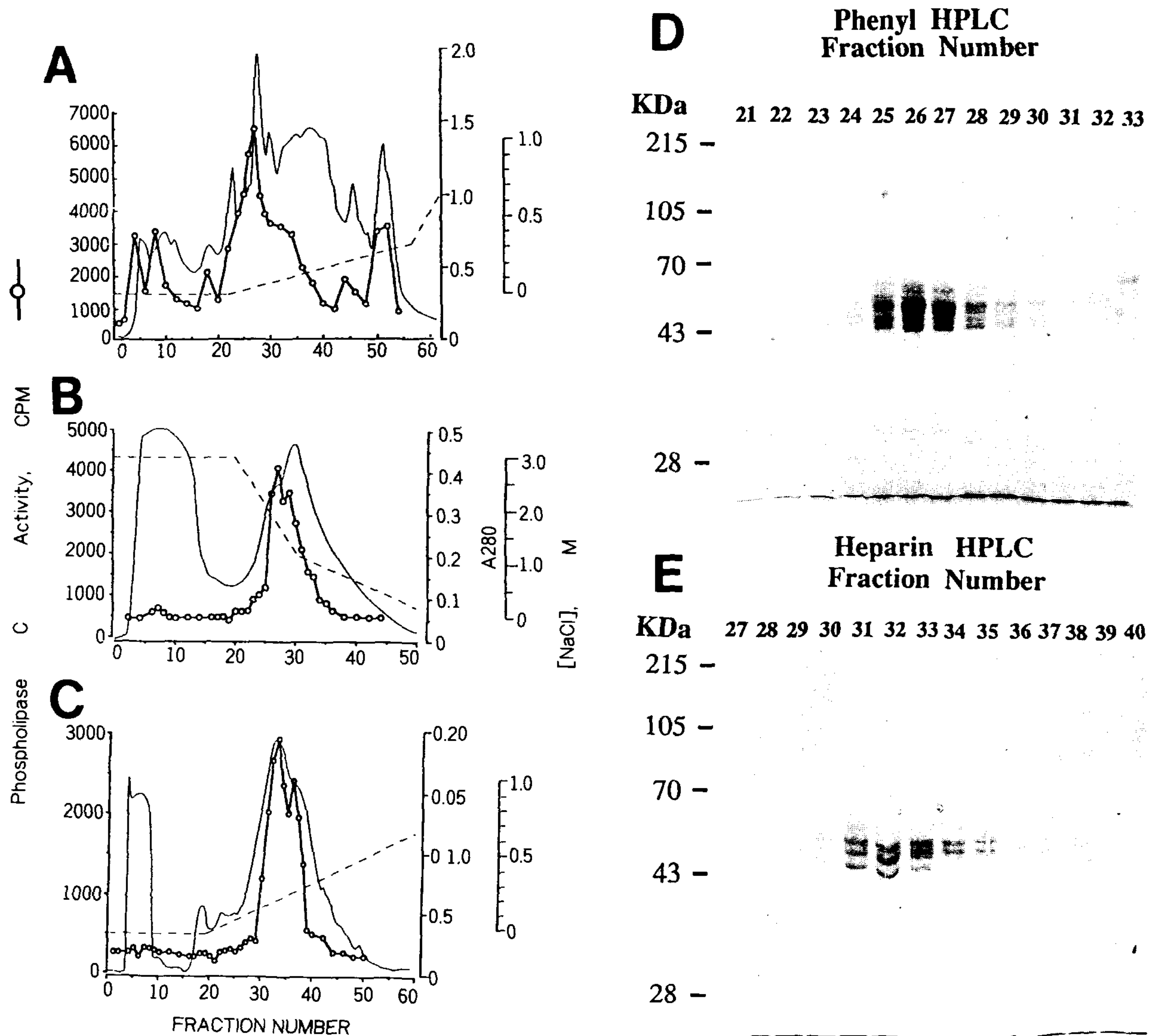


Fig. 2. Purification of the activator from bovine brain cytosol. Heat treated cytosolic fraction was purified by successive chromatographies on a preparative TSKgel DEAE-5PW HPLC column (A), a TSKgel phenyl-5PW HPLC column (B), and a TSKgel heparin-5PW HPLC column (C). The activator activity was assayed using [^{3}H] PI substrate. The peak fractions from the phenyl and heparin columns were resolved on 8% SDS-polyacylamide gel, and the proteins were stained with a Coomassie brilliant blue (D) and (E), respectively. The position of the molecular size standards is shown on the left.

the decline in the enzyme activity was non-linear, suggesting the presence of other factors which were being diluted along with PLC-γ_1 (unpublished data).

