Phosphatidylinositol 4,5-Bisphosphate Modifies Tubulin Participation in Phospholipase C β_1 Signaling

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Tubulin forms the microtubule and regulates certain G-protein-mediated signaling pathways. Both functions rely on the GTP-binding properties of tubulin. Signal transduction through G α_q -regulated phospholipase C β_1 (PLC β_1) is activated by tubulin through a direct transfer of GTP from tubulin to G α_q . However, at high tubulin concentrations, inhibition of PLC β_1 is observed. This report demonstrates that tubulin inhibits PLC β_1 by binding the PLC β_1 substrate phosphatidylinositol 4,5-bisphosphate (PIP₂). Tubulin binding of PIP₂ was specific, because PIP₂ but not phosphatidylinositol 3,4,5-trisphosphate, phosphatidylinositol 3-phosphate, phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine, or inositol 1,4,5-trisphosphate inhibited microtubule assembly. PIP₂ did not affect GTP binding or GTP hydrolysis by tubulin. Muscarinic agonists promoted microtubule depolymerization and translocation of tubulin to the plasma membrane. PIP₂ augmented this process in both Sf9 cells, containing a recombinant PLC β_1 pathway, and SK-N-SH neuroblastoma cells. Colocalization of tubulin and PIP₂ at

the plasma membrane was demonstrated with confocal laser immunofluorescence microscopy. Although tubulin bound to both G α_q and PLC β_1 , PIP₂ facilitated the interaction between tubulin and PLC β_1 but not that between tubulin and G α_q . However, PIP₂ did augment formation of tubulin–G α_q –PLC β_1 complexes. Subsequent to potentiating PLC β_1 activation, sustained agonist-independent membrane binding of tubulin at PIP₂- and PLC β_1 -rich sites appeared to inhibit G α_q coupling to PLC β_1 . Furthermore, colchicine increased membrane-associated tubulin and also inhibited PLC β_1 activity in SK-N-SH cells. Thus, tubulin, depending on local membrane concentration, may serve as a positive or negative regulator of phosphoinositide hydrolysis. Rapid changes in membrane lipid composition or in the cytoskeleton might modify neuronal signaling through such a mechanism.

Key words: tubulin; phospholipid; microtubule; cytoskeleton; G-protein; phospholipase C; muscarinic receptor; acetylcholine; G-protein-coupled receptor; calcium; protein kinase C

The phosphatidylinositol 4,5-bisphosphate (PIP₂)-specific phospholipase C (PLC) enzymes transduce signals by generating two second messengers, inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol. The β isoforms of these enzymes, PLC β_{1-3} , are activated by the α subunit of the G-protein G q (for review, see Rhee and Bae, 1997). Among the G-protein-coupled receptors linked to activation of G α_q are the m1, m3, and m5 muscarinic receptor subtypes.

The microtubule protein tubulin is involved in the control of G-protein-mediated signal transduction (Wang et al., 1990; Popova et al., 1994; Roychowdhury and Rasenick, 1994; Ravindra et al., 1996; Cote et al., 1997a,b). Association of tubulin with certain G α subunits and the subsequent regulation of adenylyl cyclase and PLC β_1 signaling has been reported (Popova et al., 1994, 1997; Yan et al., 1996, 2001; Popova and Rasenick, 2000). After m1 muscarinic receptor stimulation *in vitro* (Popova et al., 1997) and *in vivo* (Popova and Rasenick, 2000), cytosolic tubulin translocates to the plasma membrane. Membrane-associated tubulin regulates PLC β_1 activation both in a positive and negative man-

ner (Popova et al., 1997). At low (nanomolar) concentrations, tubulin activates PLC β_1 , whereas at higher concentrations, enzyme inhibition is observed.

Previous studies have indicated that transactivation of G α_q , through a direct GTP transfer from tubulin, is responsible for PLC β_1 activation by tubulin (Popova et al., 1997; Popova and Rasenick, 2000). However, the mechanism behind the inhibition of PLC β_1 , observed at high dimeric tubulin concentrations, has not been elucidated.

Tubulin binds PIP₂, and this inhibits microtubule polymerization (Popova et al., 1997). Because PIP₂ is the PLC preferred substrate, sequestration of PIP₂ by tubulin should also affect important phosphoinositide-dependent signaling pathways. By analogy, several actin-binding proteins, such as profilin, gelsolin, and CapG, have already been shown to bind PIP₂ and to modulate the activity of regulatory PLC isozymes both *in vitro* (Goldschmidt-Clermont et al., 1990, 1991; Banno et al., 1992; Steed et al., 1996; Sun et al., 1997) and *in vivo* (Sun et al., 1997). The binding of PIP₂ by the above-mentioned proteins appears to prevent phospholipase access to this substrate (Goldschmidt-Clermont et al., 1990, 1991; Banno et al., 1992; Steed et al., 1996; Sun et al., 1997). Because high tubulin concentrations inhibit PLC β_1 *in vitro*, a similar inhibitory mechanism was suggested (Popova et al., 1997).

This study was designed to evaluate the interaction between tubulin and PIP₂ and test how this interaction affects G α_q and PLC β_1 activation at the membrane. The results reveal that PIP₂ binding to tubulin is specific but does not affect the binding and

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hydrolysis of GTP by tubulin. Although activated muscarinic receptors recruit tubulin from the cytosol to the membrane, leading to $G_{\alpha q}$ transactivation, receptor-independent binding of tubulin to PIP_2 -rich sites on the membrane obstructs $PLC\beta_1$ activation. Thus, it appears that tubulin and PIP_2 interact to effect a dual regulation of $PLC\beta_1$. Such a mechanism might prove important in regulating the response and responsiveness of G-protein-mediated phospholipid signaling in neuronal and glial cells.

