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Tau Protein Mediated Activation of Phospholipase C-γ Isozyme is Dependent on the Presence of Arachidonic Acid

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We have previously reported tyrosine kinase-independent activation of phospholipase C (PLC)-γ isozymes. The proteins that activated PLC-γ 1 have been purified from bovine brain and were identified as members of the tau family of microtubule-associated proteins[1]. Activation of PLC-γ isozymes is dependent on the composition of various lipids. The presence of unsaturated fatty acids such as arachidonic acid (AA) markedly enhances tau protein induced activation of PLC-γ while saturated fatty acids being ineffective in stimulating the activation. Maximal (15- to 20-fold) activation was observed in the presence of 15 μM tau protein with 25 μM AA. The effect of tau and arachidonic acid induced activation was specific and most prominent for PLC-γ isozymes in the presence of submicromolar concentrations of Ca^{2+}. The effect was inhibited by the presence of phosphatidylcholine. Other polyunsaturated fatty acids, such as linoleic, linolenic, oleic and palmitoleic acids in the substrate mixture, induced similar activation but the effects were less prominent.

These results suggest that, in tissues where tau protein is present, the receptors and signals that are coupled to the phospholipase A_2 may activate PLC-γ isozymes indirectly, without tyrosine phosphorylation, through hydrolysis of phosphatidylcholine and by generating AA, in the presence of tau protein. This process may work to amplify the signals and provide the linkage between the two different phospholipase systems.

Key Words: Tau Protein, Arachidonic Acid Dependence, Activation of Phospholipase C-γ

INTRODUCTION

During the precess of cellular transmembrane signal transduction, it is well recognized that the hydrolysis of a relatively minor component of the phospholipid bilayer, phosphatidylinositol 4,5-biphosphate (PIP_2) is one of the earliest, yet a key initiating events following the extracellular signal stimulation of the transmembrane receptors. The activation of PLC and resulting hydrolysis of PIP_2 and subsequent regulation of cellular functions occurs in response to over 100 different extracellular signaling molecules[2]. This reaction results in generation of two important second messengers, namely inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 binds to a specific receptors and releases Ca^{2+} from intracellular storage sites such as endoplasmic reticulum[3] and diacylglycerol activates protein kinase C[4].

There are ten mammalian isozymes of PLC identified and cloned to this date. The PLC isozymes are classified into 3 separate subfamilies. These are PLC-β type (PLC-β 1, -β 2, -β 3, and -β 4), PLC-γ type (PLC-γ 1 and -γ 2), and PLC-δ type (PLC-δ 1, -δ 2, -δ 3, and -δ 4). They are divided on the basis of their amino acid sequence homologies and differences[5]. Each isozymes are products of discret genes and structurally distinct enough to be differentiated and yet share many common characteristics to be grouped into the subfamilies. The specific features of the different PLC isozymes are known to be related to specific mechanisms whereby they are activated. PLC-γ isozymes are activated by phosphorylation of their specific tyrosine residues upon receptor stimulation through receptors with intrinsic tyrosine kinase activities seen in many growth factor receptors, and by non-receptor tyrosine kinases, most of which are recognized

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as cellular proto-oncogenes. In contrast, β subfamily of PLC isozymes are activated by q family of heterotrimeric G-proteins[6] but the mechanisms of PLC-δ isozyme activation is not known.

Recently, we have reported a tyrosine kinase-independent mechanism of activation of PLC-γ isozymes and identified the activator as microtubule-associated protein, tau[1]. Tau protein was purified from the bovine brain cytosol by a series of liquid chromatographic steps based on its ability to markedly enhance the phosphatidylinositol (PI)-hydrolyzing activity of PLC-γ 1 when added to PLC reaction system. The stimulation of PLC activity by tau protein was specific for PLC-γ isozymes and was dependent on the presence of Ca^{2+} in the reaction system. Also we have described in our previous report that the enhancing activity of tau on PLC-γ was preferential toward PI substrate and the activation was markedly reduced toward its physiologic substrate PIP_2[1].

We now describe that the effect of tau on PLC-γ activity toward PIP_2 is greatly increased in the presence of arachidonic acid and other unsaturated fatty acids. The marked increase in hydrolysis of PIP_2 substrate by tau protein in the presence of AA was most prominent in PLC-γ and was inhibited by the presence of phosphatidylcholine in the system. These findings suggest that tau protein in cooperation with arachidonic acid, produced as a result of phospholipase A_2 stimulation, can serve as a link between the two different phospholipase pathways and provide the means for the signal amplification and cross-talk within the cells.