The crude cytosolic extracts from HeLa cells and also from bovine brain contained an activation factor which, when reconstituted in vitro with purified PLC-γ_1, can markedly stimulate PI hydrolyzing activity of the enzyme, 15-fold or more (Fig. 1A). This putative activation factor was heat stable retaining its original activity after heating for 5 min at 95℃. It was resistant to denaturation by trypsin, DNase, or RNase, and yet it seemed susceptible to chymotrypsin treatment (Fig. 1B). It appeared to be a macromolecule because it did not pass through the filter with a molecular weight cut-off of 30,000 Da (Fig. 1C).

Purification of activator protein : Taking the advantage of its heat stability, we were able to remove majority of

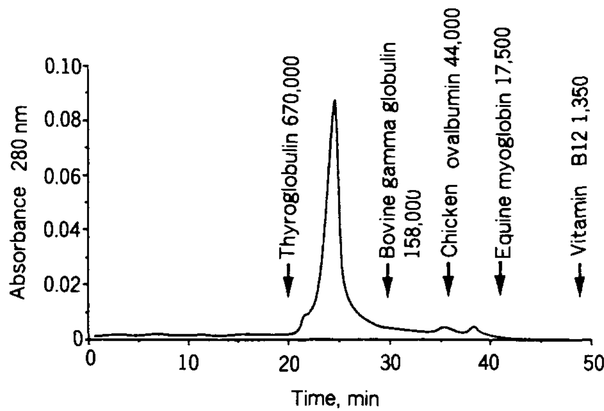
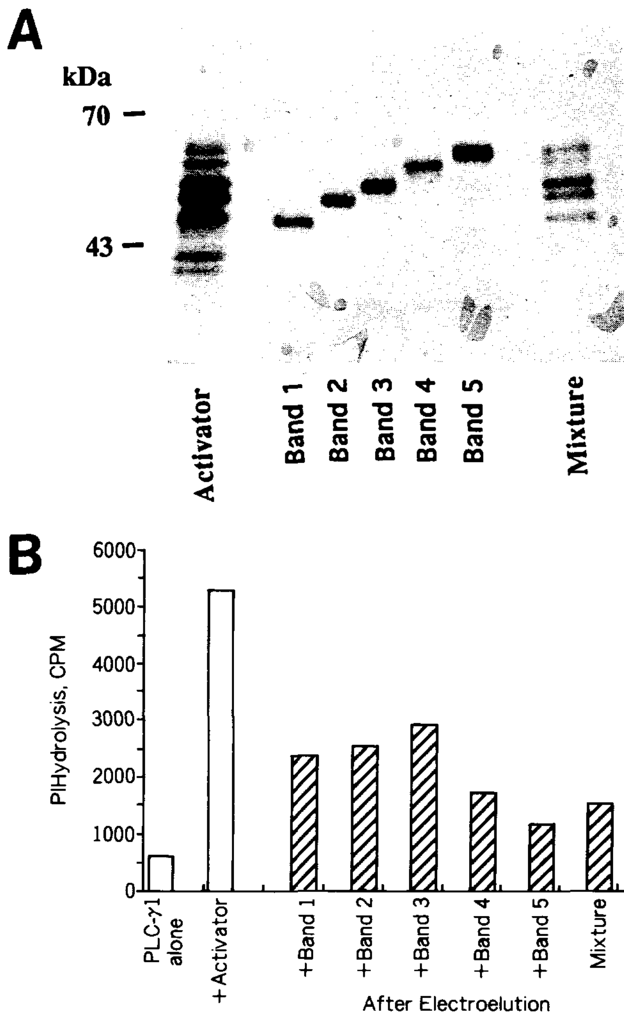