MATERIALS AND METHODS

Baculovirus-directed expression of signaling proteins in Sf9 cells. Sf9 cells were maintained in Sf-900 II SFM media (Invitrogen, Carlsbad, CA) as described previously (Popova et al., 1997). They were infected with baculoviruses bearing the m1 muscarinic receptor, $G_{\alpha q}$, or $PLC\beta_1$ cDNAs, as was done previously (Popova et al., 1997). The construction of recombinant baculoviruses was already reported previously (Parker et al., 1991; Graber et al., 1992; Boguslavsky et al., 1994). Cells were harvested after 65 hr, and membranes were prepared and frozen in liquid nitrogen for subsequent use as described previously (Popova et al., 1994, 1997). Protein concentrations were determined by Coomassie blue binding (Bradford, 1976). Bovine serum albumin was used as a standard. Protein expression was measured by immunoblotting. Antisera specific for the m1 muscarinic receptor (number 71; from G. Luthin, MCP Hahnemann University, Philadelphia, PA), $G_{\alpha q}/11$ (number 0945; from D. Manning, University of Pennsylvania, Philadelphia, PA), and $PLC\beta_1$ (anti-holoenzyme; from S. G. Rhee, National Institutes of Health, Bethesda, MD) were used at a dilution of 1:500. Biotinylated goat anti-rabbit IgG or anti-mouse IgG and streptavidin-alkaline phosphatase conjugate were used for detection. Densitometry was performed to evaluate the expression levels (Storm 840; Molecular Dynamics, Sunnyvale, CA; Popova et al., 1997; Popova and Rasenick, 2000). m1 muscarinic receptor density was determined by [3H]L-quinuclidinyl [phenyl-4(n)]benzilate ([3H]QNB) binding (Popova et al., 1997).

Tubulin preparations. Microtubule proteins were isolated (Shelanski et al., 1973), and tubulin preparations purified free of microtubule-associated proteins by phosphocellulose chromatography were prepared as described previously (Wang and Rasenick, 1991). Phosphocellulose-purified tubulin (PC-tubulin) was >95% pure as determined on SDS-PAGE.

$P^3(4\text{-azidoanilido})\text{-P1-5'-GTP}$ (AAGTP) and [^{32}P]AAGTP were synthesized as described previously (Rasenick et al., 1994). Tubulin-[^{32}P]AAGTP was made from PC-tubulin as indicated (Rasenick and Wang, 1988). The final preparations contained 0.4–0.6 mol of nucleotide bound/mol of tubulin. Tubulin-[^{32}P]AAGTP concentrations used throughout the study were based on the protein concentration.

To prepare tubulin labeled covalently with fluorescein-5-maleimide (FM-tubulin), FM (Molecular Probes, Eugene, OR) was incubated with PC-tubulin at a 5:1 molar ratio at 37°C for 30 min in polymerization buffer [100 mM 1,4-piperazinediethanesulfonic acid (Pipes), 2 mM EGTA, 4 mM $MgCl_2$, 1 mM GTP, pH 6.9, and 1 M glutamate]. The reaction was quenched with 1 mM β -mercaptoethanol, and the samples were layered onto warm 40% sucrose containing 1 mM GTP and centrifuged at $200,000 \times g$ at 37°C for 30 min. The FM-tubulin pellet was washed twice with warm buffer and depolymerized on ice, followed by chromatography through a P6-DG column (Bio-Rad, Hercules, CA) twice to remove free FM. The calculated ratio of FM labeling of tubulin was 1:1. FM-tubulin was polymerization-competent as tested by electron microscopy performed as described previously (Popova et al., 1997).

Microtubule assembly. To test the effects of various phospholipids on microtubule assembly, phosphocellulose-purified tubulin (1.5 mg/ml) was incubated in a bath sonicator for 15 min at 4°C with different phosphoinositides, IP_3 , or heparin (as indicated), at a molar ratio of 1:6 in polymerization buffer (in mM: 100 Pipes, 2 EGTA, 3 $MgCl_2$, and 1 GTP, pH 6.9). The assembly reaction was performed for 1 hr at 37°C in a shaking water bath. The polymer mass was isolated by centrifugation at $150,000 \times g$ for 30 min at 37°C, followed by separation of the pellets and the supernatants. Pellets were resuspended in identical amounts of cold polymerization buffer on ice, and protein concentrations were measured by the method of Bradford (1976) using BSA as a standard. The amount of protein in the pelleted polymer mass without any additions (control) was 0.47 ± 0.10 mg/ml. The depolymerized pellets were subjected to SDS-PAGE and immunoblotting with a monoclonal anti- α -tubulin anti-

body (Santa Cruz Biotechnology, Santa Cruz, CA) and ECL detection (Amersham Biosciences, Piscataway, NJ). The results were analyzed in a Storm 840 imaging system (Molecular Dynamics). Samples from microtubule polymerization reactions were also examined by electron microscopy (Popova et al., 1997) to evaluate the effect of lipids on microtubule assembly.

To study whether the effect of PIP_2 on microtubule assembly was concentration-dependent, polymerization of phosphocellulose-purified tubulin (2.5 mg/ml) was monitored by turbidity measurement at 350 nm in a Beckman DU 640B spectrophotometer at 37°C. PIP_2 , phosphatidylcholine (PC), or vesicles of PIP_2 and PC (at a molar ratio of 1:1) were mixed with tubulin (tubulin/lipid molar ratio of 1:6) in polymerization buffer on ice to a final volume of 300 μ l. Samples were transferred to a quartz cuvette, and the increase in absorbance was monitored at 37°C.

