Inhibition of Muscarinic Receptor-linked Phospholipase D Activation by Association with Tubulin*

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Mammalian phospholipase D (PLD) is considered a key enzyme in the transmission signals from various receptors including muscarinic receptors. PLD activation is a rapid and transient process, but a negative regulator has not been found that inhibits signal-dependent PLD activation. Here, for the first time, we report that tubulin binding to PLD2 is an inhibition mechanism for muscarinic receptor-linked PLD2 activation. Tubulin was identified in an immunoprecipitated PLD2 complex from COS-7 cells by peptide mass fingerprinting. The direct interaction between PLD2 and tubulin was found to be mediated by a specific region of PLD2 (amino acids 476–612). PLD2 was potently inhibited (IC50 <10 nM) by tubulin binding in vitro. In cells, the interaction between PLD2 and tubulin was increased by the microtubule disrupting agent nocodazole and reduced by the microtubule stabilizing agent Taxol. Moreover, PLD2 activity was found to be inversely correlated with the level of monomeric tubulin. In addition, we found that interaction with and the inhibition of PLD2 by monomeric tubulin is important for the muscarinic receptor-linked PLD signaling pathway. Interaction between PLD2 and tubulin was increased only after 1–2 min of carbachol stimulation when carbachol-stimulated PLD2 activity was decreased. The expression of the tubulin binding region of PLD2 blocked the later decrease in carbachol-induced PLD activity by masking tubulin binding. Taken together, these results indicate that an increase in local membrane monomeric tubulin concentration inhibits PLD2 activity, and provides a novel mechanism for the inhibition of muscarinic receptor-induced PLD2 activation by interaction with tubulin.

Mammalian phospholipase D (PLD) hydrolyzes membrane phosphatidylcholine to generate phosphatidic acid and choline. PLD activity is dramatically activated in response to a variety of signals, including hormones, neurotransmitters, and growth factors (1). PLD products, phosphatidic acid itself or the hydrolyzed product diacylglycerol, have been known to act as intracellular lipid second messengers and to be involved in multiple physiological events, such as, the promotion of mitogenesis, stimulation of respiratory bursts, secretory processes, and actin cytoskeletal reorganization (2–7). Therefore, signal-dependent PLD activity must be tightly controlled in cells.

Many reports have been issued about the mechanisms of receptor-mediated PLD activation. Although the mammalian PLD isozymes, PLD1, and PLD2 have some different regulatory properties, in general, agonist-induced PLD is activated by various protein kinases, including protein kinase C, protein-tyrosine kinase, and the MAP kinase family, in addition to small G proteins of the ARF, Rho, and Ras families (8–13). The signal-dependent activation of PLD is rapid and transient. Although, the activation kinetics depend on the stimulus and cell type, the PLD signal is usually diminished within 10 min (14). However, the mechanisms involved in PLD signal inhibition remain unknown. Signaling protein must be tightly regulated with respect to duration and strength (15). Some inhibitors of PLD activity have been reported (16–25), but the roles of these inhibitors in signal-dependent PLD activity has not been demonstrated, and inhibition of signal dependent PLD activity by a negative regulator has not been reported.

Members of the muscarinic acetylcholine receptor family (M1–M5) are considered to play important roles in various neurological processes such as learning, memory, emotion, perception, and cognition both in the central nervous system and the body periphery (26, 27). These receptors are members of a family of receptors that are coupled to heterotrimeric transducer G proteins. The M1, M3, and M5 acetylcholine receptor subtypes are efficiently coupled to the pertussis toxin-insensitive Goq11 and Gia subtype G proteins, leading to activations of PLC and PLD, whereas the M2 and M4 receptors are coupled to pertussis toxin-sensitive Gq and Gi subtype G proteins (27–29).

The mechanism of PLD activation by the muscarinic receptor has been mainly studied for the M1 receptor subtype. PLD activation by the M1 receptor is mediated by members of the ARF and protein-tyrosine kinase, protein kinase C, and Rho GTPase families (29–34). Muscarinic receptor-stimulated PLD activity has been reported in several cell types, including submandibular and lacrimal gland acini, neuroblastoma cells, and tracheal smooth muscle cells (35). In most cell types, carbachol-stimulated PLD activation is a very rapid and transient process, i.e. diminished within 2 min (36, 37). However, the inhibition mechanisms of muscarinic receptor-linked PLD activity have not been elucidated.