Fig. 3. Gel filtration chromatography of the activator. Purified activator (120 μg) was subjected to a gel filtration chromatography on TSKgel G3,000-SW HPLC column. The proteins were eluted at a flow rate of 0.5 ml/min with a buffer containing 20 mM sodium phosphate (pH7.0) and 100 mM NaCl. Absorbance at 280 nm was monitored. Elution positions for the molecular weight standard are marked by vertical arrows.

contaminating protein by heat treatment and yet preserve the activator activity. Initial preliminary study showed that this activator binds to a DEAE HPLC matrix. The activator activity eluted rather diffusely throughout but major portion of the activity (about 60%) was recovered in peak fractions which eluted at an apparent salt concentration of 0.15 M NaCl. Assessing from the activity of the activator, about 3 fold enrichment of the activator was achieved in this step. However, we were able to remove much of the contaminating proteins (380 mg) by this procedure (Fig. 2A).

In phenyl HPLC, activity of the activator was centered around the fractions 26 and 27 which eluted at NaCl concentration of about 2.4 M (Fig. 2B). The SDS-PAGE of the peak fractions showed 4 to 5 closely spaced protein bands with apparent molecular weight ranging from 48 to 62 kDa with about 2.5 kDa difference between each bands (Fig.2D).



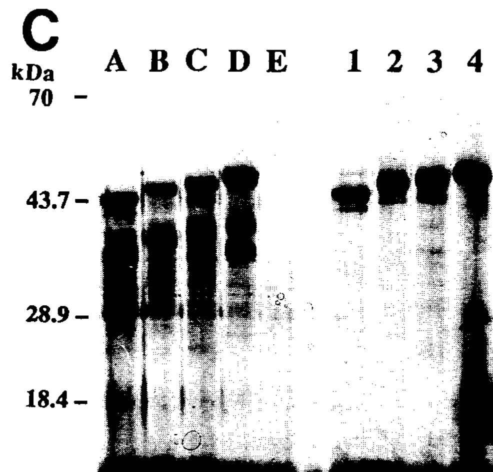


Fig. 4. Electroelution of protein bands from purified activator. Proteins from heparin column underwent electroelution from SDS-polyacylamide gels. Each electroeluted samples were mixed with 5X sample buffer and again separated by SDS-PAGE on 8% gel (A). From the *left*, AF; purified activator from heparin column (10 μg), 1to 5; each electroeluted bands (2.5 μg/lane) and the mixture of bands 1 through 5 on the *right*. Electroeluted bands were reconstituted in an assay with PLC-γ_1 to determine which band is responsible for the activation (B). Four elcetroeluted protein bands were partially cleaved with Staphylococcal V_8 protease and the cleavage pattern compared (C). 1 to 4 lanes on the right indicates undigested original protein and left 4 lanes show proteins cleaved with V_8 protease for 30 min at 37℃ prior to the electrophoresis.