GTPase activity of tubulin. To determine the amount or the species of the guanine nucleotide bound to tubulin and the extent of GTP hydrolysis, phosphocellulose-purified tubulin was made nucleotide-free by incubation with charcoal (Rasenick and Wang, 1988). This tubulin was then incubated with 0.2 mM [^{32}P]GTP for 30 min on ice. After two passes through Bio-Gel P6-DG columns to remove unbound nucleotide, tubulin GTPase activity was determined. Tubulin-[^{32}P]GTP was incubated for 30 min at 30°C, followed by nucleotide analysis by thin-layer chromatography (TLC) on polyethyleneimine cellulose as described previously (Roychowdhury and Rasenick, 1994). The chromatograms were developed in 0.35 M NH_4HCO_3 . The spots containing GTP or GDP standards were visualized with a UV lamp, and the plate was exposed to film for autoradiography or subjected directly to phosphorimage analysis (Storm 840; Molecular Dynamics). When indicated, phosphoinositides (at a molar ratio of 6:1) were added to tubulin-[^{32}P]GTP before incubation (Popova et al., 1997).

Analysis of nucleotide bound to tubulin. Phosphocellulose-purified tubulin was loaded with [^{32}P]AAGTP or [^{32}P]GTP, as described above, in the presence or absence of phosphoinositides (tubulin/phosphoinositide ratio of 1:6). [^{32}P]AAGTP-labeled samples were subjected to TLC, followed by autoradiography or phosphorimage analysis, as indicated. Tubulin samples labeled by [^{32}P]GTP were subjected to P6-DG column chromatography, and the radioactivity of 5 μ l of each tubulin-[^{32}P]GTP eluate was measured by liquid scintillation counting.

To test whether PIP_2 caused dissociation of the guanine nucleotide bound to tubulin, PIP_2 was added at the end of the binding reaction. The samples were kept on ice for an additional 30 min before being processed as described above.

Phosphoinositide preparation. Phosphoinositides were evaporated under a stream of nitrogen, sonicated for 5 min (at appropriate concentrations) in assay buffer on ice, and used immediately.

Photoaffinity labeling. Membranes from Sf9 or SK-N-SH cells were incubated with the indicated concentrations of tubulin-[^{32}P]AAGTP, PIP_2 , and carbachol as described previously (Popova et al., 1994, 1997). After UV irradiation and centrifugation, membrane pellets were dissolved in Laemmli buffer and subjected to SDS-PAGE as done previously (Popova et al., 1994, 1997). Gels were either stained (Coomassie blue) or subjected to Western blotting, followed by autoradiography (XAR-5 film; Eastman Kodak Co., Rochester, NY) or phosphorimaging. Densitometric measurements of autoradiograms and phosphorimage analysis of the gels were performed, respectively (Storm 840; Molecular Dynamics). Tubulin-[^{32}P]AAGTP and Sf9 membranes, overexpressing $G_{\alpha q}$, were run along the samples to identify the bands of tubulin and $G_{\alpha q}$. As shown previously (Popova and Rasenick, 2000), carbachol-evoked membrane association of tubulin and $G_{\alpha q}$ transactivation by tubulin were consistently reversed by atropine.

Immunoprecipitation. Sf9 cells were infected separately or simultaneously (according to the experimental design) with baculoviruses bearing the m1 muscarinic receptor, $G_{\alpha q}$, or $PLC\beta_1$ cDNA as described previously (Popova et al., 1997). Membrane preparations were extracted with 1% sodium cholate for 1 hr at 4°C and constant stirring (Popova et al., 1997). After centrifugation at $20,000 \pm g$ at 4°C the extracts (0.5 mg/ml membrane protein) were incubated with 1 μ M of tubulin-[^{32}P]AAGTP as described previously (Popova et al., 1997). When tested, PIP_2 was preincubated with tubulin-[^{32}P]AAGTP at a molar ratio of 6:1 for 15 min in a Branson (Danbury, CT) water bath sonicator at 4°C. After UV irradiation and preclearing with Pansorbin (Calbiochem, La Jolla, CA), each sample was incubated overnight with appropriate antiserum or preimmune serum (1:20 dilution) at 4°C with constant shaking. Immune complexes were precipitated with Pansorbin and subjected to SDS-PAGE

and autoradiography or phosphorimage analysis. The antisera used showed no cross-reactivity to tubulin.

Analysis of phosphoinositide hydrolysis in SK-N-SH cells. SK-N-SH neuroblastoma cells were grown in six-well plates in DMEM supplemented with 10% fetal bovine serum and 50 U/ml penicillin-streptomycin. Twenty-four hours before the experiment, inositol-free DMEM supplemented with 2 μ Ci/well myo-[3 H]inositol was added. The cells were washed three times with Locke's buffer, containing 10 mM LiCl, and incubated for 15 min with or without 33 μ M colchicine in the same buffer. After triplicate wash with Locke's buffer, 10 μ M carbachol was added as indicated, and the cells were incubated for 30 min at 37°C. Carbachol effects were routinely controlled for by addition of 1 μ M atropine. The reaction was stopped with ice-cold 10% trichloroacetic acid, and the cells were scraped from wells with a rubber policeman and transferred to tubes. After sonication (as described above) and centrifugation at 20,000 $\times$ g for 15 min (4°C), the supernatants were extracted with water-saturated ether and neutralized with 1 M NH_4HCO_3 . Ion exchange chromatography (Dowex AG 1-X8 resin, formate form; Bio-Rad) of the samples was performed as described previously (Popova and Dubocovich, 1995). Total [3 H]inositol phosphates were quantified by liquid scintillation counting. The inositol phosphate content of SK-N-SH cells at the start of the experiment (0% increase) was $1.1 \pm 0.27 \times 10^3$ dpm/ 10^6 cells.