MATERIALS AND METHODS

Materials

[^{3}H] PI and [^{3}H] PIP_2 were purchased from Du Pont-New England Nuclear, PI from Sigma, and PIP_2 from Boehringer Mannheim. Phosphatidylcholine (PC), lyso-phosphatidylcholine (Lyso-PC), phosphatidylserine (PS), phosphatidyl- ethanolamine (PE) were obtained from Avanti Polar-Lipids (Alabaster, Al). Cholesterol and AA were acquired from Calbiochem (La Jolla, CA). Other free fatty acids used in the experiment were purchased from Jansen Chimica (Geel, Belgium).

Purification of Tau Proteins

Tau protein used in this experiment was prepared and purified from fresh bovine brain by the methods described elsewhere[7] with a slight modification. All manipulations were performed at 4 °C or 6 °C in a refrigerated room or on ice unless otherwise indicated. Three fresh bovine brains (each weighing about 350 g) were used. Briefly, the purification involved 45% ammonium sulfate fractionation, CM-Sephadex 50 (Pharmacia, Piscataway, NJ) column chromatography, and 2.5% perchloric acid precipitation. The resulting purified tau protein was dialyzed extensively to change the buffer to 20 mM Hepes-NaOH (pH 7.4) 0.1 mM DTT 1 mM EGTA and was loaded onto the TSKgel heparin HPLC column (7.5 x 75 mm, TosoHaas) and was eluted with a linear NaCl gradient and active fractions collected as described previously[1]. The resulting active fractions were aliquoted and stored at -70°C for future use.

Preparation 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 isozymes as described elsewhere[8]. The purified PLC enzymes were kindly provided by Dr. Sue Goo Rhee from the Laboratory of Cell Signaling, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, U.S.A.

PLC Activity Assays and Tau Protein Activation of PLC

PLC activity assays and tau protein mediated activation of PLC enzymes were performed as previously described[1] with a minimal modification. Briefly, the substrate mixture containing different lipid compositions were dissolved in chloroform solution. It was then dried under a stream of nitrogen gas, resuspended in 50 mM Hepes-NaOH (pH7.0) and 0.1% (w/v) deoxycholate by sonication. The resulting reaction mixture (200 μl) contained mixed lipid micelles in various compositions, calcium buffer to give a desired Ca^{2+} concentration, purified PLC enzyme (20~50 ng), with or without tau proteins[9].

The reaction mixture was then incubated at 30°C for 10 minutes. After the incubation, the reaction was terminated by adding 1 ml of 1:1 (v/v) mixture of chloroform and methanol. Following termination, after brief centrifuge, 0.5 ml of upper aqueous phase was collected and was counted for [^{3}H]-radioactivity on liquid scintillation counter spectroscopy.

However when both [^{3}H]PI and [^{3}H]PIP$_2$ were used in the same substrate, aqueous supernatant was first separated on an anion-exchange chromatography column to distinguish [^{3}H]IP and [^{3}H]IP$_3$ and the resulting [^{3}H]-radioactivity counted independently as described below in more detail.

In addition, in order to maintain the stimulated PI- and PIP$_2$-hydrolyzing activity in the linear range of the assay, we adjusted the amount of PLC enzyme so that an unstimulated, basal activity will be in the range of 500 to 1,200 cpm of [^{3}H]IP$_3$ generated.

Effect of Various Fatty Acids on the Activation of PLC-γ 1 by Tau

In order to investigate the effect of AA on PI and PIP$_2$ hydrolysis catalyzed by PLC-γ 1 mediated by tau protein, we included PE, PS, and cholesterol in the substrate to mimick the composition of cell membranes. Moreover, an equal concentration of both [^{3}H] PI and [^{3}H] PIP$_2$ were used in the same substrate. So the substrate system contained PI, PIP$_2$, PS, cholesterol, and PE in a molar ratio of 1:1:1:1:4. After the hydrolysis of the substrate by PLC-γ 1, the resulting [^{3}H] inositol 1-phosphate ([^{3}H]IP) and [^{3}H]IP$_3$ generated were separated by the anion-exchange chromatography on Dowex AG1X-2 resin (BioRad) and counted separately in order to study the substrate preference of tau and AA when both PI and PIP$_2$ were present in the substrate simultaneously. PC was not included in the substrate system because it was found to inhibit tau-dependent activation of PLC-γ 1. AA in the absence of tau was tested for its effect on the PLC-γ 1 as a negative control.