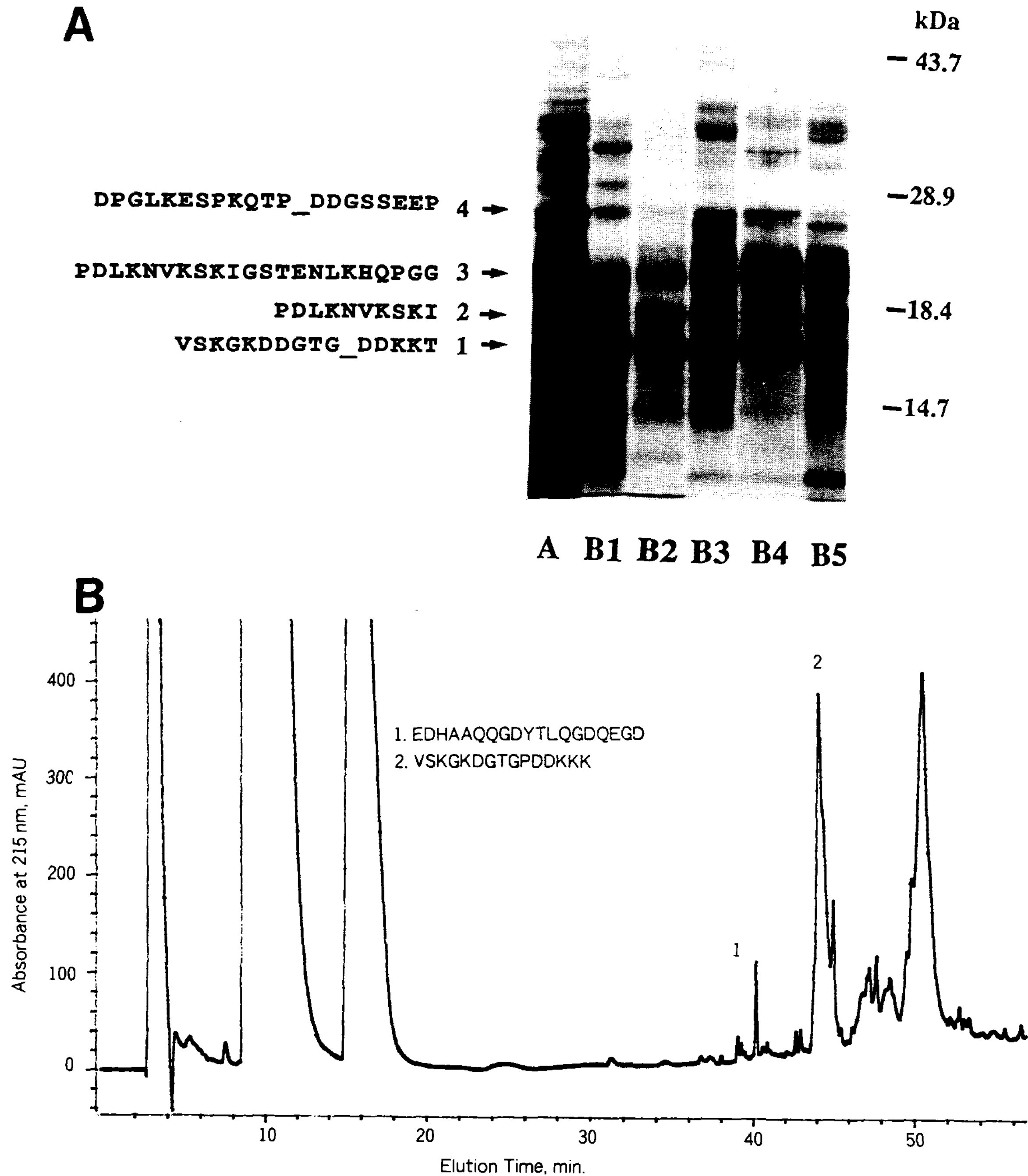


Fig. 5. Separation and sequencing of CNBr cleavage peptides from activator protein. Purified activator from heparin column (*lane* 1, original) and each of the 5 electroeluted bands (lanes 2 to 6) were cleaved with 100 mM CNBr in the presence of 70% formic acid for 16 hours. After the cleavage, each bands were again subjected to analysis on 16% SDS-polyacrylamide gel with silver stain (A). Four major bands digested from 60 μg of purified activator were blotted onto a PVDF membrane and was subjected to amino acid sequencing as shown on the left. The positions for the low range molecular weight standards are indicated on the right. Two peptide peaks, resulting from CNBr cleavage of 100 μg of purified activator, were isolated on C_{18} HPLC column and were subjected to the amino acid sequencing (B). Peptides that underwent amino acid sequencing are indicated by the numbers and their sequences are shown. The peptide sequences found from the activator were superimposed on the entire amino acid sequences of bovine brain tau2 isoform (C).

