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Activation of Phospholipase C-γ by the Concerted Action of Tau Proteins and Arachidonic Acid*

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Phospholipase C-γ (PLC-γ) isozymes are thought to be activated by receptor-induced tyrosine phosphorylation. Proteins that activate PLC-γ1 have now been purified from bovine brain and identified as members of the tau family of microtubule-associated proteins. Activation of PLC-γ by tau was enhanced in the presence of unsaturated fatty acids such as arachidonic acid, saturated fatty acids being ineffective. Maximal (15–20-fold) activation was apparent in the presence of 0.15 μM tau and 25 μM arachidonic acid (AA). The effect of tau and AA was specific to PLC-γ isozymes in the presence of submicromolar concentrations of Ca^{2+} and was markedly inhibited by phosphatidylcholine. These results suggest that in cells that express tau, receptors coupled to cytosolic phospholipase A_2 may activate PLC-γ isozymes indirectly in the absence of tyrosine phosphorylation through the hydrolysis of phosphatidylcholine to generate AA.

The hydrolysis of a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2),[1] by a specific phospholipase C (PLC) is one of the earliest key events in the regulation of cellular function by more than 100 different extracellular signaling molecules (reviewed by Noh *et al.* (1995)). This reaction generates two intracellular messengers: inositol 1,4,5-trisphosphate (IP_3), which induces the release of Ca^{2+} from internal stores, and diacylglycerol, which activates protein kinase C.

Ten mammalian isoforms of PLC have been identified to date, and these can be divided into β type (PLC-β1, -β2, -β3, and -β4), γ type (PLC-γ1 and -γ2), and δ type (PLC-δ1, -δ2, -δ3, and -δ4) enzymes on the basis of amino acid sequence (Noh *et al.*, 1995). The distinct structural features of the different PLC types have been related to specific mechanisms of receptor-mediated enzyme activation. Thus, PLC-γ isozymes are activated by tyrosine phosphorylation, and PLC-β isozymes are activated by heterotrimeric G proteins (Noh *et al.*, 1995); the mechanism of PLC-δ isozyme activation is not known.

PLC hydrolyzes phosphatidylinositol (PI) and phosphatidylinositol 4-phosphate as well as the physiological substrate, PIP_2, *in vitro*, with PI-hydrolyzing activity often measured during PLC purification. While purifying PLC-γ1, the most abundant PLC isoform in brain cytosol, we observed that the PI-hydrolyzing activity of crude brain cytosol decreased more than expected on dilution. Furthermore, addition of crude cytosol to purified PLC-γ1 markedly enhanced PI-hydrolyzing activity. These observations suggested that brain cytosol contains a component that can enhance the activity of PLC-γ1 toward PI.

We now describe the purification of this activator and its identification as the microtubule-associated protein tau. We also show that tau enhances the activity of PLC-γ1 toward PIP_2 to a markedly lesser extent than that apparent with PI and that the effect of tau on PLC-γ activity toward PIP_2 is greatly increased in the presence of arachidonic acid (AA). Of the three types of PLC, the γ type isozymes are most sensitive to activation by tau and AA. These observations suggest that AA, the generation of which is mediated by receptor-activated phospholipase A_2 (PLA_2), can serve as a link between the PLA_2 and PLC pathways and, together with tau, activate PLC-γ isozymes in the absence of receptor-mediated tyrosine phosphorylation.

EXPERIMENTAL PROCEDURES

Materials

Phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylcholine (PC) were purchased from Avanti Polar Lipids. Arachidonic acid and cholesterol were purchased from Calbiochem. PI and PIP_2 were purchased from Sigma and Boehringer Mannheim, respectively. [^{3}H]PI and [^{3}H]PIP_2 were purchased from New England Nuclear. Preparative DEAE-5PW (21.5 × 150 mm), analytical phenyl-5PW (7.5 × 75 mm), and analytical heparin-5PW (7.5 × 75 mm) were purchased from Toso Haas Inc.

PLC Isozymes

PLC isozymes (PLC-β1, -β2, -γ1, -γ2, -δ1, and -δ2) were purified from HeLa cells that had been transfected with recombinant vaccinia virus containing the entire coding sequence of the respective enzyme as described (Park *et al.*, 1992).

Purification of Activator

All manipulations were performed at 4–6 °C in a refrigerated room or on ice, unless otherwise indicated. During purification, PLC-γ1-activating activity was measured at 37 °C for 5 min in 200 μl of a reaction mixture containing 20,000 cpm of [^{3}H]PI (DuPont NEN), 150 μM soybean PI (Sigma), PLC-γ1 (20–50 ng), 3 mM $CaCl_2$, 2 mM EGTA, 0.1% (w/v) sodium deoxycholate, 50 mM Hepes-NaOH (pH 7.0), and a source of activator. To maintain the stimulated activity in the linear range of the assay, we adjusted the amount of PLC to obtain an unstimulated, basal activity in the range of 500-1200 cpm of inositol 1-phosphate generated. The purification procedure consisted of the following steps.

(i) Preparation of Bovine Brain Cytosolic Extracts—Fifteen fresh bovine brains (total of 4.5 kg of tissue) were obtained from a local slaughter house and homogenized in 10 liters of a solution containing 10 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 1 mM phenylmeth-

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[1] The abbreviations used are: PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; PI, phosphatidylinositol; AA, arachidonic acid; PLA_2, phospholipase A_2; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DTT, dithiothreitol; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; $cPLA_2$, cytosolic PLA_2; PH, pleckstrin homology.

ylsulfonyl fluoride, 1 mM dithiothreitol (DTT), leupeptin (2 μg/ml), and aprotinin (2 μg/ml) in a Waring blender. The homogenate was centrifuged at 1000 × *g* for 10 min, and the resulting supernatant was centrifuged further at 13,000 × *g* for 1 h. The second supernatant was adjusted to 60% saturation with ammonium sulfate and then centrifuged for 30 min at 13,000 × *g*. The resulting precipitate was stored at −70 °C.

(ii) Heat and Acid Treatment—The frozen ammonium sulfate precipitate was thawed and resuspended by adding 2 volumes of distilled water, heated at 95 °C for 5 min, and centrifuged at 1000 × *g* for 15 min. The resulting supernatant was filtered through Whatman no. 1 paper, and the filtrate was adjusted to 5% (w/v) trichloroacetic acid and centrifuged at 17,000 × *g* for 10 min. The resulting pellet was immediately resuspended in 20 ml of 1 M Tris-HCl (pH 8.4) and dialyzed extensively against a solution containing 50 mM Tris-HCl (pH 7.4), 1 mM EGTA, and 0.1 mM DTT. After dialysis, insoluble materials were removed by centrifugation at 75,000 × *g* for 10 min.