Recording of enhanced green fluorescent protein-tubulin-containing SK-N-SH cells by immunofluorescence microscopy. Cells plated on 35-mm-diameter Delta T dishes (Biotech, Inc.) were transiently transfected with 5 μ g enhanced green fluorescent protein (EGFP)-tubulin cDNA (Clontech, Cambridge, UK) using Lipofectin reagent as described by the manufacturer (Invitrogen, Gaithersburg, MD). The cells were observed 24 hr later using fluorescence microscopy. A Nikon fluorescence microscope equipped with a 100 W mercury arc lamp was used. Before observation, the medium in the dish was changed to serum-free DMEM containing 20 mM HEPES, and the cells were maintained in this media for at least 30 min before the recording. The cells were transferred to the microscope stage and maintained at 37°C during the entire period of observation. Images were acquired with an interline charge-coupled device camera (1300 YHS; Roper Scientific, Trenton, NJ) driven by IP Lab imaging software (Scanalytics, Inc., Suitland, VA). Fluorescent images for EGFP were recorded every 15 sec and the recorded images were processed with IP Lab.

Confocal immunofluorescence microscopy. SK-N-SH neuroblastoma cells were plated onto glass coverslips in 12-well culture plates at a density of 1×10^5 . After 24 hr, cells were incubated for 15 min with or without 33 μ M colchicine. After a PBS wash, the cells were treated for 2 min with 1 mM carbachol, 10 μ M atropine, or both. The cells were immediately fixed in -20°C methanol for 3 min and washed three times, 10 min each, in PBS containing 0.1% Triton X-100. The cells were blocked for 40 min in PBS containing 5% milk and washed in PBS. Subsequently the cells were incubated for 1 hr with a polyclonal anti-tubulin antibody (raised against the C-terminal 422–431 amino acid region of β -tubulin; Popova and Rasenick, 2000) and a monoclonal anti-PIP₂ antibody (Assay Designs, Inc.), both at a dilution of 1:100. After a PBS wash, secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit and Texas Red-conjugated horse anti-mouse antibodies (Vector Laboratories, Burlingame, CA; 1:100 dilution) were applied for 1 hr, followed by washing and mounting of the coverslips. Images were acquired using a Zeiss (Thornwood, NY) LSM 510 laser scanning confocal microscope equipped with a 63 $\times$ water immersion objective. A 488 nm beam from an argon-krypton laser was used for the excitation of FITC, whereas a 543 nm beam was used for Texas Red excitation. Emission from FITC was detected through a BP505 filter, whereas emission from Texas Red was detected through an LP560 filter. Areas of antibody colocalization appeared in yellow. Differential interference contrast images of the cells were regularly acquired as well. Coverslips were examined at random. For each experimental condition, a total of 90 randomly selected cells over three consecutive experiments were evaluated for tubulin and PIP₂ distribution and colocalization. Final image composites were created using Adobe Photoshop 5.0. No specific FITC or Texas Red labeling was observed in cells treated with rabbit or mouse preimmune serum instead of anti- β -tubulin or anti-PIP₂ antibodies, respectively. FITC labeling was not observed when the anti-tubulin antiserum was preincubated overnight at 4°C with PC-tubulin (1:1 ratio), and Texas Red labeling was not detected when the anti-PIP₂ antiserum was preincubated with PIP₂ (1:1 ratio), both conditions tested at the same antibody dilutions (1:100) afterward. Although colchicine

treatment changed the shape of the treated cells, it did not affect the membrane localization and intracellular distribution of G α_q (Ibarrondo et al., 1995).

Materials. [α - 32 P]GTP was from ICN Biomedicals (Cleveland, OH). [3 H]QNB was from Amersham Biosciences. Carbachol and all phosphoinositides used were from Sigma (St. Louis, MO). Fluorescein-5-maleimide was from Molecular Probes. *p*-Azidoaniline was synthesized by Dr. William Dunn III (University of Illinois at Chicago). All other reagents were of analytical grade.

RESULTS

Specific interaction with PIP₂ decreases tubulin polymerization

Previous experiments demonstrated that PIP₂ bound to tubulin and inhibited microtubule assembly (Popova et al., 1997). The specificity of tubulin-PIP₂ interaction was not addressed in that study. Phosphatidylinositol 3,4,5-trisphosphate (PIP₃) as well as the second messenger IP₃ can bind to certain PIP₂-binding protein domains (Ferguson et al., 1995; Kavran et al., 1998), and the negative charge of these molecules was presumed responsible for such interaction. The anionic phospholipid constituents of hepatic membranes have also been reported to account for membrane binding of brain microtubule protein and inhibition of assembly (Reaven and Azhar, 1981). A hydrophobic interaction of tubulin (Andreu, 1986) with the uncharged phospholipid phosphatidylcholine at the lipid phase transition temperature (Klausner et al., 1981; Kumar et al., 1981) has been found as well.

To investigate the specificity of tubulin interaction with the anionic phospholipid PIP₂, several charged and neutral phospholipids as well as IP₃ were included in microtubule polymerization assays. PC-tubulin, purified free of microtubule-associated proteins, was preincubated with the phospholipids tested or IP₃. These tubulin preparations were allowed to polymerize under conditions that favor microtubule assembly (see Materials and Methods). In each case, the amount of tubulin distributed between the pelleted polymer mass and the supernatant was measured. The results obtained demonstrated that PIP₂ inhibited tubulin polymerization by $39.9 \pm 3.6\%$ (SEM; $n = 5$) compared with the control not containing this phosphoinositide (Fig. 1A). Other closely related anionic phosphoinositides, such as PIP₃, phosphatidylinositol 3-phosphate (PIP), and phosphatidylinositol (PI), as well as the negatively charged inositol phosphate IP₃, had no significant effect on the microtubule assembly process. When tested under the same conditions, the polyanion heparin also had no effect on tubulin assembly. The neutral phospholipids PC and phosphatidylethanolamine (PE) did not significantly affect polymerization either (Fig. 1A). Electron microscopy and light scattering of tubulin samples was also done. As was the case with microtubule pellets, PIP₂ but not PIP₃, PC, PE, PI, or IP₃ inhibited microtubule formation. Thus, it is suggested that the regulatory phosphoinositide PIP₂ inhibits microtubule polymerization through a specific interaction with tubulin.