We have also tested the effect of other saturated and unsaturated fatty acids other than AA on PIP$_2$ hydrolysis of PLC-γ 1 either in the absence or presence of 0.3 μM concentration of tau proteins. In place of AA, other fatty acids in 25 μM concentration, such as arachidic, linolenic, linoleic, oleic, stearic, palmitoleic, and plmitic acids were added in the micellar substrate and the tau-mediated stimulation of PIP$_2$ hydrolyzing activity of PLC-γ 1 was compared.

Effect of Tau Concentration on the PIP$_2$ Hydrolyzing Activity of PLC-γ 1

The dependence of the PIP$_2$ hydrolyzing activity of PLC-γ1 on the concentration of tau protein was examined with the mixed micellar substrate containing 25 μM AA. Steadily increasing concentrations of tau proteins (0 to 0.4 μM) were added to the reaction system described above and tau protein mediated activation of PIP$_2$ hydroysis by PLC-γ 1 was counted. The concentration of tau to achieve a maximal activation of PIP$_2$ hydrolysis, thus found, was compared with the optimal tau concentration required for the stimulation of PI hydrolysis in the absence of AA and other lipids as we have previously reported[1].

Isozyme Specificity of Tau- and AA-Dependent Activation of PIP$_2$ Hydrolysis by PLC-γ 1

Combined effect of tau protein and AA on PIP$_2$ hydrolysis of PLC-β 1, -β 2, -γ 1, -γ 2, -δ 1, and -δ 2 isozymes were compared in the lipid micellar substrate as described above. The reactions were performed at 0.1 and 1.0 μM Ca^{2+} in either absence or presence of tau proteins.

Effect of PC on Tau- and AA-Dependent PLC-γ 1 Activity

Various concentrations of PC were introduced into the mixed micellar substrate containing [^{3}H] PI, [^{3}H]PIP$_2$, PS, AA, cholesterol, AA, and PE in a molar ratio of 1:1:1:1:1:4, where one part corresponds to a concentration of 30 μM in either presence or absence of tau proteins. PI and PIP$_2$ hydrolysis were measured separately as described above. In addition, the effect of Lyso-PC on tau protein-mediated activation of PLC-γ 1 was also tested.

In order to study the influence of increasing AA concentration resulting from an activation of phospholipase A$_2$ and subsequent conversion of PC into AA and Lyso-PC, the effect of a stepwise increase in AA and Lyso-PC concentrations with corresponding complementary decrease in PC concentration was tested in the fixed mixed micellar substrate consisting of 30 μM each of [^{3}H] PIP$_2$, PS, cholesterol, and 120 μM of PE. The resulting [^{3}H] PIP$_2$ was counted for PIP$_2$-hydrolyzing activity.

RESULTS

Effect of AA on Tau-Mediated Activation of PLC-γ 1

AA affected neither the PI- nor PIP$_2$-Hydrolyzing activity of PLC-γ 1 when added alone to the reaction mixture in the absence of tau protein. However, AA greatly enhanced both PI- and PIP$_2$-hydrolysis of PLC-γ 1 in a concentration