In this report, we found that a major component of the cytoskeleton proteins, tubulin, is a PLD2 binder and inhibitor. Furthermore, we show the dynamic inhibition of PLD2 activity

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The abbreviations used are: PLD, phospholipase D; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PIP2, phosphatidylinositol 4,5-bisphosphate; GST, glutathione S-transferase; PBS, phosphate-buffered saline; Taxol, paclitaxel; Pipes, 1,4-piperazinediethanesulfonic acid.

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by tubulin in muscarinic receptor signaling, suggesting a new mechanism for a G-protein-coupled receptor-PLD linkage.

**EXPERIMENTAL PROCEDURES**

**Materials**—Enhanced chemiluminescence kits (ECL system), glutathione-Sepharose 4B, and chelating-Sepharose were purchased from Amersham Biosciences; [H]myristic acid from PerkinElmer Life Sciences; dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylethanolamine, rhodamine-conjugated goat anti-rabbit IgG, fluorescein isothiocyanate-conjugated goat anti-mouse IgG, anti-β-tubulin monoclonal antibody, nocodazole, Taxol, and carbachol from Sigma. Anti-β-tubulin monoclonal antibody was purchased Hybridoma Bank (Iowa, IA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgA, IgM, and IgG were obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD); Silica Gel 60 thin-layer chromatography plates from Merck (Darmstadt, Germany); Protein A-Sepharose from RepliGen (Needham, MA); Dulbecco’s modified Eagle’s medium from Invitrogen; and purified bovine brain tubulin from Cytoskeleton (Denver, CO). The polyclonal antibody recognizing PLD1 and PLD2 was generated as described previously (10).

**Cell Culture and Transfection—**COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum and wild type PC12 cells were maintained in RPMI medium supplemented with 10% (v/v) equine serum and 5% (v/v) fetal bovine serum at 37 °C in a humidified, CO2-controlled (5%) incubator. PLD2 overexpressing PC12 cells were prepared using the tetracycline-regulated expression system (TET-OFF, Invitrogen), as described previously (38). Clonal cell lines were usually maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) equine serum and 5% (v/v) fetal bovine serum. For transfection and transient expression of PLD, cells were plated at a density of 1 × 10⁶ cells/well in 100-mm dishes and transfected using Lipofectamine (Invitrogen), as described previously (39).

**Co-immunoprecipitation of PLD-binding Proteins—**Cultured cells were harvested and lysed with PLD assay buffer (50 mM Hepes/NaOH, pH 7.4, 2 mM CaCl₂, 0.5 mM EGTA, 50 mM KCl, 80 mM NaCl, 0.5% Triton X-100, 1% cholic acid, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 5 μg/ml aprotinin. After a brief sonication, the lysates were centrifuged at 100,000 × g for 1 h, and the cell extracts were incubated, respectively, with immobilized anti-PLD antibody over night. The precipitates were washed four times and subjected to SDS-PAGE followed by immunoblot analysis. For silver staining, PLD and binding proteins were eluted from the immune complexes with antigen peptide of PLD antibody, as previously reported (10).

**Protein Identification by Peptide Mass Fingerprinting—**The technique used was as described previously (26). In brief, the fraction containing the 55-kDa protein (p55) after co-immunoprecipitation from COS-7 cells was separated by SDS-PAGE, and the band corresponding to p55 was excised and digested with trypsin (Roche Molecular Biochemicals) for 4 h and washed twice with phosphate-buffered saline (PBS) and scraped. The cells were then incubated with 0.2 ml of 0.5% Triton X-100 containing microtubule stabilizing buffer (2% glycerol, 0.1 mM Pipes, pH 7.1, 1 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 0.1 mM dithiothreitol, 250 mM sucrose, 2 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin), and immediately sonicated. Each sample was centrifuged at 600 × g at 4 °C. Supernatants were centrifuged at 20,000 × g at 4 °C. The pellet were collected and used as the membrane fraction.