(iii) Preparative HPLC on a DEAE-5PW Column—The final supernatant (420 mg of protein) from the previous step was divided into two equal portions, each of which was applied to a preparative TSKgel DEAE-5PW HPLC column (21.5 × 150 mm) that had been equilibrated with 50 mM Tris-HCl (pH 7.4), 1 mM EGTA, and 0.1 mM DTT. Proteins were eluted at a flow rate of 5 ml/min by washing with equilibration buffer for 5 min and then applying a linear gradient of 0–0.3 M NaCl over 35 min and a second linear gradient of 0.3–1 M NaCl over 5 min. Fractions (5 ml) were collected and assayed for PLC-γ1-activating activity. Peak fractions (25–27) from the two identical runs were combined.

(iv) Reverse Phase HPLC on a TSKgel Phenyl-5PW Column—Solid KCl was added to the pooled fractions (40 mg of protein) from the previous step to a final salt concentration of 3 M. Insoluble material was removed by centrifugation, and the resulting supernatant was injected into an analytic TSKgel phenyl-5PW HPLC column (7.5 × 75 mm) that had been equilibrated with 20 mM Hepes-NaOH (pH 7.0), 3 M NaCl, 1 mM EGTA, and 0.1 mM DTT. Proteins were eluted at a flow rate of 1 ml/min by sequential application of equilibration buffer for 5 min and consecutive decreasing linear gradients of 3–1.2 M NaCl over 10 min and 1.2 to 0 M NaCl over 25 min. Fractions (1 ml) were collected and assayed for activator activity. Peak fractions (26 and 27) were pooled and washed with 20 mM Hepes-NaOH (pH 7.0), 1 mM EGTA, and 0.1 mM DTT in a Centriprep-30 (Amicon) concentrator to lower the salt concentration to <0.1 M.

(v) HPLC on a TSKgel Heparin-5PW Column—The washed fraction (4 mg of protein) from the previous column was applied to a TSKgel heparin-5PW column (7.5 × 75 mm) that had been equilibrated with 20 mM Hepes-NaOH (pH 7.0), 1 mM EGTA, and 0.1 mM DTT. Proteins were eluted at a flow rate of 1 ml/min by sequential application of equilibration buffer for 15 min and linear NaCl gradients of 0–0.64 M over 40 min and 0.64 to 1 M over 10 min. Fractions (1 ml) were collected and assayed for activator activity. Peak fractions (32 and 33) were pooled, concentrated, divided into portions, and stored at −70 °C.

(vi) Electroelution of Proteins from SDS-Polyacrylamide Gels—Purified proteins from the heparin column (700 μg) were separated by preparative SDS-PAGE on an 8% gel (3-mm thickness, single-well comb). The gel was stained lightly with Coomassie Brilliant Blue, and visualized protein bands were excised from the gel with a razor blade. The proteins were subsequently eluted with an Electro-Eluter (C.B.S. Scientific, Del Mar, CA), after which Coomassie Brilliant Blue was extracted with isobutanol and SDS was removed by precipitation with ice-cold acetone.

Cyanogen Bromide Cleavage and Amino Acid Sequencing

Proteins (10 μg each) electroeluted from three different bands (bands 1, 2, and 3 in Fig. 3*A*) were subjected to chemical cleavage with 100 mM CNBr in the presence of 70% (v/v) formic acid for 16 h. The reaction was quenched by adding excess methionine crystals to the reaction mixture. The cleaved products were dried under vaccum, resuspended in 50 mM Tris-HCl (pH 8.4) and subjected to HPLC analysis on Vydac C_{18} column (4.6 × 250 mm) that had been equilibrated with 0.05% (w/v) trifluoroacetic acid. Peptides were eluted at a flow rate of 1 ml/min by sequential application of equilibration buffer for 20 min and consecutive linear acetonitrile gradients of 0 to 50% (v/v) in 0.05% trifluoroacetic acid over 50 min and 50–100% over 10 min. Two peptides that eluted at 40.2 and 44.5 min and were common to the three elution profiles obtained with bands 1, 2, and 3 were subjected to sequence analysis.

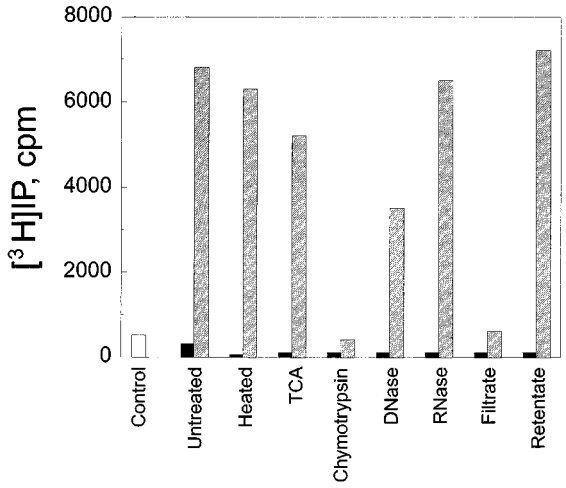

FIG. 1. **Effect of bovine brain cytosol on PLC-γ1 activity.** PI-hydrolyzing activity of purified PLC-γ1 was measured before and after the addition of bovine brain cytosol that had been subjected to various treatments. The assay was performed as described under "Experimental Procedures" for the purification of activator protein. PI-hydrolyzing activity was measured with 50 ng of purified PLC-γ1 alone (control, *open bar*), with brain cytosol alone (*black bars*), or with 50 ng of purified PLC-γ1 plus brain cytosol (*gray bars*). Before the addition to the PLC assay, brain cytosol (400 μg of protein in 200 μl) was untreated; heated at 95 °C for 5 min; precipitated with 5% (w/v) trichloroacetic acid (*TCA*), redissolved in 50 mM Tris, and adjusted to pH 7.4 with NaOH; or treated at 37 °C for 1 h with 5 μg of chymotrypsin, 0.2 μg of DNase, or 0.2 μg of RNase, as indicated. Samples that had been treated with chymotrypsin, DNase, or RNase were heated at 100 °C for 3 min to inactivate the added enzymes before addition to the PLC assay. Brain cytosol (400 μg) was also filtered through a membrane with a molecular size cut-off of 30 kDa; the retained protein was adjusted to the initial volume, and both filtrate and retentate were assayed for PLC activity in the absence or the presence of PLC-γ1. In the case of untreated cytosol, 2 μg of protein were used per PLC assay. In the case of cytosol that had been subjected to the various treatments, a volume of sample that initially corresponded to 2 μg of protein was added to PLC assay without further protein quantitation. Activity is expressed as counts per minute of inositol 1-phosphate (IP) generated.