To test whether the effect of PIP₂ on tubulin was concentration-dependent, tubulin polymerization was studied in the absence or presence of PIP₂, PC, or the mixture of both in light-scattering experiments (Fig. 1B). When the concentration of PIP₂ was reduced by half (PIP₂ mixed with PC at a molar ratio of 1:1), the inhibition of microtubule formation was also half that seen with PIP₂ alone.

PIP₂ does not affect the binding and hydrolysis of GTP by tubulin

Several possible mechanisms exist through which PIP₂ binding could interfere with tubulin polymerization. One possibility is

Figure 1. PIP₂ inhibits tubulin polymerization. *A*, Comparison of the effects of various phosphoinositides on microtubule polymerization. Where indicated, PIP₃, PIP₂, PIP, PI, PC, PE, and the inositol phosphate IP₃ (final molar concentration of 75 μ M) were preincubated with tubulin (*Tub*) as described in Materials and Methods. Microtubule polymerization reactions were performed for 1 hr at 37°C. Pellets were resuspended in cold polymerization buffer and subjected to SDS-PAGE and immunoblotting with a monoclonal anti- α -tubulin antibody. Values are means $\pm$ SEM of five independent experiments performed in triplicate. *Significantly different from the control (tubulin, subjected to polymerization without any addition); $p < 0.05$, one-way ANOVA. Colorimetric measurements of the protein content of depolymerized pellets (performed before SDS-PAGE) corroborated these findings. *B*, PIP₂ inhibition of microtubule formation is concentration-dependent. PIP₂, PC, mixed vesicles of PIP₂ and PC (at the ratio of 1:1), and vehicle were added to microtubule polymerization reactions. Polymerization was performed for 30 min as described in Materials and Methods. Absorbance at 350 nm was monitored. Values were obtained after 20 min, when the polymerization reactions were at equilibrium. Values are means $\pm$ SEM of three separate experiments done in triplicate. The net absorbance of microtubule polymerization reactions without added phosphoinositides was 1.09 ± 0.12 (control). * $p < 0.05$; ** $p < 0.01$.

Figure 2. PIP₂ does not alter binding and hydrolysis of GTP by tubulin. *A*, Effects of PIP₂ on [³²P]AAGTP binding to tubulin (*Tub*). Phosphocellulose-purified tubulin, stripped of nucleotide, was incubated with [³²P]AAGTP in the absence or presence of phosphoinositides. *First lane*, No addition to the binding reaction; *second lane*, PIP₂ added at the start of the binding reaction; *third lane*, PIP₂ added after the end of the binding reaction, as described. A representative of two identical experiments performed in triplicate with similar results is shown. *B*, Effects of PIP₂ on GTPase activity of tubulin. Tubulin-[³²P]GTP was incubated for 30 min at 30°C, in the absence or presence of phosphoinositides, and the nucleotide bound to tubulin was analyzed as described in Materials and Methods. A representative of two identical experiments performed in triplicate with similar results is shown.

that PIP₂ affects the binding of GTP to tubulin, because PIP₂ has been reported to promote dissociation of GTP from the small GTP-binding proteins Arf, CDC 42, and Rho (Terui et al., 1994; Glaven et al., 1996). Another scenario is that GTP hydrolysis on tubulin is activated by PIP₂. Because tubulin must bind GTP to assemble, PIP₂ could block the process by activating tubulin GTPase.

PIP₂ did not modify the amount of [³²P]AAGTP or [³²P]GTP bound to tubulin, estimated at 0.49 ± 0.08 mol bound/mol of tubulin (Fig. 2*A*). This was independent of whether PIP₂ was added to tubulin before or after the course of the guanine nucleotide binding reaction. Densitometry revealed relative absorbance values of 100.0 ± 14.6 for the tubulin-[³²P]AAGTP band obtained when PIP₂ was not present in the binding reaction, as well as 94.41 ± 10.2 and 103.3 ± 15.1 for the bands obtained when PIP₂ was added before or after the binding reaction, respectively ($p > 0.05$; $n = 6$ for each experimental condition).

Tubulin contains an intrinsic GTPase, which is not activated until the microtubule is formed (Carrier and Pantaloni, 1981). If PIP₂ activated the GTPase of tubulin dimers, polymerization would be blocked, because those dimers would be binding GDP. However, PIP₂ did not promote hydrolysis of GTP by tubulin. The amount of GTP bound per mole of tubulin remained at 0.55 ± 0.05 mol/mol during the course of these experiments regardless of the presence or absence of phospholipids ($p > 0.05$; $n = 6$ for each experimental condition; Fig. 2*B*).

PIP₂ promotes association of tubulin with the membrane but does not promote Gαq transactivation by tubulin

PIP₂ is normally membrane-associated. Although it is not clear how tubulin associates with membranes (a subject of some controversy), it is possible that PIP₂ is involved in the process (Reaven and Azhar, 1981). This was investigated using both membranes prepared from Sf9 cells expressing recombinant m1 muscarinic receptors, Gαq, and PLCβ₁ (Popova et al., 1997) and membranes from SK-N-SH neuroblastoma cells, which normally

Figure 3. PIP₂ binding increases membrane-associated tubulin (*Tub*). *A*, Membranes from Sf9 cells expressing m1 muscarinic receptors, Gαq, and PLCβ₁ (20 μg of membrane protein) were incubated with 300 nM tubulin-[³²P]AAGTP with or without 100 μM carbachol, 30 μM PIP₂, or both for 5 min at 23°C, followed by UV irradiation, SDS-PAGE (50 μg of membrane protein in each lane), and autoradiography. A representative of three similar experiments performed in triplicate is shown. *B*, Membranes from SK-N-SH cells (40 μg of membrane protein) were incubated with tubulin-[³²P]AAGTP or carbachol, PIP₂, or both under the conditions described above. A representative of two identical experiments performed in triplicate with similar results is shown.

contain m3 muscarinic receptors, Gαq, and PLCβ₁ (Fisher and Heacock, 1988).