**Monomeric Tubulin Extraction—**Monomeric tubulin was extracted from vector and PLD₂-transfected COS-7 cells plated on 100-mm dishes, as described previously (43). Following treatments as indicated, cell monolayers were washed twice with phosphate-buffered saline (PBS) and scraped. The cells were then incubated with 0.2 ml of 0.5% Triton X-100 containing microtubule stabilizing buffer (2% glycerol, 0.1 mM Pipes, pH 7.1, 1 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 0.1 mM dithiothreitol, 250 mM sucrose, 2 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin), and immediately sonicated. Each sample was centrifuged at 600 × g at 4 °C. Supernatants were centrifuged at 20,000 × g at 4 °C. The pellet were collected and used as the membrane fraction.

**In Vitro PLD Activity Assay—**In vitro PLD activity was determined, as described previously (39). In brief, vector or human PLD₂-transfected COS-7 cells were cultured for 48 h. The cells were then loaded with [H]myristic acid (10 μCi/ml) for 4 h and washed twice with Dulbecco’s modified Eagle’s medium. The loaded cells were treated carbachol with 0.4% butanol, scraped into 0.8 ml of methanol and 1 ml NaCl (1:1), and mixed with 0.4 ml of chloroform. The organic phases were dried, and the lipids were separated by thin-layer chromatography on silica gel plates. The PLD activity of PLD, overexpressing PC12 cells was determined using the same procedures. The amount of [H]phosphatidylbutanol formed was expressed as a percentage of total [H]-lipid to account for cell labelling efficiency differences.

**Immunoblot Analysis—**Proteins were denatured by boiling for 5 min at 95 °C in a Laemmli sample buffer (42), separated by SDS-PAGE, and immunoblot analysis was performed as described previously (17). Delayed ion extraction resulted in peptide masses with better than 50 parts/million mass accuracy on average. Using the amino acid sequence of the 55-kDa protein (p55) after co-immunoprecipitation from COS-7 cells was separated by SDS-PAGE, and the band corresponding to p55 was excised and digested with trypsin (Roche Molecular Biochemicals) for 4 h. Masses tryptic peptides were measured to p55 was excised and digested with trypsin (Roche Molecular Biochemicals) for 4 h and washed twice with phosphate-buffered saline (PBS) and scraped. The cells were then incubated with 0.2 ml of 0.5% Triton X-100 containing microtubule stabilizing buffer (2% glycerol, 0.1 mM Pipes, pH 7.1, 1 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 0.1 mM dithiothreitol, 250 mM sucrose, 2 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin), and immediately sonicated. Each sample was centrifuged at 600 × g at 4 °C. Supernatants were centrifuged at 20,000 × g at 4 °C. The pellet were collected and used as the membrane fraction.

**Confocal Microscopy—**Cells were treated as above and washed with PBS. Cells were resuspended in lysis buffer (20 mM Tris, pH 7.5, 5 mM dithiothreitol, 250 mM sucrose, 2 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin), and immediately sonicated. Each sample was centrifuged at 600 × g at 4 °C. Supernatants were centrifuged at 20,000 × g at 4 °C. The pellet were collected and used as the membrane fraction.

**Immunocytochemistry—**Immunocytochemical analysis was performed as described previously (44). In brief, PLD₂-transfected PC12 cells plated on coverslips were rinsed with cold PBS four times, and fixed with 4% (w/v) paraformaldehyde overnight at 4 °C. After rinsing with PBS and blocking with PBS containing 1% goat serum and 0.1% Triton X-100 for 30 min at room temperature, the cells were incubated with 2 μg/ml mouse β-tubulin monoclonal antibodies and rabbit PLD polyclonal antibodies for 2 h at room temperature. After washing six times with PBS, fluorescein isothiocyanate-conjugated goat anti-mouse antibodies and rhodamine-conjugated goat anti-rabbit antibodies were incubated with the cells for 1 h to allow visualization of tubulin and PLD. After a further six washings with PBS, the slides were examined under a confocal microscope (Zeiss, Germany).