RESULTS

PLC-γ1-activating Protein in Bovine Brain Cytosol—The addition of crude bovine brain cytosol to purified PLC-γ1 increased the PI-hydrolyzing activity of the enzyme 15-fold (Fig. 1), suggesting the presence of an activator in brain extract. This putative activator appeared to be a protein that is stable to treatment with heat or acid. Activator activity in the cytosol was (i) largely unaffected by heating for 5 min at 95 °C; (ii) recovered in the 5% (w/v) trichloroacetic acid precipitate of the extract; (iii) resistant to treatment with DNase or RNase; (iv) susceptible to chymotrypsin treatment; and (v) retained by a membrane with a size cut-off of 30 kDa. (Fig. 1).

Purification of Activator Protein—The heat and acid stability of the PLC-γ1-activating protein allowed us to obtain a preparation highly enriched in the activator by submitting a 60% saturated ammonium sulfate fraction of crude brain cytosol to these treatments. This enriched preparation was then subjected to HPLC on successive DEAE, phenyl, and heparin columns (Fig. 2). SDS-PAGE of the peak fractions from the final column revealed five closely spaced protein bands with apparent molecular sizes of 48–62 kDa and two additional bands of <43 kDa (Fig. 3*A*). Pooling peak fractions more narrowly or further purification of the heparin column fractions on an HPLC gel filtration column (TSKgel G3000-SW) or an HPLC Mono Q column also resulted in multiple protein bands similar to those shown in Fig. 3*A* on SDS-PAGE analysis (data not shown).

Each of the five protein bands between 48 and 62 kDa was

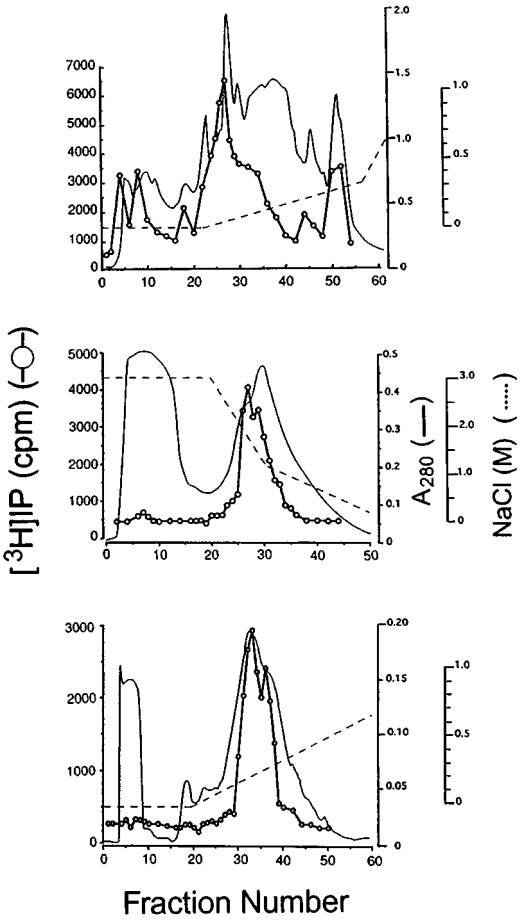

FIG. 2. **Purification of PLC-γ1-activating protein.** Bovine brain cytosolic proteins, after ammonium sulfate precipitation and treatment with heat and trichloroacetic acid, were subjected to HPLC on preparative TSKgel DEAE-5PW (*top panel*), TSKgel phenyl-5PW (*middle panel*), and TSKgel heparin-5PW (*bottom panel*) columns. Fractions were assayed for PLC-γ1-activating activity.

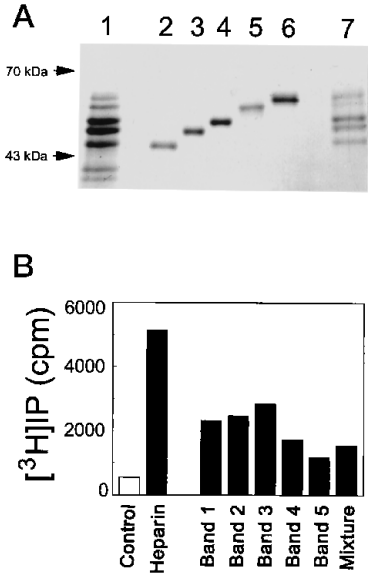

FIG. 3. **SDS-PAGE and electroelution of PLC- 1-activating protein.** *A*, the peak fractions (700 μg of protein) pooled from the heparin column chromatography shown in the bottom panel of Fig. 2 were fractionated on a preparative SDS-polyacrylamide gel, and each of the five protein bands (bands 1, 2, 3, 4, and 5, beginning with the smallest protein) with molecular sizes between 48 and 62 kDa was excised from the gel and electroeluted. The pooled peak fraction (30 μg of protein) from the heparin column (*lane 1*), band 1 (*lane 2*), band 2 (*lane 3*), band 3 (*lane 4*), band 4 (*lane 5*), band 5 (*lane 6*), and a recombined mixture of the five bands (*lane 7*) were then subjected to SDS-PAGE on an 8% gel and visualized by staining with Coomassie Brilliant Blue. The positions of molecular size standards are shown on the *left*. *B*, the PLC-γ1-activating activity of the electroeluted proteins was assayed with 50 ng of purified PLC-γ1 alone (*Control*) or in the presence of 200 ng of the heparin column peak fraction (*Heparin*), band 1, band 2, band 3, band 4, band 5, or a recombined mixture of the five bands (*Mixture*).

excised from the polyacrylamide gel and electroeluted. After removal of SDS, each eluted protein was tested for PLC-γ1-activating activity. All of the eluted proteins activated PLC-γ1 (Fig. 3*B*).

Identification of the PLC-γ1 Activator as Tau—Three of the electroeluted proteins (bands 1, 2, and 3) were individually cleaved with CNBr and analyzed on a C_{18} HPLC column. The three elution profiles were similar (data not shown), suggesting that the three proteins are related. Two peptides that eluted at 40.2 and 44.5 min and that were common to all three proteins were sequenced. The peptides yielded sequences of EDHAQGDYTLQDQEGD and VSKGKDGTGPDDKKTK, respectively, both of which showed a perfect match to bovine brain tau sequences encoded by exon 1 and 5, respectively (Fig. 4*A*).

The pooled heparin column peak fraction and electroeluted proteins were subjected to immunoblot analysis with a monoclonal antibody to tau (Fig. 4*B*). Not only the proteins with molecular sizes between 48 and 62 kDa (bands 1 to 5) but also proteins smaller than 43 kDa were recognized by the antibody, suggesting that nearly all of the proteins in the heparin column fraction are either tau isoforms or their proteolytic fragments. Furthermore, tau proteins purified from bovine brain by the standard procedure (Baudier *et al.*, 1987) yielded multiple bands on SDS-PAGE and activated PLC-γ1 with a potency and efficacy similar to those of the heparin column fraction (Fig. 4*C*). The concentration dependence profile showed that half-maximal activation of PLC-γ1 was achieved at ~0.2 μM tau (data not shown).