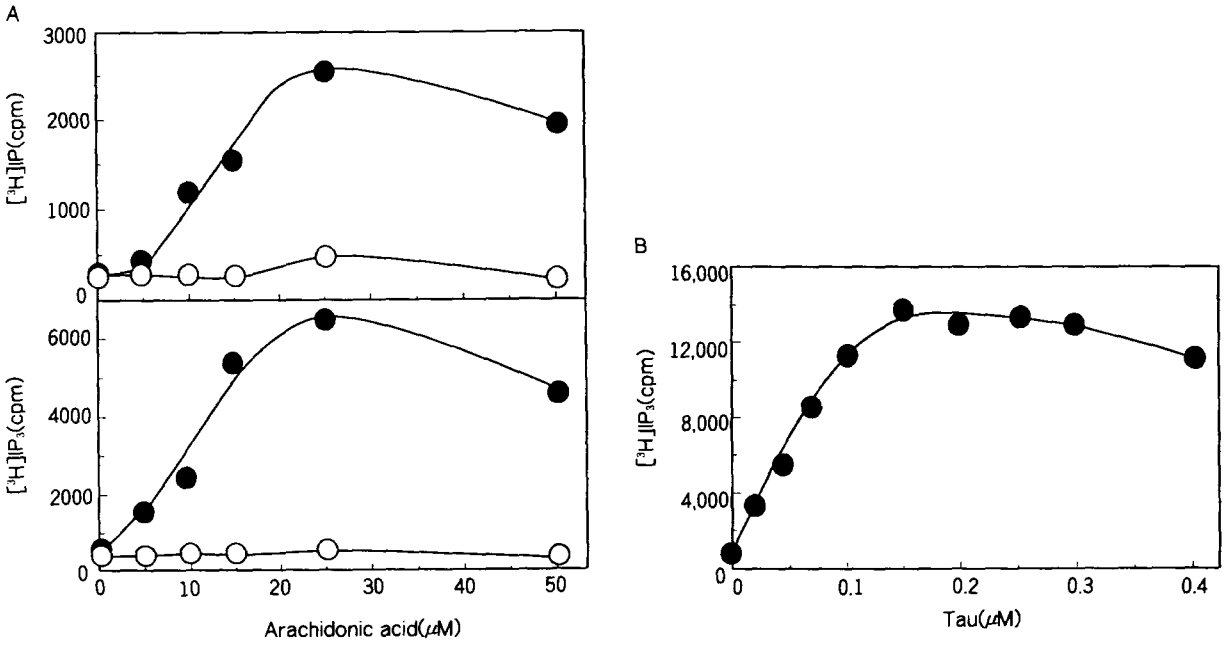

Fig. 1. Effects of AA and Tau on the Activity of PLC-γ 1 toward a Mixed Micellar Substrate Containing PS, Cholesterol, and PE. (A) The PI-hydrolyzing (*upper panels*) and PIP_2-hydrolyzing (*lower panels*) activities of PLC-γ 1 were measured in the absence (*open circles*) or presence (*closed circles*) 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 0.1% deoxycholate. The final assay mixture (200 μl) contained 50 ng of PLC-γ 1, 0.3 μM tau, 30 μM each of [^{3}H]PI (30,000 cpm), [^{3}H]PIP2 (30,000 cpm), PS, and cholesterol, 120 μM PE, and the indicated concentrations of AA in 50 mM Hepes-NaOH (pH7.0), 0.1% deoxycholate, 2 mM $MgCl_2$, 2 mM EGTA, and 1 μM free Ca^{2+}. After incubatin for 10 min at 30 ° C, the reaction was terminated by an addition of 1 ml of 1:1 mixture of chloroform and methanol. After a brief centrifugation, upper aqueous phase was the applied to a 0.5 ml column of Dowex AG1X-2 anion-exchange resin (formate form). The column was then washed with 3 ml of distilled water. [^{3}H]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. Then the resulting [^{3}H]-radioactivity was independently counted for [^{3}H]PI and [^{3}H]PIP_2 with a liquid scintillation counter spectroscopy. (B) Dependence of the PIP_2-Hydrolyzing Activity of PLC-γ 1 in the Presence of AA on Tau Concentration. The PIP_2-hydrolyzing activity of PLC-γ 1 (50 ng per assay) was measured at various concentrations of tau proteins as described above in panel A, with the exception that the micellar substrate contained a fixed concentration of AA and was devoid of [^{3}H] PI.

dependent manner when introduced into the mixed micellar substrate in the presence of tau. For both hydrolyzing activities, the maximal intensity of activation was achieved with 25 μM AA concentration but at higher AA concentrations the effect of activation was decreased (Fig. 1A.). A similar decrease in PLC-γ 1 activation at higher concentrations of unsaturated fatty acids was also observed in crude cytosolic extracts of brain tissue in the experiment of Irvine et al[10].

Effect of Tau Concentration on the AA Dependent Activation of PLC-γ 1

The dependence of PIP_2-hydrolyzing activity of PLC-γ 1 on tau concentration was examined with mixed micellar substrate system containing 25 μM AA (Fig. 1B.). Maximal activation was obtained with 0.15 μM tau concentration. The requirement for the tau protein was significantly less when we compare the results to 0.6 μM tau protein that was needed to obtain maximal activation of PI-hydrolysis in 0.1% deoxycholate substrate in the absence of AA and other lipids, as we have previously reported[1].