**RESULTS**

**55-kDa Protein Precipitated with PLD₂ from COS-7 Cell Was Identified as α,β-Tubulin—**To find the binding partners of PLD, we started our investigation by looking for cellular PLD-binding proteins in transiently PLD₂-overexpressing COS-7 cells. To find the specific binding partner of PLD₂ after the precipitation with anti-PLD antibody, PLD₂ were eluted with antigen peptide of PLD antibody, and resolved by SDS-PAGE, and visualized by silver staining. As a result, we found that the co-precipitate contained PLD₂-binding proteins with relative molecular masses of 55,000 (p55) and some other binding proteins (Fig. 1A). To identify the PLD₂-interacting protein, p55 was excised from the polyacrylamide gel, digested with trypsin, and the cleaved peptide mixture was then subjected to
peptide mass fingerprinting by MALDI-TOF mass spectrometry. Fig. 1B shows the mass spectrum of the digested peptides of p55. The masses obtained were compared with proteins in the Swiss-Prot data base using the MS-Fit peptide mass search program. The peptides were found to have molecular masses that were almost identical to the calculated molecular masses of the corresponding theoretically predicted tryptic peptides of α- and β-tubulin. This peptide search result was performed at an accuracy of 50 parts/million, and the analyzed peptides covered 24% of the α-tubulin, and 50% of the β-tubulin sequence. To substantiate the identity of these proteins further, the presence of tubulin in the PLD2 precipitate was confirmed using a monoclonal antibody specific to β-tubulin. As shown in Fig. 1C, p55 was blotted by β-tubulin monoclonal antibody. Based on these results, we concluded that the p55 interacting with PLD2 from COS-7 cells is a α,β-tubulin heterodimer (monomeric tubulin).

**Tubulin Directly Interacts with PLD2**—To determine whether tubulin associates directly with PLD2, 99% pure monomeric tubulin was incubated with the PLD2. As shown in Fig. 2A, tubulin coprecipitated with PLD2 in a concentration-dependent manner, demonstrating that tubulin binds directly to PLD2. To identify the PLD2 sequence involved in tubulin binding, we constructed GST fusion proteins as shown in Fig. 2B, and tested them for their ability to bind to purified tubulin. GST-PLD2 (amino acids 476–612) was identified as the region that most potently bound to tubulin (Fig. 2C). Therefore, it appears that the region of the protein between amino acids 476 and 612 is important for direct interaction with tubulin.

**Tubulin Inhibits PLD2 Activity in Vitro**—Because tubulin binds to PLD2, the effect of tubulin on the activity of PLD2 was examined. As shown in Fig. 3, tubulin inhibits PLD2 activity in vitro in a concentration-dependent manner. The concentration required for half-maximal inhibition was determined to be ~2 nM in a PIP2-dependent PLD activity assay. To exclude the possibilities that the inhibition of PLD2 activity by tubulin is caused by PIP2 sequestration, we also performed a PLD2 activity assay in the absence of PIP2. Previously, we reported that PLD2 activity is stimulated by oleate in vitro (40). PLD2 activation by oleate was found to be progressively inhibited by increasing the tubulin concentration with an IC50 of ~10 nM. These results suggest that PLD2 activity is inhibited by direct interaction with tubulin.

**Microtubule Dynamics Affect the Interaction of PLD2 with Tubulin and PLD2 Activity in COS-7 Cells**—To test whether microtubule dynamics affect interaction between PLD2 and tubulin and that of PLD activity in cells, we transfected the PLD2 genes into COS-7 cells, and treated them with nocodazole or Taxol to change the cellular monomeric tubulin concentrations. Nocodazole depolymerizes microtubules and causes the monomeric tubulin concentration to increase. On the other hand, Taxol polymerizes microtubules and causes a monomeric tubulin concentration to decrease. These results suggest that PLD2 activity is inhibited by direct interaction with tubulin.