Effect of Tau on the Activity of PLC Isozymes toward PI and PIP_2 at Various Ca^{2+} Concentrations—All PLC isozymes require Ca^{2+} for catalysis, but the sensitivity to Ca^{2+} varies with specific isozyme and substrate. The effect of tau on PLC-β1, PLC-γ1, and PLC-δ1, representatives of each type of PLC, was evaluated with PI or PIP_2 as substrates at various Ca^{2+} concentrations (Fig. 5). At Ca^{2+} concentrations of >0.1 μM, tau proteins increased the PI-hydrolyzing activity of PLC-γ1 by up to 15–20-fold; in contrast, tau had no marked effect on the PI-hydrolyzing activity of PLC-β1 and induced only a 3–4-fold increase in that of PLC-δ1 at Ca^{2+} concentrations above 10 μM. Tau did not have a marked effect on the PIP_2-hydrolyzing activity of any of the three PLC isozymes; only an approximately 2-fold activation was observed for all three enzymes at high Ca^{2+} concentrations.

Effects of Various Fatty Acids on the Activation of PLC-γ1 by Tau—Irvine *et al.* (1979) showed that unsaturated fatty acids such as oleic acid and AA stimulated PLC activity in crude brain cytosol with a [^{3}H]inositol-labeled microsomal fraction from rat liver as substrate. However, such unsaturated fatty acids do not directly affect the activities of purified PLC isozymes, including that of PLC-γ1.[2] The brain cytosol preparation used by Irvine *et al.* (1979), likely contained tau proteins in addition to PLC-γ1, the most abundant PLC isozyme in brain cytosol, and tau may thus have mediated the effect of unsaturated fatty acids on PLC activity.

We therefore investigated the effect of AA on PI and PIP_2 hydrolysis catalyzed by PLC-γ1. We included PE, PS, and cho-

[2] S. C. Hwang, D.-Y. Jhon, Y. S. Bae, J. H. Kim, and S. G. Rhee, unpublished data.

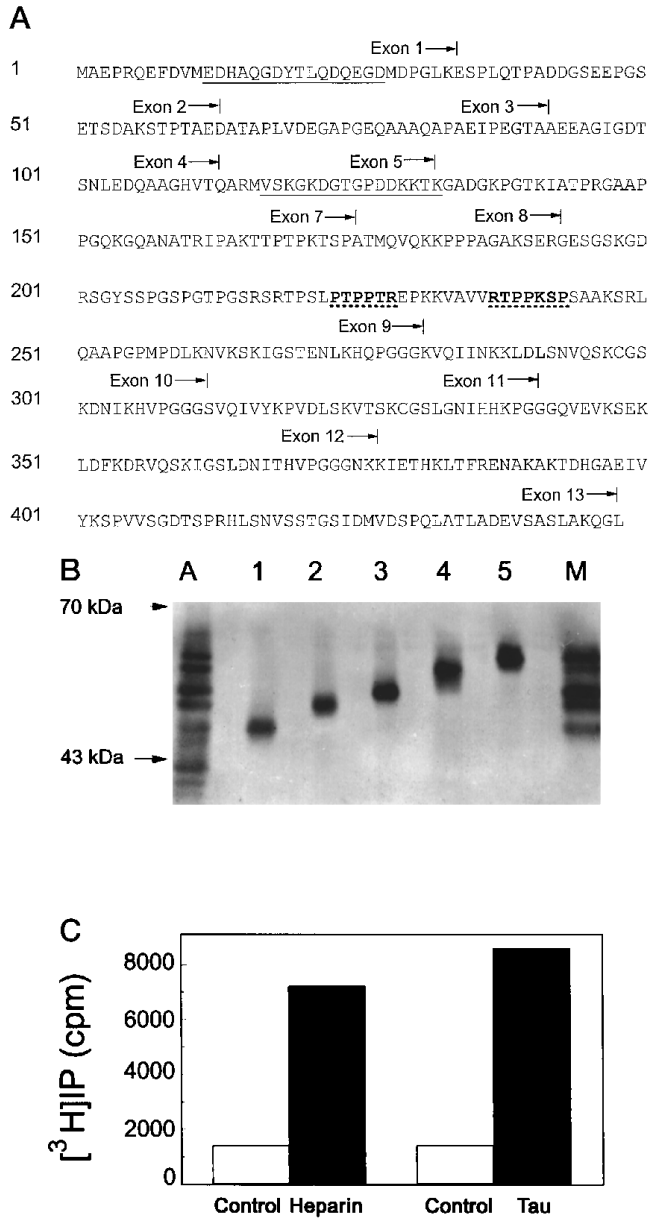

FIG. 4. **Identification of the PLC-γ1 activator as tau.** *A*, the sequences of two CNBr petides (*underlined*) derived from the electro-eluted bands 1, 2, and 3 in Fig. 3 are contained within a tau sequence that was deduced from mRNA containing all 13 exons except exon 6. The ends of each exon ends are indicated (*arrows* and *vertical lines*). Two proline-rich sequences, which are potential SH3 binding sites, are also shown (*bold letters* and *dotted underline*). Residue numbers are shown on the *left*. *B*, immunoblot analysis of PLC-γ1-activating proteins with monoclonal antibody to tau. The pooled peak fraction from the heparin column (*lane A*), band 1 (*lane 1*), band 2 (*lane 2*), band 3 (*lane 3*), band 4 (*lane 4*), band 5 (*lane 5*), and a recombined mixture of the five bands (*lane M*) were subjected to SDS-PAGE on an 8% gel, transferred to a nitrocellulose membrane, and incubated with a monoclonal antibody to tau. Approximately 200 ng of protein were loaded in each lane. Immune complexes were detected with alkaline phosphatase-conjugated rabbit antibodies to mouse immunoglobulin. *C*, comparison of the PLC-γ1-activating activities of the pooled peak fraction from the hepain column (*Heparin*) and tau proteins purified from bovine brain by the standard procedure, which includes precipitation with 2.5% (w/v) perchloric acid (Baudier *et al.*, 1987). The PI-hydrolyzing activities of 50 ng of PLC-γ1 in the absence (*Control*) and the presence of the heparin column fraction (3.5 μg of protein, equivalent to 0.3 μM tau) or 0.3 μM of tau are indicated.