Effect of Various Fatty Acids on Tau-Dependent PLC-γ 1 Activation

We have also examined the effect of other fatty acids other than AA on PIP_2-hydrolysis by PLC-γ 1 carried out in the presence of 0.3 μM tau (Fig. 2.). Unsaturated fatty acids such as AA, linolenic acid, linoleic acid, oleic acid, and palmitoleic

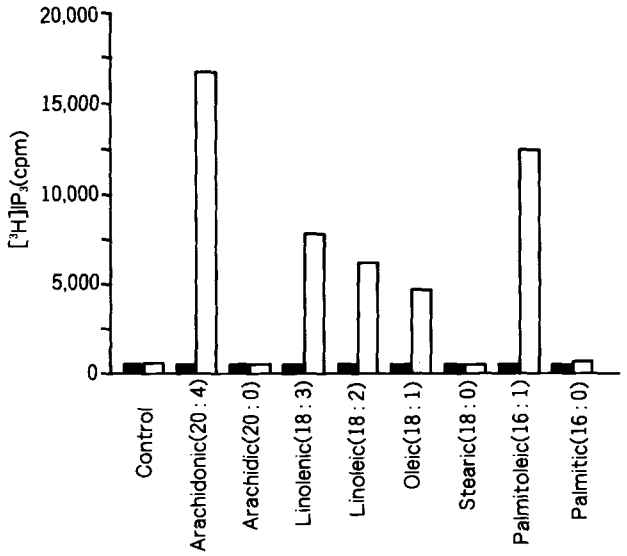

Fig. 2. Effects of Various fatty Acids on the PIP2-Hydrolyzing Activity of PLC-γ 1. The PIP_2-hydrolyzing activity of PLC-γ 1 (50 ng per assay) was measured in the absence (*open bars*) or presence (*solid bars*) of 3 μM tau with mixed micellar substrates containing the indicated fatty acid at a final concentration of 30 μM (control, no fatty acid). Otherwise, the assay conditions were the same as described in Fig. 1B. Data shown above are the means of duplicate and are representative of two similar experiments.

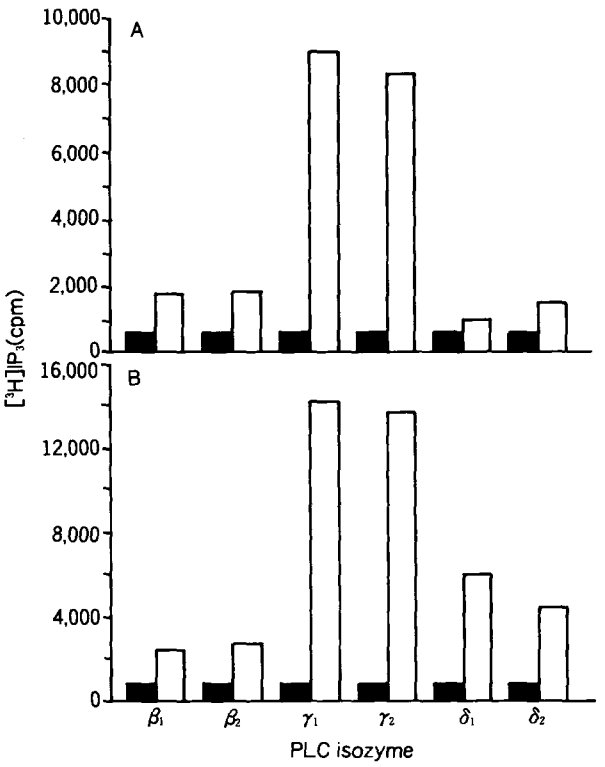

Fig. 3. Combined effects of Tau and AA on the PIP_2-Hydrolyzing Activity of Various PLC Isozymes. The PIP_2-hydrolyzing activities of the indicated PLC isozymes (20 to 100 ng per assay) were measured either at 0.1 μM (A) or 1 μM (B) free Ca^{2+} in the absence (*open bars*) or presence (*solid bars*) of 0.3 μM tau with a mixed micellar substrate containing 30 μM AA, as described in Fig. 1B. The results are means of triplicate determinations and are a representation of two similar experiments.

acid stimulated PIP_2-hydrolyzing activity in the presence of tau proteins. However, no activation was observed in the absence of tau protein. In contrast to these findings, corresponding saturated fatty acids counterpart including arachidic acid, stearic acid, and palmitic acid had no effect on PIP_2-hydrolyzing activity of PLC-γ 1 either in absence or in presence of tau protein (Fig. 2).