C

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                                   4. DPGLKESPKQTP_DDGSSEEP
1     MAEPRQEFDVMEDHAQGDYTLQDQEGDMDPGLKESPLQTPADDGSEEPGS
          C18 1. EDHAQGDYTLQDQEGD

51    ETSDAKSTPTAEDATAPLVDEGAPGEQAAAQAPAEIPEGTAAEEAGIGDT

                        1. VSKGKDGTG_DDKKT
101   SNLEDQAAGHVTQARMVSKGKDGTGPDDKKTKGADGKPGTKIATPRGAAP
                    C18 2. VSKGKDGTGPDDKK K

151   PGQKGQANATRIPAKTTPTPKTSPATMQVQKKPPPAGAKSERGESGKSGD

201   RSGYSSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSAAKSRL

        2&3. PDLKNVKSKIGSTENLKHQPGG_ V
251   QAAPGPMPDLKNVKSKIGSTENLKHQPGGGKVQIINKKLDLSNVQSKCGS

301   KDNIKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEK

351   LDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAEIV

401   YKSPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAKQGL
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Fig. 5. Continued

Further purification of the activator protein was achieved with an additional chromatogrpahy on heparin column. The peak activity was centered around the fractions 32 and 33 (Fig. 2C). SDS-PAGE analysis of the peak fractions gave similar results to that of phenyl column fractions. (Fig. 2E). Further attempts to separate each individual bands by chromatography on FPLC Mono Q column or C_{18} reverse phase HPLC column was unsuccessful (data not shown).

Gel filtration chromatography : On gelfiltration chromatography of the activator, the activity and the protein peak resolved as a single peak between thyroglobulin (M.W. 620,000 Da) and chicken ovalbumin (M.W. 150,000 Da) giving an apparent molecular weight of 350,000 Da (Fig. 3).

Electroelution and peptide mapping : Electroelution of the heparin fraction yielded 5 distinct bands of protein on SDS-PAGE analysis, free from contamination (Fig. 4A). After SDS removal and Coomassie blue extraction, these proteins were reconstituted in the activator assay. Although we were not able to fully recover its original activity due to several factors including denaturation during electroelution, contaminating impurities, and limited amount of proteins, but we were able to observe that all of these 5 bands had a remaining activity to stimulate PI hydrolysis of PLC-γ_1 to a varying degree, by the factor of 1.5- to 4- fold (Fig. 4B).

Partial peptide mapping : The partial digestion of electroeluted bands generated a single cleavage product from

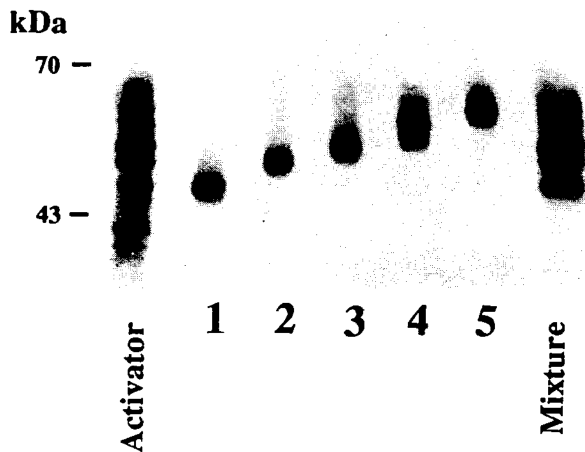

Fig. 6. Immunoblot analysis of the purified activator. Activator protein and its electroeluted components were separated on 8% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, incubated with a specific anti-tau monoclonal antibody, and visualized with alkaline phosphatase conjugated secondary antibody. Each lane received 200 ng of proteins per band. The positions for the molecular size marker is shown on the left.

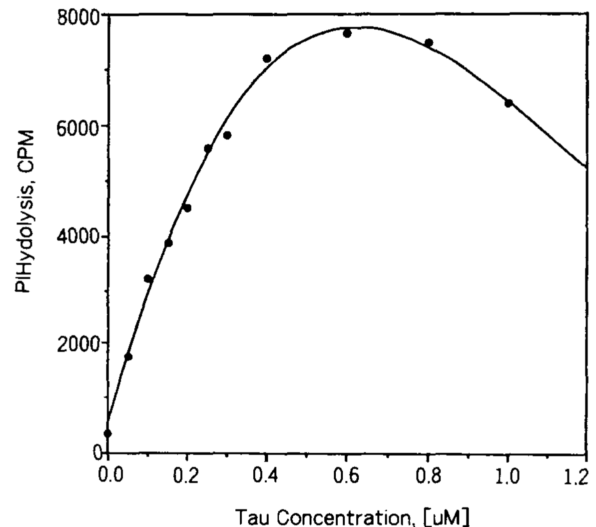

Fig. 7. Effect of tau concentration on PLC-γ1 activity. Various concentrations of tau protein was reconstituted with a fixed amount of PLC-γ1 (50ng). PLC activity was measured using [^{3}H]PI as the substrate with 10^{-6} free calcium concentration. The result shown are representative of three similar experiments.