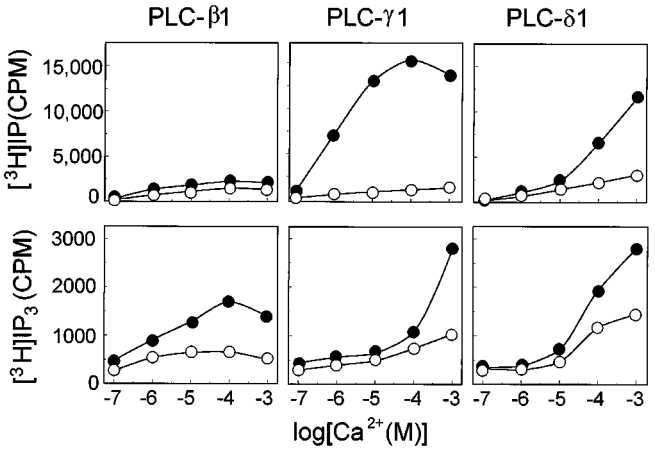

FIG. 5. **Effect of tau on the activity of PLC isozymes toward micellar PI or PIP_2 substrate at various Ca^{2+} concentrations.** The activities of PLC-β1, PLC-γ1, and PLC-δ1 toward PI (*upper panels*) or PIP_2 (*lower panels*) were measured at the indicated free Ca^{2+} concentrations in the absence (*open circles*) or the presence (*closed circles*) of tau. The assay mixture contained 30 μM [^{3}H]PI (30,000 cpm) or 30 μM [^{3}H]PIP_2 (30,000 cpm), PLC (20–100 ng/assay), 0.5 μM tau in 50 mM Hepes-NaOH (pH 7.0), 0.1% sodium deoxycholate, 2 mM EGTA, and various concentrations of $CaCl_2$. The data are the means of duplicate measurements and are representative of three similar experiments.

lesterol in the substrate to mimic the composition of cell membranes. In addition, equal concentrations of both [^{3}H]PI and [^{3}H]PIP_2 were added in the substrate, which thus comprised PI, PIP_2, PS, cholesterol, and PE in a molar ratio of 1:1:1:1:4, and [^{3}H]inositol 1-phosphate and [^{3}H]IP_3 generated by PLC-γ1 were separated by ion-exchange chromatography in order to compare directly the effects of tau and AA on PI hydrolysis and on PIP_2 hydrolysis. PC was not included in the substrate because it inhibits tau-dependent activation of PLC-γ1 (see below). AA affected neither the PI- nor the PIP_2-hydrolyzing activity of PLC-γ1 in the absence of tau, but it increased both activities in a concentration-dependent manner in the presence of tau (Fig. 6). For both activities, the extent of activation was a maximal at 25 μM AA and decreased at higher concentrations; a similar decrease at higher concentrations of unsaturated fatty acid was also observed in the experiment of Irvine *et al.* (1979).

The dependence of the PIP_2-hydrolyzing activity of PLC-γ1 on tau concentration was examined with the mixed micellar substrate containing AA. Maximal activation was apparent at 0.15 μM tau, in contrast to the tau concentration of 0.5 μM required for maximal activation of PI hydrolysis in the absence of AA and other lipids (data not shown). We also examined the effects of fatty acids other than AA on PIP_2 hydrolysis by PLC-γ1 in the presence of 0.3 μM tau proteins (Fig. 7). Unsaturated fatty acids, including AA, linolenic acid, linoleic acid, oleic acid, and palmitoleic acid, stimulated PIP_2-hydrolyzing activity in the presence, but not in the absence, of tau. In contrast, the corresponding saturated fatty acids (arachidic acid, stearic acid, and palmitic acid) had no effect on PIP_2-hydrolyzing activity in the absence or the presence of tau.

Isozyme Specificity of Tau- and AA-dependent Activation of PIP_2 Hydrolysis by PLC-γ1—The combined effects of tau and AA on PIP_2 hydrolysis by PLC-β1, -β2, -γ1, -γ2, -δ1, and -δ2 were compared (Fig. 8). At 0.1 μM Ca^{2+}, marked effects of tau and AA were apparent only with PLC-γ1 and PLC-γ2 (Fig. 8*A*); however, at 1 μM Ca^{2+}, tau and AA stimulated the PLC-δ isozymes to activity levels 30–50% of those apparent with the PLC-γ isozymes (Fig. 8*B*).

Effect of PC on Tau- and AA-dependent PLC-γ1 Activity—The addition of PC to the mixed micellar substrate containing [^{3}H]PI, [^{3}H]PIP_2, PS, cholesterol, AA, and PE in a molar ratio of 1:1:1:1:1:4 (where 1 corresponds to a concentration of 30 μM) resulted in a concentration-dependent inhibition of both PI and

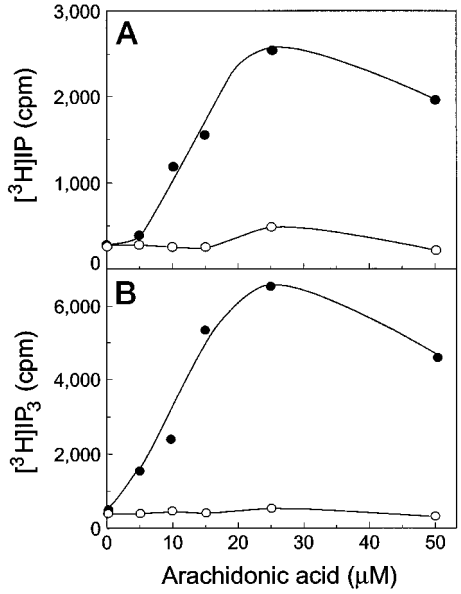

FIG. 6. **Effects of AA and tau on the activity of PLC-γ1 toward a mixed micellar substrate containing PI, PIP_2, PS, cholesterol, and PE.** The PI-hydrolyzing (*A*) and PIP_2-hydrolyzing (*B*) activities of PLC-γ1 were measured in the absence (*open circles*) or the presence (*closed circles*) of tau. The mixed micellar substrate was prepared by mixing [^{3}H]PI, [^{3}H]PIP_2, PS, cholesterol, and PE in a molar ratio of 1:1:1:1:4 together with various amounts of AA in 0.066% deoxycholate. The final assay mixture (100 μl) contained 50 ng of PLC-γ1, 0.3 μM tau, 30 μM each of [^{3}H]PI (30,000 cpm), [^{3}H]PIP_2 (30,000 cpm), PS, and cholesterol, 120 μM PE, and the indicated concentrations of AA in 50 mM Hepes-NaOH (pH 7.0), 0.033% deoxycholate, 2 mM $MgCl_2$, 2 mM EGTA, and 1 μM free Ca^{2+}. After incubation for 10 min at 30 °C, the reactions were terminated by addition of 1 ml of a 1:1 (v/v) mixture of chloroform and methanol and centrifugation. The resulting aqueous phase was applied to a 0.5-ml column of Dowex AG1X-2 anion-exchange resin (formate form), which was then washed with 3 ml of distilled water. [^{3}H]Inositol 1-phosphate (IP) was eluted with 3 ml of 100 mM ammonium formate, and [^{3}H]IP_3 was eluted with 3 ml of 1 M ammonium formate.