In membranes from infected Sf9 or SK-N-SH cells, both carbachol and PIP₂ increased the association of tubulin with the membrane (Fig. 3). At the experimental conditions used (Fig. 3*A*), the average increase in association of tubulin-[³²P]AAGTP with the Sf9 cell membranes was $99.6 \pm 22.4\%$ (SD; $n = 3$) in the presence of carbachol and $96.1 \pm 17.7\%$ ($n = 3$) in the presence of PIP₂. When both of them were present, association of tubulin with the membrane was $115.1 \pm 29.2\%$ ($n = 3$) greater than that of the control. Comparable results were obtained when membranes from SK-N-SH cells were tested (Fig. 3*B*).

Gαq activation by tubulin was also assessed in these experiments by examining the transfer of [³²P]AAGTP from tubulin to Gαq. Although Gαq transactivation by tubulin increased by $124.0 \pm 23.0\%$ (SD; $n = 3$) after muscarinic receptor stimulation (Fig. 3*A*), it was not affected by PIP₂. Atropine inhibited the membrane association of tubulin evoked by carbachol, but it failed to suppress the PIP₂-promoted membrane association of tubulin. These findings were corroborated when SK-N-SH membranes were tested under similar experimental conditions (Fig. 3*B*).

Concentration-response experiments were performed to inspect the effect of PIP₂ on tubulin regulation of Gαq. PIP₂ increased the binding of exogenous tubulin to Sf9 membranes containing the recombinant proteins over a range of tubulin concentrations. Both FM-tubulin (Fig. 4*A*) and tubulin-[³²P]AAGTP gave similar results. The effects of PIP₂ on tubulin-[³²P]AAGTP membrane association were also concentration-dependent (Fig. 4*B*). However, as shown in Figure 4*C*, over a range of tubulin-[³²P]AAGTP concentrations, transactivation of Gαq by tubulin was independent of PIP₂. In fact, a decrease in the carbachol-evoked [³²P]AAGTP transfer from tubulin to Gαq was observed at PIP₂ concentrations of $>40 \mu\text{M}$ (Fig. 4*D*). Thus, although PIP₂ promoted tubulin association with the membrane, it did not evoke the rapid process of Gαq transactivation by tubulin. These results are consistent with the notion that PIP₂ binding to tubulin interfered with both tubulin polymerization properties and the ability to transactivate Gαq.

Figure 4. PIP₂ does not increase Gαq transactivation by tubulin. *A*, The effects of carbachol and PIP₂ on the membrane association of tubulin are additive. Recruitment of tubulin to the membrane was studied using increasing concentrations of FM-tubulin. Membranes from Sf9 cells expressing m1 muscarinic receptors, Gαq, and PLCβ₁ were incubated with carbachol and FM-tubulin (at the indicated concentrations), with or without PIP₂. SDS-PAGE (50 μg of membrane protein in each lane) was followed by measurement of the fluorescence of membrane-associated tubulin with a fluorescence imaging system (Storm 840; Molecular Dynamics). The results represent one of three similar experiments performed in triplicate. *Open circles*, Membranes treated with 1 mM carbachol; *filled circles*, membranes treated with 1 mM carbachol and 30 μM PIP₂. *B*, PIP₂-assisted recruitment of tubulin-[³²P]AAGTP to the membrane is concentration-dependent. Membranes from Sf9 cells containing m1 muscarinic receptors, Gαq, and PLCβ₁ were incubated with 1 mM carbachol, 1 μM tubulin-[³²P]AAGTP, and increasing concentrations of PIP₂, as described in Figure 1. After SDS-PAGE (50 μg of membrane protein in each lane) [³²P]-labeled protein bands were measured by phosphorimager analysis. One of three identical experiments done in triplicate with similar results is shown. *C*, Carbachol-evoked Gαq transactivation by tubulin is not affected by PIP₂. The experiments were done as described in *A*, except that tubulin-[³²P]AAGTP was used. Proteins were resolved by SDS-PAGE (50 μg of membrane protein in each lane), and the radioactivity of the Gαq bands ([³²P]AAGTP was transferred from tubulin) was measured by phosphorimager analysis (Storm 840; Molecular Dynamics). One of five independent experiments done in triplicate with similar results is shown. *Open circles*, Membranes treated with 1 mM carbachol; *filled circles*, membranes treated with 1 mM carbachol and 30 μM PIP₂. *D*, The increased membrane association caused by PIP₂ was not linked to Gαq transactivation. The percent ratios of [³²P]AAGTP-labeled Gαq and tubulin-[³²P]AAGTP at the various PIP₂ concentrations are derived from the experiment described in *B*. One of three identical experiments done in triplicate with similar results is shown. Control values for *B* and *D* represent the amount of tubulin associated with the plasma membrane in the absence of carbachol or PIP₂.

PIP₂ increases the association of tubulin with PLCβ₁

Because PIP₂ is the natural substrate for PLCβ₁, the relevance of PLCβ₁ to the process of PIP₂-mediated association of tubulin with the membrane was tested. In the absence of PLCβ₁, PIP₂ had no effect on the association of tubulin with the Sf9 cell membranes (Fig. 5*A*). Thus, it appeared that PLCβ₁ was involved in the PIP₂-promoted membrane association of tubulin.