each of the first 3 lanes of protein, and 2 fragments from protein on the lane 4 as the major cleavage product. All these fragments have migrated an equal distance from the original protein giving a "step-ladder" appearance, suggesting a possible presence of a common cleavage sites generating fragments with differing length (Fig. 4C).

Amino acid sequencing of CNBr peptide : CNBr cleavage of both the heparin fraction and that of each electroeluted-bands have yielded 4 major peptides common to all when analyzed on 16% SDS-polyacrylamide gel. The molecular weight of each fragments were approximately 16, 18.5, 22, and 26 kDa. From the results of peptide mapping and comparison of CNBr cleavage pattern, we came to a conclusion that all 5 bands may be closely related proteins. The amino acid sequences identified from four CNBr cleaved peptides transferred on PVDF membrane were as follows, (Fig. 5A)

Peptide 1: VSKGKDGTG_DDKKT

Peptide 2: PDLKNVKSKI

Peptide 3: PDLKNVKSKIGSTENLKHQPGG_V

Peptide 4: DPGLKESPKQTP_DDGSSEEP

Also, the amino acid sequnces from 2 peptide peaks isolated on C_{18} HPLC were shown below.(Fig. 5B)

Peak 1: EDHAQGDYTLQDQEGD

Peak 2: VSKGKDGTGPDDKKK

The peptide sequences were searched in Swiss-Pro data base and all of the peptide sequences either perfect or near pefect match for the bovine brain tau-2 isoform (Fig. 5C).

Confirmation of the activator as tau-protein : The immunoblot analysis of the activator with commercially available monoclonal anti-tau antibody, specifically recognized all 5 bands of the activator which we had purified (Fig. 6). Also tau protein purified by an alternative method[2] caused a marked activation of PLC-γ_1 when reconstituted (data not shown).

PLC isozyme specificity, calcium effects, and substrate preference in deoxycholate system : When activation of PLC-γ_1 was measured at 1 μM free calcium concentration (Fig.7), half maximal stimulation of 60 nM PLC-γ_1 was observed at approximately 300 nM of tau concentration in the assay. Nearly saturating concentration of Tau (600 nM) produced about 23-fold stimulation of PI hydrolyzing activity