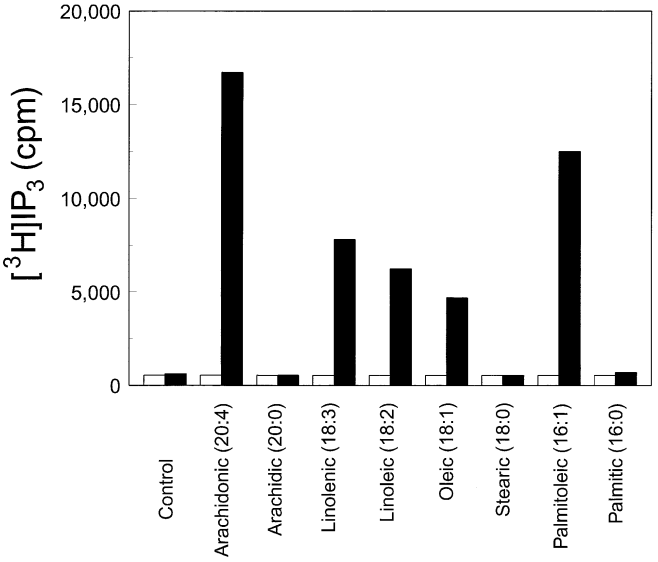

FIG. 7. **Effects of various fatty acids on the PIP_2-hydrolyzing activity of PLC-γ1.** The PIP_2-hydrolyzing activity of PLC-γ1 (50 ng/assay) was measured in the absence (*open bars*) or the presence (*solid bars*) of 0.3 μM tau with mixed micellar substrates containing the indicated fatty acid at a final concentration of 30 μM (control, no fatty acid). Otherwise, the assay conditions were as described in the legend to Fig. 6. The data are the means of duplicate determinations and are representative of two similar experiments.

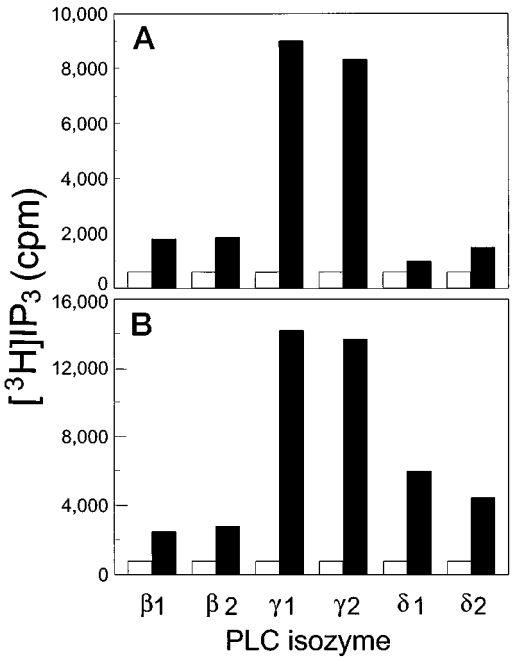

FIG. 8. **Combined effects of tau and AA on the PIP_2-hydrolyzing activity of various PLC isozymes.** The PIP_2-hydrolyzing activities of the indicated PLC isozymes (20–100 ng/assay) were measured at 0.1 μM (*A*) or 1 μM (*B*) free Ca^{2+} in the absence (*open bars*) or the presence (*solid bars*) of 0.3 μM tau with a mixed micellar substrate containing 30 μM AA, as described in the legend to Fig. 6. The data are the means of triplicate determinations and are representative of three similar experiments.

PIP_2 hydrolysis by PLC-γ1 in the presence of tau (Fig. 9*A*). Half-maximal inhibition was apparent at 30–40 μM PC. No inhibiting effect of PC was observed in the absence of tau. Lyso-PC (50 μM) inhibited PIP_2 hydrolysis by PLC-γ1 in the absence or the presence of tau by <10% (data not shown).

Thus the conversion of inhibitory PC to lyso-PC and AA by a PLA_2 enzyme might constitute a signal for the activation of PLC-γ in the presence of tau. We evaluated this hypothesis with mixed micellar substrates containing 30 μM each of [^{3}H]PIP_2, PS, and cholesterol, 120 μM PE, and various concentrations of PC and AA (Fig. 9*B*). The initial concentrations of AA and PC in the mixed micelle were 0 and 90 μM, respectively. The concentration of AA was then increased incrementally to 90 μM and that of PC was decreased to 0 μM, with the total concentration of both agents maintained constant at 90 μM. Marked activation of PIP_2 hydrolysis was not apparent until the PC concentration decreased below 70 μM and the AA concentration increased above 20 μM. Maximal activity was apparent when both PC and AA were present at 45 μM; at higher concentrations of AA, the activity decreased.

DISCUSSION

We have purified proteins that enhance the activity of PLC-γ1 toward a micellar substrate containing PI and deoxycholate and identified them as tau isoforms. Tau comprises a family of microtubule-associated proteins that are generated from alternatively spliced transcripts derived from a single gene with 13 exons (reviewed by Lee (1990)). Tau expression is largely restricted to brain and is developmentally regulated. Six different cDNAs capable of encoding isoforms comprised of between 304 and 448 residues have been isolated for bovine tau; these correspond to mRNA species lacking one or more of exons 3, 6, 8, and 10 (Himmer, 1989).

Tau proteins did not markedly increase PLC-γ1 activity toward micellar PIP_2. Furthermore, when common lipid components of membranes (PE, PS, and cholesterol) were incorpo-