Isozyme Specificity of Tau and AA Dependent Activation of PIP2 hydrolysis by PLC-γ 1

At 0.1 μM Ca^{2+} concentration, only the activation of PLC-γ 1 and -γ 2 were apparent while with other PLC isozymes the activation was not noticeable (Fig. 3A). However, at 1 μM Ca^{2+} concentration, tau and AA was able to stimulate PLC-δ isozymes but to a somewhat less extent, amounting to 30 to 50% of thoses seen in the PLC-γ isozymes at same Ca^{2+} concentration (Fig. 3B).

Effect of PC and Lyso-PC on Tau and AA Dependent Activation of PLC-γ 1 Activity

Adding PC in the mixed micelle substrate consisting of [^{3}H]PI, [^{3}H]PIP_2, PS, cholesterol, AA, and PE in a molar ratio of 1:1:1:1:1:4 resulted in concentratin dependent inhibition of both PI and PIP_2 hydrolyzing activity of PLC-γ 1 when tested even with the maximal activating concentration of tau proteins (Fig. 4A.).

Half-maximal inhibition was apparent at 30 to 40 μM concentration of PC. However, no inhibitory effect of PC was observed in the absence of tau protein (Fig. 4A). Lyso-PC in 30 μM concentration inhibited PIP_2 hydrolysis of PLC-γ 1 either in the absence or in the presence of tau protein by less than 10% (Data not shown).

Conversion of PC by phospholipase A_2 will release AA and an equimolar concentration of Lyso-PC while producing a corresponding decrease in PC concentration. In order to mimick this effect, a mixed micellar susbstrate which con-

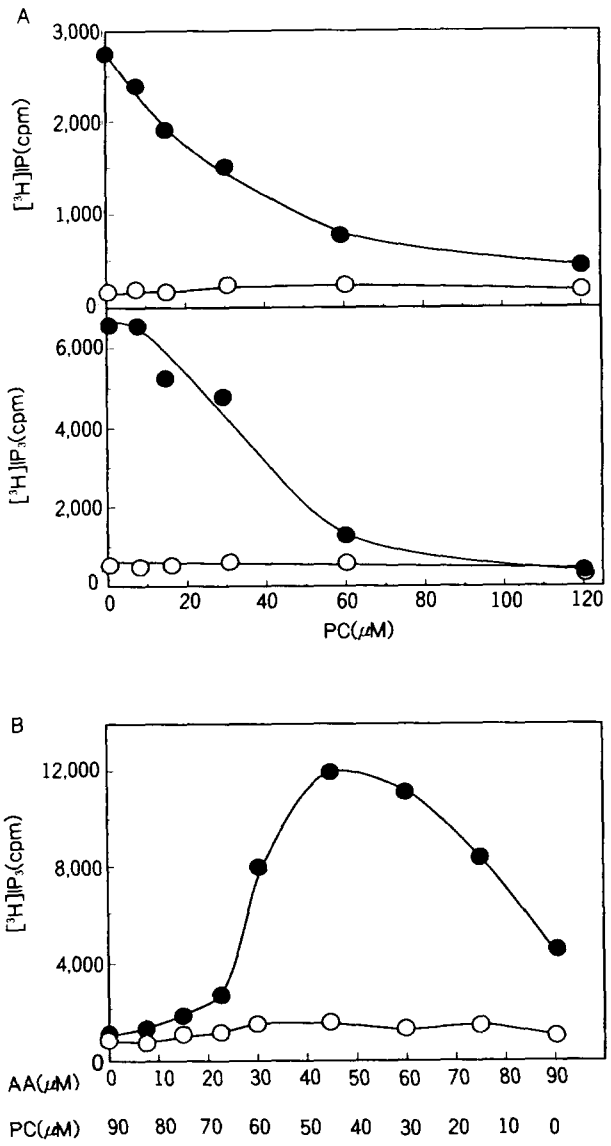

Fig. 4. Effects of PC on Tau- and AA-Dependent PLC-γ 1 Activity. (A) The PI-hydrolyzing (*upper panel*) and PIP_2-hydrolyzing (*lower panel*) activities of PLC-γ 1 were measured in the absence (*open circles*) or presence (*closed circles*) of 0.3 μM tau with mixed micellar substrates containing 30 μM AA and various concentrations of PC in addition to [^{3}H]PI, [^{3}H]PIP_2, PS, cholesterol, and PE as described in Fig. 1A. (B) The PIP_2-hydrolyzing activity of PLC-γ 1 (50 ng per assay) was measured in the absence (*open circles*) or 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 Fig. 1B. The data are the means of duplicate determinations and are a representation fo three similar experiments.

tained a stepwise increments in concentrations of AA (eg. 0, 10, 20, 30 μM, etc) and a corresponding stepwise decrease in PC concentration (eg. 90, 80, 70 μM, etc) while maintaining the total concentration of both agents at constant 90 μM was devised. Noticeable activation of PLC-γ 1 by tau protein was not apparent until PC concentration fell below 70 μM and corresponding AA release was above 20 μM. Maximal activation was achieved when both agents, PC and AA, were present in equimolar ratio at 45 μM concentration. With higher concentrations of AA, the tau protein mediated activation of PLC-γ 1 tend to decline (Fig. 4B).