This was tested by coimmunoprecipitation. Tubulin coimu-

Figure 5. PIP₂ is involved in the association of tubulin with PLCβ₁. *A*, PIP₂ does not potentiate the membrane association of tubulin when PLCβ₁ is not present. Membranes from Sf9 cells containing m1 muscarinic receptors and Gαq but not PLCβ₁ were incubated with tubulin-[³²P]AAGTP and the indicated concentrations of PIP₂ as described in Figure 4*B*. One of two identical experiments done in triplicate with similar results is shown. Tubulin associated with the membrane in the absence of carbachol represents the control. *B*, PIP₂ increases coimmunoprecipitation of PLCβ₁ with tubulin (*Tub*). Membrane preparations of Sf9 cells containing PLCβ₁ were extracted with 1% sodium cholate. Where indicated, tubulin-[³²P]AAGTP was preincubated with PIP₂ as described in Materials and Methods. After UV irradiation, each sample was incubated overnight with anti-PLCβ₁ antiserum or preimmune serum, as indicated, and immunoprecipitated as described. The immunoprecipitates were subjected to SDS-PAGE and autoradiography. An autoradiogram from one of four independent experiments with identical results is shown. *C*, PIP₂ increased coimmunoprecipitation of Gαq and tubulin when PLCβ₁ was present. Membrane preparations of Sf9 cells, expressing either Gαq or Gαq and PLCβ₁, were tested as described in *B*, except that anti-Gαq antiserum was used to test Gαq coimmunoprecipitation with tubulin. Note that Gαq expression level decreased when Sf9 cells were cotransfected with Gαq and PLCβ₁ baculoviruses, as revealed by immunoblotting with anti-Gαq antiserum. An autoradiogram from one of three similar experiments is shown.

noprecipitates with Gαq and, to a lesser extent, PLCβ₁ (Popova et al., 1997). However, the mechanism whereby PIP₂ affects these interactions has not been evaluated. Extracts from Sf9 membranes, containing PLCβ₁, Gαq, or both, were tested (Fig. 5*B,C*).

PIP₂ increased coimmunoprecipitation of tubulin-[³²P]AAGTP with PLCβ₁ by approximately twofold [$204 \pm 11.0\%$ (SD)], suggesting stabilization of tubulin-PLCβ₁ interaction (Fig. 5*B*). PIP₂ did not alter the coimmunoprecipitation of tubulin and Gαq (Fig. 5*C*, left). However, when Gαq and PLCβ₁ were both present on the membrane, PIP₂ increased Gαq-tubulin coimmunoprecipitation by twofold [$216 \pm 10.0\%$ (SD); Fig. 5*C*, right]. These results suggested that PIP₂ might promote the formation of tubulin-Gαq-PLCβ₁ complexes.

Carbachol stimulation causes redistribution and colocalization of intracellular tubulin with PIP₂ at the plasma membrane

If tubulin-PIP₂ interaction modulates a related membrane signaling event, we would expect to see colocalization of tubulin and PIP₂ at regions of the cell specialized for signaling. To examine this, SK-N-SH cells were transiently transfected with pEGFP-tubulin. Immunofluorescence microscopy was used to confirm *in vivo* the microtubule depolymerization and redistribution of tubulin in SK-N-SH cells in response to carbachol stimulation. Although the appearance of the microtubules in cells treated with vehicle did not change, rapid microtubule depolymerization was observed in the carbachol-treated cells (Fig. 6).

Confocal laser immunofluorescence microscopy was used to compare the patterns of localization of tubulin and PIP₂ in carbachol-treated and untreated SK-N-SH cells. A monoclonal antibody shown to bind specifically to endogenous PIP₂ and to inhibit the intracellular breakdown of this phosphoinositide was used (Fukami et al., 1988). This antibody blocked the PIP₂-mediated increase in tubulin binding to isolated SK-N-SH membranes. Because Lipofectin treatment compromised membrane PIP₂, EGFP-tubulin-transfected cells could not be used in this study. Anti-tubulin antibody raised against the C-terminal 422–431 amino acid region of β-tubulin was used to visualize tubulin (Popova and Rasenick, 2000). In both carbachol-treated and untreated SK-N-SH cells, anti-PIP₂ antibody labeling (seen in red) was detected along the cell surface and in the cytoplasm, but it was mostly enriched in the membrane and submembrane regions of the cell (Fig. 7). In the untreated cells, tubulin (seen in green) was found in microtubules, bundles, and throughout the cytoplasm. Some tubulin colocalized with PIP₂ in areas close to the plasma membrane (Fig. 7*A*, yellow). (Note that because confocal images of cell areas that are 1 μm thick are presented, filamentous microtubule arrays are not obvious.) When SK-N-SH cells were stimulated with carbachol, microtubule depolymerization and redistribution of tubulin along the plasma membrane was observed (Fig. 7*B*). Tubulin colocalized with PIP₂ in regions along the plasma membrane. Tubulin and PIP₂ did not colocalize in areas distal to the plasma membrane in control and carbachol-treated cells. All effects of carbachol were blocked by atropine.

Microtubule depolymerization inhibits phosphoinositide hydrolysis in SK-N-SH cells

Exogenous tubulin regulates PLCβ₁ signaling when added to membranes from engineered Sf9 or SK-N-SH neuroblastoma cells (Popova and Rasenick, 2000). To test whether endogenous tubulin affected phosphoinositide hydrolysis, SK-N-SH cells were pretreated with colchicine before analysis of inositol phosphate production. Colchicine is a well known pharmacologic agent that binds to microtubules and causes microtubule depolymerization (Wilson and Jordan, 1994). Colchicine also activates tubulin GTPase in the absence of polymerization (David-Pfeuty et al., 1979; Andreu and Timasheff, 1981). Colchicine would be ex-

Figure 6. Microtubule depolymerization and redistribution of tubulin in response to carbachol stimulation in GFP-tubulin-expressing SK-N-SH cells. SK-N-SH cells were transfected with EGFP-tubulin cDNA as described. Twenty four hours after transfection, cells treated with either vehicle (control) or 100 μ M carbachol were observed on a heated (37°C) microscope stage, and images were collected at 15 sec intervals as described. Arrowheads indicate microtubule depolymerizing in response to carbachol treatment.