**Carbachol Increase PLD2-Tubulin Interaction when Carbachol-induced PLD Activity Was Decreased**—Previous studies have indicated that the muscarinic receptor agonist, carbachol, rapidly causes microtubule depolymerization and translocation of tubulin to the plasma membrane (45). To elucidate whether PLD2 activity can be regulated by tubulin by stimulating the muscarinic receptor signaling pathway, we monitored the carbachol-induced PLD2 activity in M3 acetylcholine receptor-expressed COS-7 cells. PLD2 activity was saturated in the presence of 100 μM carbachol (Fig. 5A). Under this condition,
maximal PLD activity was obtained after 1 min but PLD activity was rapidly inhibited and reached baseline levels 2 min after carbachol was treated (Fig. 5B). Interestingly, the interaction between PLD2 and tubulin was weak at baseline, but increased after 2 min of carbachol stimulation when PLD activity was inhibited (Fig. 5C). These results suggest that carbachol increases the association between PLD2 with tubulin to inhibit PLD activity in COS-7 cells.

**Tubulin Binding Inhibits Muscarinic Receptor-linked PLD Activity**—Tubulin directly interacts with PLD2 via the F3 region of PLD2 (Fig. 2B). The F3 region of PLD2 can interfere with the interaction between PLD2 and tubulin in vitro but the F2 region of PLD2 as a negative control cannot interfere with this interaction (Fig. 6A). To demonstrate muscarinic receptor-induced PLD activity inhibition by tubulin, we transfected the F3 region to mask the interaction between PLD2 and tubulin in COS-7 cells. We found that the F3 region expression did not affect the basal PLD activity of COS-7 cells, but that it prolonged carbachol-induced PLD activation (Fig. 6B). In vector and the F2 region of PLD2-transfected cells, maximal PLD activity was obtained after 1 min of carbachol stimulation and this was rapidly diminished within 2 min. In cells expressing the F3 region of PLD2, maximal PLD activity occurred at the same time, but the later decline in PLD activity was retarded. These results suggest that carbachol-stimulated PLD activity is inhibited by tubulin-PLD2 interaction and that the F3 region of PLD2 inhibits the interaction between PLD2 and tubulin.

**Carbachol Induces PLD2-Tubulin Interaction in Concert with PLD Activity Inhibition in PC12 Cells**—To examine whether the interaction between PLD2 and tubulin is changed by stimulating endogeneous muscarinic receptor, we used the PLD2 inducible PC12 cell line (38). PC12 cells have endogeneous muscaric receptor and PLD activation by carbachol stimulation has been reported in PC12 cells (37). In these cells, PLD activity rapidly increased up to 0.5 min and then was reduced to basal level within 1 min after carbachol stimulation (Fig. 7A). Interestingly, interaction between PLD2 and tubulin was elevated 1 min after carbachol stimulation in PC12 cells, showing the same PLD activity decreasing time as COS-7 cells (Fig. 7B).
These results indicate that the PLD₂ activity regulating mechanism via tubulin interaction exists in endogenous muscarinic receptor possessing cells.

**Carbachol Stimulation Causes the Translocation and Co-localization of Tubulin with PLD₂ at the Plasma Membrane**—Next, to confirm whether co-localization between PLD₂ and tubulin can be induced by activating muscarinic receptors, we analyzed the cellular localization of tubulin by confocal laser microscopy. Because endogenous PLD₂ was not seen in PC12 cells, we transfected the PLD₂ gene into wild type PC12 cells and found that in these cells, PLD₂ was localized at the plasma membrane (Fig. 8A2). Tubulin was not seen at the plasma membrane, and most of the tubulin was localized in the cytosol (Fig. 8A1). However, after carbachol stimulation, some tubulin redistributed to areas along the plasma membrane and co-localized with PLD₂ (Fig. 8B). To test whether carbachol-induced tubulin redistribution was caused by microtubule depolymerization, PC12 cells were treated with either nocodazole or Taxol and tubulin localization was checked. In nocodazole-treated PC12 cells, tubulin colocalized with PLD₂ at the plasma membrane region (Fig. 8C), but in Taxol-treated cells, tubulin was absent at the plasma membrane and was not colocalized with PLD₂ (Fig. 8D). To clarify whether tubulin was translocated toward the membrane in response to carbachol stimulation, we
isolated membrane fractions and quantified membrane-associated tubulin by immunoblotting. Carbachol was found to induce a rapid and time-dependent increase in tubulin recruitment to the membranes of the PC12 cells (Fig. 8E), and this recruitment increased after 1 min of carbachol stimulation. Taken together, these results suggest that carbachol stimulation induces rapid microtubule depolymerization and tubulin translocation to the plasma membrane.