DISCUSSION

Irvine et al[10] showed that unsaturated fatty acids such as oleic acid and AA stimulated PLC activity in crude brain cytosol with a [^{3}H] inositol-labelled 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-γ 1 (unpublished data of the authors). The brain cytosol preparation used by Irvine et al[10] most likely contained tau proteins in addition to PLC-γ 1, the most abundant PLC isozymes in brain cytosol, and tau proteins thus may have mediated the effect of unsaturated fatty acids on PLC activity.

We have previously reported the purification of tyrosine kinase-independent PLC-γ activators from bovine brain cytosol and identified them as tau isoforms[1]. It had a preferential activity toward PI hydrolysis and tau-mediated activation of PLC was specific for PLC-γ 1 and -γ 2. Tau comprises a family of microtubule-associated proteins that are generated from alternatively spliced transcripts derived from a single gene with 13 exons[11]. Tau expression is largely restricted to brain and neuronal tissues 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[12].

Tau proteins themselves did not have any catalytic activity and did not show any PLC activity toward micellar PIP_2. Furthermore, when common lipid components of membranes, such as PE, PS, and cholesterol, were incorporated into micelles, activation of PLC-γ 1 by tau observed toward PI in 0.1% deoxycholate was not apparent with either the PI or PIP_2

as substrate. 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 most effective activator. The Efficacy of activation appeared to decrease in the rank order of palmitoleic acid (16:1) > linolenic acid (18:3) > oleic acid (18:1). The corresponding saturated counter parts such as arachidic acid (20:0), stearic acid (18:0), and palmitic acid (16:0) were ineffective. Maximal (15- to 20-fold) activation of PLC-γ 1 toward PIP_2 in micelles containing 30 μM each of PIP_2, PS, and cholesterol with 120 μM PE was observed at 0.15 μM tau in the presence of 25 μM AA concentration.

Several studies have examined the effects of lipids on PLC activity. Unsaturated fatty acids were shown to increase PLC activity in rat brain cytosol about 10 fold[10]. The effect probablely attributable to the presence of both tau and PLC-γ 1 in the brain cytosol. For both PLC-γ 1 and tau proteins are abundant in rat brain cytosol. An approximately three- to four-fold activation by unsaturated fatty acids of a 68-kDa PLC purified from rat liver cytosol was also observed when hydrolysis of micellar PI was measured in the presence of 2 mM Ca^{2+} [13]. It is now thought that the 68-kDa enzyme was a proteolytic fragment of PLC-δ 1 [14]. Saturated fatty acids had no effect on brain or liver PLC activity.

Abundant membrane phospholipids such as PC, PE, and PS were shown to have no marked effect on the activities of PLC-β isozymes [15] and PLC-γ 1 [16] in detailed kinetic studies performed with mixed micellar PIP_2 substrates. An approximately three-fold activation by PS was observed for PLC-β 1, PLC-γ 1 and PLC-δ 1 with a monolayer substrate containing PIP_2, whereas PC did not have any effect[17]. However, marked inhibition of the PLC-δ 1 activity by PC was observed with PI presented as small unilamellar vesicles[18]. These studies suggest that the activity of PLC, like that of many enzymes that act on lipid substrates, depends on the composition and physical conditions of the substrate.

In our present study, PC had no significant effect on basal PLC-γ 1 activity but markedly inhibited activity stimulated by tau and AA. This observation suggested that the activation of PLC-γ 1 by tau proteins might be facilitated by a concomitant decrease in PC concentration and increase in AA concentration. These changes occur in cells upon activation of the 85-kDa cytosolic PLA_2 ($cPLA_2$) that is known to be coupled to a variety of receptors[19~21]. 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 [22]. In contrast, secreted PLA_2 enzymes with molecular sizes of 13 to 18 kDa require millimolar concentrations of Ca^{2+} for the catalytic activity. they are known to show a preference for PE and are non-selective with regard to sn-2 fatty acids. A 40-kDa Ca^{2+}-independent PLA_2 identified in myocardium preferentially hydrolyzes AA-containing PC[23], whereas an 80-kDa Ca^{2+}-independent PLA_2 from macrophages lacks specificities for AA-containing lipids[19].