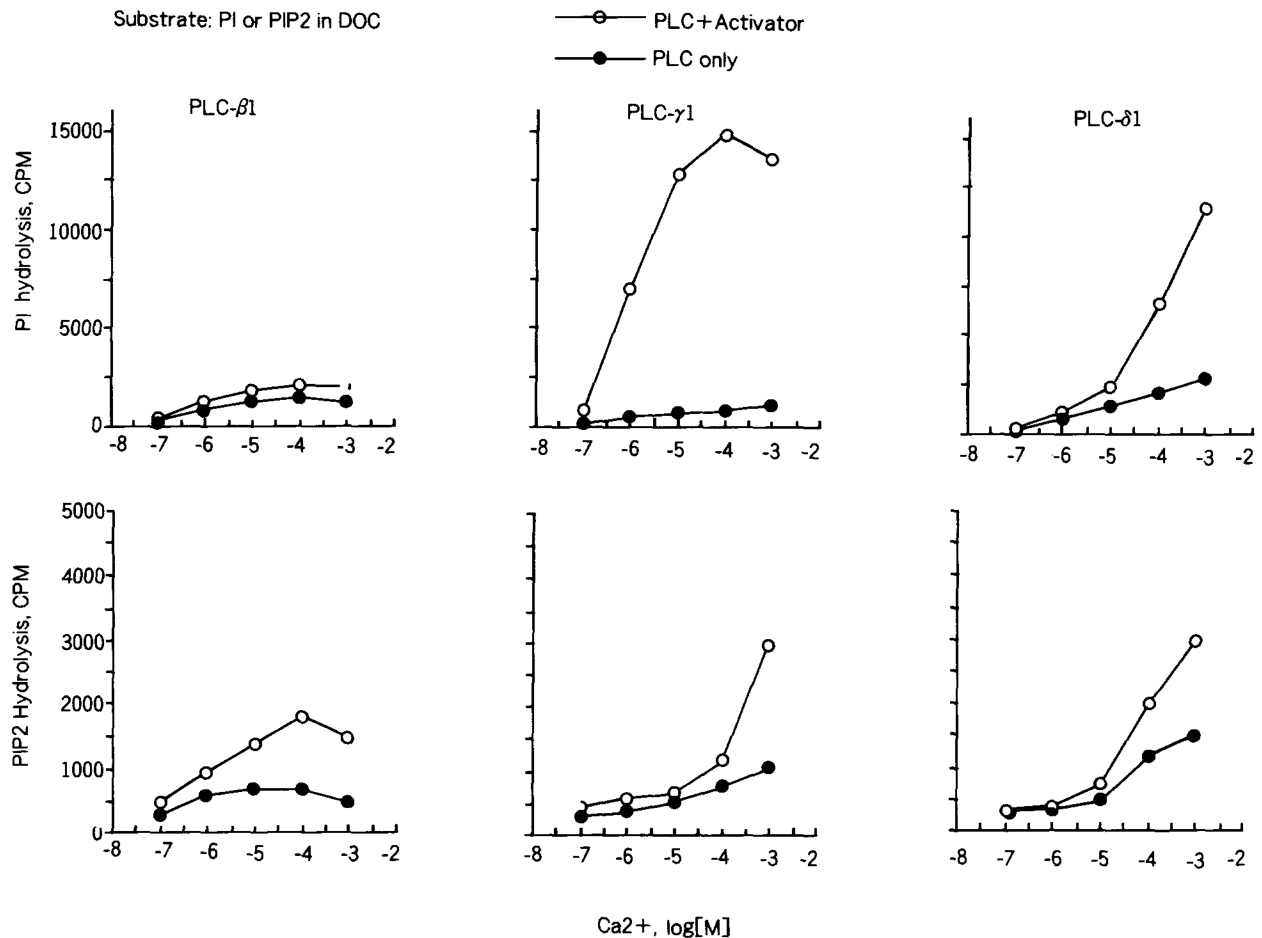

Fig. 8. ***PLC isozyme specific effects of tau protein in varying calcium concentrations.*** Purified tau protein(1,0 μg) was reconstituted with various PLC isozymes(each 20 to 100 ng per assay) in varying free calcium concentration from 10^{-7} to 10^{-3} M using either [^{3}H] PI or [^{3}H] PIP2 in deoxycholate as substrate. *Top panel* ; activation of PI hydrolysis, *Bottom panel*; PIP2 hydrolysis, *Closed circle*; PLC enzyme alone, and *Open circle*; PLC enzyme plus tau. The results are the mean of duplicate values and the data is the representation of three similar experiments.

of the PLC-γ_1.

Tau protein activation of PLC was calcium dependent. In the absence of free calcium, no activation was observed. Moreover, as the free calcium concentration increases, activation increased dramatically reaching maximal level at 10^{-5} to 10^{-3} M of free calcium for PLC-γ_1. The degree of activation by tau-proteins for PLC isozymes in deoxycholate substrate system was PLC-γ1> PLC-γ2 PLC-δ1 PLC-β1 in decreasing order (Fig. 8).

While marked activation was seen for PI substrate in deoxycholate lipid micelle, activation of PIP_2 hydrolysis by tau-protein was less prominent (1.5 to 2.5 fold) for all isozymes of PLC tested (Fig. 8).