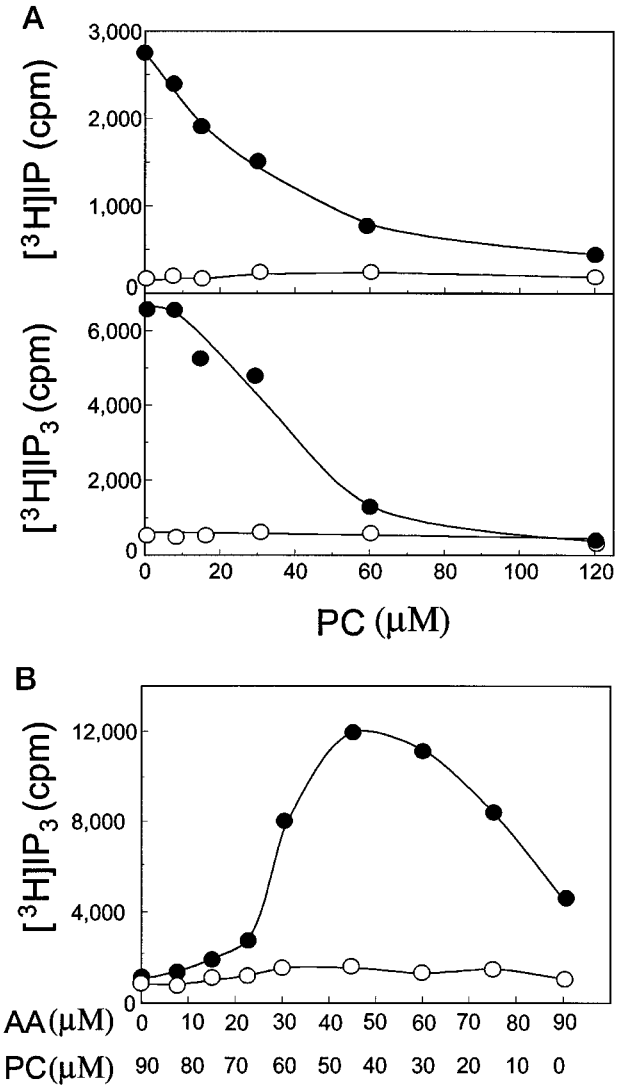

FIG. 9. **Effect of PC on tau- and AA-dependent PLC-γ1 activity.** *A*, the PI-hydrolyzing (*upper panel*) and PIP_2-hydrolyzing (*lower panel*) activities of PLC-γ1 were measured in the absence (*open circles*) or the presence (*closed circles*) of 0.3 μM tau with mixed micellar substrates containing 30 μM AA and various concentrations of PC in addition to [^{3}H]PI, [^{3}H]PIP_2, PS, cholesterol, and PE as described in the legend to Fig. 7. *B*, the PIP_2-hydrolyzing activity of PLC-γ1 (50 ng/assay) was measured in the absence (*open circles*) or the presence (*solid circles*) of 0.3 μM tau with mixed micellar substrates containing the indicated final concentrations of AA and PC, in addition to [^{3}H]PIP_2, PS, cholesterol, and PE, as described in the legend to Fig. 8. The data are the means of duplicate determinations and are representative of two similar experiments.

rated into micelles, activation of PLC-γ1 by tau was not apparent with either the PI or PIP_2 as substrate. The addition of an unsaturated fatty acid to the substrate restored tau-dependent activation of both PI and PIP_2 hydrolysis at low Ca^{2+} concentrations. Of the unsaturated fatty acids tested, AA was the efficacious activator, and efficacy decreased in the rank order palmitoleic acid (16:1) > linolenic acid (18:3) > linoleic acid (18:2) > oleic acid (18:1). The corresponding saturated fatty acids, arachidic acid (20:0), stearic acid (18:0), and palmitic acid (16:0) were ineffective. Maximal (15–20-fold) activation of PLC-γ1 toward PIP_2 in micelles containing 30 μM each of PIP_2, PS, and cholesterol and 120 μM PE was observed at 0.15 μM tau and 25 μM AA.

Several studies have examined the effects of lipids on PLC activity. Uunsaturated fatty acids were shown to increase PLC activity in rat brain cytosol ~10-fold (Irvine *et al.*, 1979). The effect was probably attributable to the presence of both tau and PLC-γ1 in the brain cytosol. An approximately 3–4-fold activation by unsaturated fatty acids of a 68-kDa PLC purified from rat liver cytosol was observed when the hydrolysis of micellar PI was measured in the presence of 2 mM Ca^{2+} (Takenawa and Nagai, 1981). It is now thought that the 68-kDa enzyme was a proteolytic fragment of PLC-δ1 (Taylor *et al.*, 1992). Saturated fatty acids had no effect on brain or liver PLC activity.

Abundunt membrane phospholipids such as PC, PE, and PS were shown to have no marked effect on the activities of PLC-β isozymes (James *et al.*, 1995) and PLC-γ1 (Jones and Carpenter, 1993) in detailed kinetic studies performed with mixed micellar PIP_2 substrates. An approximately 3-fold activation by PS was observed for PLC-β1, PLC-γ1, and PLC-δ1 with a monolayer substrate containing PIP_2, whereas PC had no effect (Boguslavsky *et al.*, 1994). However, marked inhibition of the PLC-δ1 activity by PC was observed with PI presented as small unilamellar vesicles (Hofmann and Majerus, 1982). These studies suggest that the activity of PLC, like that of many enzymes that act on lipid substrates, depends on the composition and physical condition of the substrate.

In the present study, PC had no significant effect on basal PLC-γ1 activity but markedly inhibited activity stimulated by tau and AA. This observation suggested that the activation of PLC-γ1 by tau might be facilitated by a concomitant decrease in PC concentration and increase in AA concentration, both of which occur in cells on activation of the 85-kDa cytosolic PLA_2 ($cPLA_2$) that is known to be coupled to various receptors (reviewed by Dennis (1994) and by Kramer (1994); Clark *et al.* (1991)). This enzyme requires submicromolar concentrations of Ca^{2+} and preferentially hydrolyzes PC with unsaturated fatty acids in the *sn*-2 position: The rank order of preference for *sn*-2 acyl chains is 20:4 > 18:3 > 18:2 > 18:1 > 16:1, and the preference order for C_{20} acyl chains is 20:4 > 20:3 > 20:2 > 20:1 > 20:0 (Hanel *et al.*, 1993). In contrast, secreted PLA_2 enzymes with molecular sizes of 13–18 kDa require millimolar concentrations of Ca^{2+} for catalytic activity, show a preference for PE, and are nonselective with regard to *sn*-2 fatty acids. A 40-kDa Ca^{2+}-independent PLA_2 identified in myocardium preferentially hydrolyzes AA-containing PC (Hazen *et al.*, 1990), whereas an 80-kDa Ca^{2+}-independent PLA_2 from macrophages lacks specificity for AA-containing lipids (Dennis, 1994).

Submicromolar concentrations of Ca^{2+} are required for the translocation of $cPLA_2$ to membranes rather than for catalytic activity and this translocation is a prerequisite for activation (Clark *et al.*, 1991; Sharp *et al.*, 1991). Activation of $cPLA_2$ may occur secondarily to receptor-mediated activation of a PLC that results in an increase in the cytosolic Ca^{2+} concentration (Kramer, 1994). Initial activation of a PLC-β isozyme, for example, in response to ligand occupancy of a G protein-coupled receptor may thus result in an increase in intracellular Ca^{2+}, which in turn results in activation of $cPLA_2$ and subsequent activation of PLC-γ isozymes. Therefore, activation by the combined action of tau and AA may represent a mechanism by which PLC-γ isozymes can be activated independently of tyrosine phosphorylation. Jones and Carpenter (1993) observed that incorporation of phosphatidic acid into a micellar substrate containing PIP_2 and Triton X-100 enhanced PLC-γ1 activity 40-fold; they therefore proposed that PLC-γ1 can be activated independently of tyrosine phosphorylation if phosphatidic acid is generated by the action of phospholipase D.