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[21,24]. 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[20]. 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[16] observed that incorporation of phosphatidic acid into a micellar substrate containing PIP_2 and Triton X-100 enhanced PLC-γ 1 activity 40-fold; they therefore proposed that PLC-γ 1 can be activated independently of tyrosine phosphorylation if phosphatidic acid is generated by the action of phospholipase D.

Evidences also suggest that the activation of $cPLA_2$ may occur at basal cytosolic Ca^{2+} concentration; that is, independently of PLC-mediated IP_3 generation[25,26]. Addition of bombesin to Swiss 3T3 cells resulted in the rapid (within 2 seconds) release of AA and concomitant depletion of PC, without effects on other phospholipids[25]. The initial AA release was dependent on neither the influs of extracellular Ca^{2+} nor the mobilization of intracellular 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 treated with tumor necrosis factor-α were all independent of PLC activation[26]. 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 many cells and tissues. Incubation of human trophoblasts with AA stimulates PLC activity[27]. Further studies with these cells suggested that the stimulation of phosphoinositide metabolism and placental lactogen release are mediated by initial activation of PLA2[28]. AA, but not other biologically important fatty acids, stimulates phosphoinositide metabolism and catecholamine release from bovine adrenal chromaffin cells[29]. AA was also shown to increase phosphoinositides breakdown and glutamate release in rat hippocampal tissue[30], to induce phosphoinositide breakdown and diacylglycerol generation in human platelets[31], and to increase intracellular Ca^{2+} by mobilizing an IP_3-sensitive Ca^{2+} pool in an isolated rat pancreatic islets[32] and a human leukemic T cell lines[33]. The AA-induced Ca^{2+} release was shown to be not due to the metabolites of AA[32].

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[34]. AA and other unsaturated fatty acids activate protein kinase C directly[4,35,36]. Furthermore, AA has been shwon to modulate both guanlyate cyclase and Ca^{2+}, calmodulin-dependent protein kinase. 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 to 100 μM have been measured in activated cells.

Tau proteins are predominantly expressed in neuronal tissues[11,40]. However, the above examples of the potential linkage between AA and PLC activation include both neuronal and non-neuronal cells[32,39]. Moreover, we have found that non-neuronal tissues also contain protein components that can activate PLC-γ1 (unpublished data of the authors). Such activating proteins purified from bovine lungs 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 (unpublished data of the authors). However the lung activator-poteins are larger than tau proteins and are not recognized by the antibodies to tau. These obsevations suggest that the putative linkage of $cPLA_2$ activation may not be restricted to the neuronal cells.

Activation by tau and AA was relatively specific for PLC-γ isozymes at physiological, submicromolar concentrations of Ca^{2+}. In the absence of Ca^{2+}, no activation was observed. This specificity may be attributable to the unique structural features of PLC-γ isozymes. Unlike PLC-β and PLC-δ isozymes, PLC-γ isozymes contain a *src* homology 3 (SH3) domain, which is characterized by the ability to bind proline-rich sequences. Tau protein possess several sequences rich in prolines; two sequences, PTPPTR and RTPPKSP, encoded by exon 9 are similar to the two classes of consensus SH3-binding sequences, PPLPXR and RXLPPXP (critical prolines are underlined; X, any aminoacid; other residues are partially conserved), respectively[41]. 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 succesful (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 and insoluble, probablely by mitogen-activated protein kinase and glycogen synthase kinase 3, at Ser-Pro and Thr-Pro motifs[40,42]. 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 may alter interaction of tau with PLC-γ, thereby causing derangement of the PLA_2 and PLC linkage, in brains affected by Alzheimer's disease.

All PLC isozymes have a pleckstrin homology (PH) domain near their amino terminus[2]. PLC-γ isozymes, unlike other PLC isoforms, possess another PH domains that is split by the SH domain. Although one function of PH domains appears to be bind PIP_2[43], alignment of 92 such domains identified to date revealed marked sequence diversities, 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[44]. The overall topology of the PH domain has been suggested to be similar to those of fatty acid-binding proteins[45]. 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.

CONCLUSION

In conclusion, our observation that tau proteins in concerted action 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 and tissues. Such a link between the two phospholipase pathway, 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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