**DISCUSSION**

Although the precise time frame of PLD activation is dependent on stimulus and cell type, transient PLD activation has been commonly observed. PLD activation has largely been studied in the context of the activation mechanism of PLD; however, the inhibition mechanism of agonist-induced PLD activity has not been elucidated. Although, some inhibitors of PLD activity have been reported, the signal-dependent inhibition of PLD activity by its negative regulator after agonist stimulation has not been previously reported. In the present study, we report that tubulin dynamically interacts with PLD2 to inhibit the carbachol-induced PLD2 activation. This is the first example of the inhibition of agonist-induced PLD signal-

**Inhibition of Carbachol-induced PLD2 Activity by Tubulin**

![Figure 6](http://www.jbc.org/)

**Figure 6.** The F3 region (amino acids 476–612) of PLD2 blocked the later decrease in carbachol-induced PLD2 activity. A, the same amounts of PLD2 (0.2 μg)-bound immune complex were incubated with 0.3 μM purified tubulin. The F3 and F2 region of PLD2 were then added in increasing amounts (0, 0.05, 0.2, 1, 3 μM) and incubation was continued at 37°C for 20 min, as described under “Experimental Procedures.” 3 μM GST was added as 0 control. After precipitation and washing, the final precipitates were subjected to immunoblot (I.B.) analysis using antibody-directed anti-PLD2 or anti-tubulin. Data are representative of three independent experiments. B, COS-7 cells were co-transfected with M3 muscarinic receptor and GFP-vector or M3 muscarinic receptor and the GFP-F3 region of PLD2 or M3 muscarinic receptor and the GFP-F2 region of PLD2. After serum starvation for 24 h, cells were stimulated with carbachol (100 μM) for 0, 0.5, 1, 2, or 5 min, then 1-butanol was added, and incubation was continued for an additional 30 s, as described under “Experimental Procedures.” The data shown are the mean ± S.E. of three independent experiments.

Muscarinic acetylcholine receptor signaling is inhibited by the uncoupling of this receptor from its G protein and receptor internalization to intracellular compartments (46–48). This type of down-regulation is usually mediated by the phosphorylation of the activated receptor by members of the G protein-coupled receptor kinases. Phosphorylated receptors then interact with cytoplasmic proteins termed β-arrestins, which interfere with receptor-G protein interaction, favoring receptor endocytosis, thus inhibiting the signal (49, 50). Generally muscarinic receptor-linked PLD activity is inhibited rapidly within 2 min, but the completion of muscarinic receptor phosphorylation and internalization events are required for a longer time (51, 52). Therefore, muscarinic receptor-linked PLD activity might be inhibited by another mechanism. In this work, we report for the first time that tubulin acts as a negative regulator on the muscarinic receptor in association with PLD2 signaling. Several lines of evidence support this notion. First, tubulin purified from bovine brain directly interacted with PLD2 and inhibited its activity in a concentration-dependent manner in
activity by inhibiting tubulin binding to PLD2 (Fig. 6). These results suggest that tubulin plays an inhibitory role in the inhibition of carbachol-induced PLD2 activity.

Several studies have suggested that PLD activity is regulated by negative regulators. Many negative regulators of PLD activity have been identified, including fodrin (16), α-actinin (17), actin (19), gelsolin (18), amphiphysin (21), aldolase (20), α-β-synuclein (24), synaptotagmin (22), clathrin assembly protein 3 (23), and collapsin response mediator protein-2 (25). However, the roles of these inhibitors in signal-dependent PLD activity has not been demonstrated. Recently our group reported that Munc-18-1 directly inhibits PLD activity (53). In this report, epidermal growth factor treatment was found to trigger the dissociation of Munc-18-1 from PLD, and the inhibitory role of Munc-18-1 upon basal PLD activity, in a signal-dependent manner, was suggested. However, until now, no negative regulator for the inhibition of signal-dependent transient PLD activation has been reported. In the present report, we suggest that tubulin is the first identified inhibitor to inhibit signal-dependent PLD activity by dynamic interaction.