DISCUSSION

Evidences presented in this study suggest that bovine brain cytosol contains a heat-stable, microtubule-associated protein tau which may function as a constitutively active, tyrosine kinase-independent activator for PLC isozymes, especially for PLC-γ family of isozymes. Amino acid sequene of CNBr cleaved peptides giving a perfect match for tau_2 isoform, immunoblotting using a specific anti-tau monoclonal antibody recoguizing all 5 bauds of the activator, general physical

and chemical characteristics of tau protein as described by others[31~32] agreeing completely with our purified activator, and alternatively purified tau protein acting as the activator of PLCs, all of these results provide the supports for the observation that the activator protein found in bovine brain cytosol is indeed tau.

Tau protein is one of the first microtubule-associated protein to be identified through cycled assembly purification[32], based on its ability to bind and stabilize tubulin polymers[33~35]. Tau protein is found in abundance in the nervous system of all vertebrates. Most published observations indicate that tau is largely an axonal protein[36~38], although others have demonstrated its presence within somatodendritic compartment of neurons, astrocytes, interfascicular oligodendrocytes, cultured primate cells, and neuroblastoma cells[39~42]. Taken together, these reports indicate that tau is found in most cell types within central nervous system of mammals.

Tau protein consists of multiple isoforms that are developmentally regulated and are produced by alternative mRNA splicing[43]. Carboxy-terminal half of tau protein is well conserved across species, and is also homologous to other microtubule associated proteins[44]. The most striking features of its primary structure is that it contains 3 (fetal form) or 4 (adult form) stretches of 31- or 32-amino acid residues that are shown to provide the microtubule binding sites[45]. Amino-terminal half of the protein is more variable, however, it contains multiple serine residues that are phosphorylated[46] and a region rich in amino acid proline[47]. PLC-γ1 has been shown to be activated and translocated to the cytoskeletal compartment in rat hepatocytes by EGF and its association with actin microfilaments has been demonstrated by immunofluorescence techniques in rat embryoblasts[15]. It is also shown that SH3 domain of PLC-γ1 directs its localization to cytoskeleton[16]. Moreover SH3 domains of other cellular proteins have been shown to selectively bind proline-rich sequences . In our study, activation of PLC-γ1 by tau protein was most prominent. In light of the fact that PLC-γ1 is known to associate with cytoskeletal compartment, there is a good possibility that the proline-rich region of tau could serve to mediate the interaction with PLC-γ1 through SH3 domain[48,49].

It also seems that, in tau activated PLC, PI appears to be preferred substrate over PIP_2 since PI hydrolysis was markedly enhanced (4- to 23-fold) where as PIP_2 hydrolysis was relatively slightly increased (2.5-fold). However, the facts that PIP_2 hydrolysis by PLC depends on multiple factors such as phospholipid composition, calcium and magnesium ions in the substrate system, that tau exhibits differential behavior in interaction with the cytoskeletal components in the presence of different phospholipid[50], and that phosphorylation of tau can be modulated by phospholipid[51], indicate that the activation of PLC by tau protein could be modified by the phospholipid composition of the membrane. Therefore, the substrate preference and conditions of activation needs to be studied further under more diverse conditions.

Upon stimulation, PLC-γ1 becomes phosphorylated on specific tyrosine residues 472, 771,783 and 1254, by receptor tyrosine kinase and also in serine residues. PLC-γ2 has also equivalent tyrosine phosphorylation sites but serine phosphorylation sites are different[7]. Tau also becomes phosphorylated on its multiple serine sites by various kinases which changes its electrophoretic mobility and interaction with microtubules[51]. In Alzheimer's disease, abnormally hyperphosphorylated and altered form of tau protein exists as a major component of paired helical filaments in neurofibrillary tangles[52]. It is also known that in neurofibrillary tangle of the Alzheimer's disease, intense staining with anti-PLC-δ1 antibody was observed and the state of PLC-δ1 was reported to be as insoluble as the abnormal tau[53,54]. It is highly speculative, however the state of phosphorylation of either PLC or tau protein could be an important determining factor in controlling the degree of activation in both physiologic and pathologic conditions such as Alzheimer's disease. Obviously, these aspects of tau-PLC interaction need to be pursued further in more detail.

In conclusion, microtubule-associated protein tau markedly activates PI hydrolysis of PLC-γ1 in calcium dependent and tyrosine kinase-independent manner *in vitro,* and it could have an important implications in signal transduction pathways in both normal and pathologic states.

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