Evidence also suggests that the activation of $cPLA_2$ may occur at basal cytosolic Ca^{2+} concentration; that is, independently of PLC-mediated IP_3 generation (Currie *et al.*, 1992; Kast *et al.*, 1993). The addition of bombesin to Swiss 3T3 cells re-

sulted in the rapid (within 2 s) release of AA and concomitant depletion of PC, without effects on other phospholipids (Currie *et al.*, 1992). The initial AA release was dependent on neither the influx of extracellular Ca^{2+} nor the mobilization of intracelluar Ca^{2+} by IP_3. Furthermore, the increased concentration of AA was sustained over several minutes, whereas the increase in lyso-PC was more transitory. In another study, the association of $cPLA_2$ with membranes, the increase in $cPLA_2$ activity, and the liberation of AA in HEL-30 cells treated with tumor necrosis factor-α were all independent of PLC activation (Kast *et al.*, 1993). Thus, ligation of receptors that are directly coupled to $cPLA_2$ but not to PLC may induce PIP_2 breakdown by stimulation of PLC-γ isozymes indirectly through tau and AA.

Several studies are consistent with the notion that stimulation of PLC by endogenously released AA occurs in cells. Incubation of human trophoblasts with AA stimulates PLC activity (Zeitler and Handwerger, 1985). Further studies with these cells suggested that the stimulation of phosphoinositide metabolism and placental lactogen release are mediated by initial activation of PLA_2 (Zeitler *et al.*, 1991). AA but not other biologically important fatty acids stimulates phosphoinositide metabolism in and catecholamine release from bovine adrenal chromaffin cells (Negishi *et al.*, 1990). AA was also shown to increase phosphoinositide breakdown and glutamate release in rat hippocampal tissue (Lynch and Voss, 1990), to induce phosphoinositide breakdown and diacylglycerol generation in human platelets (Siess *et al.*, 1983), and to increase intracellular Ca^{2+} by mobilizing an IP_3-sensitive Ca^{2+} pool in isolated rat pancreatic islets (Wolf *et al.*, 1986) and a human leukemic T cell line (Chow and Jondal, 1990). The AA-induced Ca^{2+} release was shown to be not due to the metabolites of AA (Wolf *et al.*, 1986)

In addition to serving as a precursor for the biosynthesis of prostaglandins, thromboxanes, leukotrienes, and other eicosanoids, AA has been proposed to act as a modulator or second messenger in signal transduction (reviewed by Sumida *et al.*, 1993). AA and other unsaturated fatty acids activate protein kinase C directly (reviewed by Nishizuka (1992); McPhail *et al.* (1984); Touny *et al.* (1990)). Furthermore, AA has been shown to activate guanylate cyclase (Gerzer *et al.*, 1986) and to inhibit both Ca^{2+}-calmodulin-dependent protein kinase (Piomelli *et al.*, 1989) and guanine nucleotide binding to the α subunit of G_z (Glick *et al.*, 1996). The effective concentrations of AA in these studies were in the range of 10^{-4} M. Although it is difficult to determine the local concentration of released AA at a precise moment in time, intracellular concentrations of 50–100 μM have been measured in activated cells (Wolf *et al.*, 1986; Nishikawa *et al.*, 1988).

Tau proteins are predominantly expressed in neuronal tissue (Lee 1990; Mandelkow and Mandelkow, 1993). However, the above examples of the potential linkage between AA and PLC activation include both neuronal and non-neuronal cells. Furthermore, we have shown that non-neuronal tissues also contain protein components that can activate PLC-γ1.[2] Such activating proteins purified from bovine lung are also resistant to heat and acid treatment, exhibit extensive size heterogeneity, and activate PLC-γ isozymes relatively specifically in the presence but not in the absence of AA.[2] However, the lung proteins are larger than tau proteins and are not recognized by antibodies to tau. These observations suggest that the putative linkage of $cPLA_2$ activation to PLC-γ activation may not be restricted to neuronal cells.

Activation by tau and AA was relatively specific for PLC-γ isozymes at physiological (submicromolar) concentrations of Ca^{2+}. This specificity may be attributable to the unique structural features of PLC-γ isozymes. Unlike PLC-β and PLC-δ isozymes, PLC-γ isozymes each contain a Src homology 3 (SH3) domain, which is characterized by the ability to bind proline-rich sequences. Tau proteins possess several sequences rich in proline; two sequences, PTPPTR and RTPPKSP, encoded by exon 9 are similar to the two classes of consensus SH3-binding sequences, PPLP*X*R and RXLPP*X*P (critical prolines are underlined; *X* indicates any amino acid; other residues are partially conserved), respectively (Feng *et al.*, 1994). The two consensus sequences were derived for the Src and phosphatidylinositol 3-kinase SH3 domains and may differ from that for the PLC-γ SH3 domain. However, attempts to co-immunoprecipitate PLC-γ1 and tau from bovine brain cytosol were not successful (data not shown). It is also of interest that the neurofibrillary tangles typical of the brains of individuals with Alzheimer's disease consist largely of tau proteins that are abnormally phosphorylated, probably by microtubule-associated protein kinase and glycogen synthase kinase 3, at Ser-Pro and Thr-Pro motifs (reviewed by Mandelkow and Mandelkow (1993)). Tau contains 17 Ser-Pro and Thr-Pro motifs, three of which are present in the putative SH3-binding sequences PTPPTR and RTPPKSP. It is possible that phosphorylation of these sites alters interaction of tau with PLC-γ, thereby undermining the PLA_2-PLC linkage, in brains affected by Alzheimer's disease.

All PLC isozymes have a pleckstrin homology (PH) domain near their amino terminus (Noh *et al.*, 1995). PLC-γ isozymes, unlike other PLC isoforms, possess another PH domain that is split by the SH domain. Although one function of PH domains appears to be to bind PIP_2 (Harlan *et al.*, 1994), alignment of 92 such domains identified to date revealed marked sequence diversity, and there is neither a conserved surface patch nor a cavity in the known structures that could help identify regions crucial for a common function (Hyvönen *et al.*, 1995). The overall topology of the PH domain has been suggested to be similar to those of fatty acid-binding proteins (Yoon *et al.*, 1994). It is therefore possible that AA interacts with one of the two PH domains of PLC-γ and cooperates with tau bound to the SH3 domain to enhance enzyme activity.

In conclusion, our observation that tau proteins together with AA activate PLC-γ activity *in vitro* suggests that receptor-mediated activation of $cPLA_2$ might result in the activation of PLC-γ in neuronal cells. Such a link between the two phospholipase pathways could provide for activation of phosphoinositide metabolism in the absence of or in coordination with direct receptor-mediated stimulation of a PLC enzyme.

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