PIP2 has been established as an allosteric activator of PLD in vivo and in vitro (41, 54). Although many proteins inhibit PLD activity via direct interaction, some inhibitory proteins, such as fodrin and synaptotagmin may sequester or hydrolyze PIP2 to suppress PLD activity (16, 22). The inhibitory effect of tubulin on PLD2 is affected by the presence or absence of PIP2 (Fig. 5). In PIP2-dependent PLD activity assays, tubulin inhibits PLD2 at lower concentrations than in oleate dependent assays. These results are consistent with the inhibition by tubulin being caused, at least in part, by PIP2 sequestration. However, this may not be the case, because nanomolar tubulin is insufficient to sequestrate PIP2 in assay vesicle (2.33 μM). Tubulin interacts directly with amino acids 476–612 (between CR II and CR III including a part of CR III) regions of PLD2 (Fig. 4B). Previously, it has been reported that the Arg545 and Arg558 motifs between II and III are important for PIP2 binding (54). In the report, PLD2 mutants R545G and R558G cannot interact with PIP2 and cannot be activated by PIP2. From this result, we speculate that interaction of tubulin with PLD2 may block to the PIP2 binding of PLD2 so tubulin more potently inhibits PLD2 activity in the presence of PIP2.

In cells, tubulin exists in a polymerized form (microtubule) and monomeric tubulin in an αβ-heterodimer form, and PLD2 can bind to both the polymerized form and the monomeric tubulin form in vitro (data not shown). However, microtubule is a cytosolic structure protein, and PLD2 a membrane-bound protein. Although, membrane- and phospholipid-associated tubulin have been reported (55–57), it appears that this membrane tubulin is similar to the monomeric form (55, 57, 59). This notion is supported by observations of the COS-7 cells treated with the microtubule stability regulating pharmacological agents nocodazole and Taxol. Nocodazole promotes microtubule depolymerization and increases monomeric tubulin concentrations, whereas Taxol induces microtubule assembly and stabilizes the microtubule structure, reducing monomeric tubulin concentrations. In nocodazole-treated COS-7 cells, as the interaction between PLD2 and tubulin increased, basal PLD2 activity was inhibited, and in Taxol-treated cells the opposite results were obtained (Fig. 4). In fact, in unstimulated PC12 cells, PLD2 shows minimal colocalization with tubulin at the plasma membrane (Fig. 8A), but nocodazole treatment induced its translocation to the plasma membrane and colocalization of tubulin and membrane PLD2, whereas in the case of Taxol treatment no tubulin translocation to the membrane or colocalization with membrane PLD2 was observed (Fig. 8, C and D). These data suggest that in cells, PLD2 interacts with mono-
meric tubulin and that its activity is regulated by changes in cellular monomeric tubulin concentration. It has been reported that the acetylcholine muscarinic receptor can regulate microtubule dynamics (45, 49). These studies show that microtubule depolymerization and rapid tubulin translocation to the plasma membrane occur within 1 min of carbachol treatment in SK-N-SH cells. In PC12 cells, we also observed an increased translocation of tubulin to the membrane after carbachol treatment (Fig. 8, B and E). It is suggested that Gαs and Gαi, G-proteins under the control of muscarinic receptors, bind tubulin and stimulate GTPase activity to destroy the GTP cap on G-proteins under the control of muscarinic receptors, bind tubulin and stimulate GTPase activity to destroy the GTP cap on microtubules (60). Moreover, neurotransmitter-mediated activation of PLC would increase local Ca2+ concentrations (58, 61, 62). These reports explain why the interaction between PLD2 and tubulin increases after carbachol stimulation.

In conclusion, the present study identifies a novel signaling pathway between PLD2 and the microtubule structure, it is also the first example of the inhibition mechanism of agonist-induced PLD activation. Although the precise cellular meaning of this action remains to be elucidated, these findings may provide new insight into the regulation of PLD activity in a variety of cellular processes related to microtubule structure.

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