Figure 7. Carbachol stimulation causes microtubule depolymerization and translocation of tubulin to PIP₂-enriched membrane regions of SK-N-SH-neuroblastoma cells. Cells were untreated (*A*) or treated with 1 mM carbachol for 2 min (*B*) before fixation, followed by FITC labeling of tubulin and Texas Red labeling of PIP₂, as described. Carbachol-induced concentration of tubulin in the PIP₂-enriched membrane and submembrane areas of the cells (*B*) is apparent. Tubulin-PIP₂ colocalization appears in yellow. Representative images of cells obtained in one of three independent experiments with similar results are shown.

pected to increase the cellular concentration of tubulin-GDP dimers, which do not activate G α_q .

Endogenous phosphoinositide pools of SK-N-SH cells were prelabeled with *myo*-[³H]inositol (Popova and Dubocovich, 1995), and carbachol-induced inositol phosphate generation was studied in colchicine-treated or control cells (Fig. 8). Confocal immunofluorescence microscopy demonstrated significant microtubule depolymerization in colchicine-pretreated cells (Fig. 8*A*). Retraction of cellular projections and change in cell shape were also observed. Colocalization of tubulin and PIP₂ in regions close to the membrane was also seen.

Colchicine treatment of SK-N-SH cells inhibited carbachol-stimulated inositol phosphate generation by 40% (Fig. 8*B*). Colchicine treatment did not affect the basal PLC activity of SK-N-SH cells. When present in the incubation medium, colchicine

Figure 8. Colchicine-evoked microtubule depolymerization inhibits PLC β signaling in SK-N-SH neuroblastoma cells. *A*, Confocal immunofluorescence image of an SK-N-SH cell treated for 15 min with 33 μ M colchicine as described in Materials and Methods. Microtubule depolymerization as well as colocalization (yellow) of tubulin (green) with PIP₂ (red) is demonstrated. *B*, Myo-[³H]inositol-prelabeled SK-N-SH cells were treated for 15 min with 33 μ M colchicine (col). Carbachol (carb; 10 μ M) was added, the samples were incubated for 30 min at 37°C, and the total inositol phosphate production was measured as described. **Significantly different from control cells ($p < 0.01$); *significantly different from carbachol-treated cells ($p < 0.05$).

did not increase the association of purified tubulin with SK-N-SH membranes, suggesting that the increase in membrane-associated tubulin was attributable to the increase in tubulin dimer concentration.

Colchicine pretreatment did not affect the interaction of tubulin with G α q or PLC β ₁. When Sf9 membranes containing these proteins were pretreated for 15 min with colchicine (10 μ M), the membrane association of 1 μ M tubulin-[³²P]AAGTP, induced by carbachol or PIP₂, was unaltered. Carbachol (100 μ M) and guanosine 5'-(β , γ -imido)triphosphate (10 μ M) activation of PLC β ₁ were also unaffected when Sf9 membranes were pretreated with colchicine (2.71 ± 0.2 nmol $\cdot$ min⁻¹ $\cdot$ mg of protein⁻¹ and 2.79 ± 0.6 nmol $\cdot$ min⁻¹ $\cdot$ mg of protein⁻¹ before and after colchicine, respectively).

DISCUSSION

The purpose of this study was to determine whether and how PIP₂ contributes to the regulation of PLC β ₁ signaling by tubulin. Although PIP₂ is the preferred substrate for PLC β ₁, it also binds to tubulin and shortens microtubules *in vitro* (Popova et al., 1997). Furthermore, low (nanomolar) concentrations of tubulin activate, whereas high (micromolar) concentrations inhibit, PLC β ₁ (Popova et al., 1997). Tubulin binding to G α q, followed by transactivation of G α q attributable to the transfer of GTP from tubulin, appears responsible for the activation phase (Popova and Rasenick, 2000). The mechanism by which tubulin inhibits PLC β ₁ has not been revealed, but it appeared to involve PIP₂.

A previous study (Popova et al., 1997) speculated that, at high tubulin concentrations, association of PIP₂ with tubulin might render PIP₂ unavailable to PLC β ₁, decreasing PLC β ₁ activity. However, this previous study left open another possibility, which is that the binding of PIP₂ might also affect the GTP-binding or -hydrolyzing properties of tubulin (Davis et al., 1994) and might render tubulin unable to transactivate G α q (Popova et al., 1997). Data in Figures 1 and 2 showed that although PIP₂ interacted with tubulin in a specific manner, it did not affect either GTP binding or GTP hydrolysis by tubulin.

The present results also demonstrate that, although PIP₂ had no direct effect on G α q transactivation by tubulin, it supported the membrane association of tubulin in both Sf9 cells, which ectopically express recombinant muscarinic receptors, G α q, and PLC β ₁, and SK-N-SH neuroblastoma cells, which normally contain these proteins (Figs. 3, 4). Colocalization of tubulin and PIP₂ along the plasma membrane of SK-N-SH neuroblastoma cells was also observed (Fig. 7). These results are concordant with the idea that PIP₂-enriched regions of the membrane might be sites for tubulin association. Examples of such regions are lipid rafts enriched in sphingolipids and cholesterol, which sequester certain proteins but exclude others. They are considered platforms for initiation of signal transduction processes, membrane trafficking, and molecular sorting. PIP₂ is present in these rafts (Laux et al., 2000). It has been shown recently that in differentiated rat cerebellar granule cells, glycerophospholipids represent 45–75% of the constituents of sphingolipid-enriched membrane domains, of which PIP₂ is ~3% (Prinetti et al., 2001). Because the protein content of these domains is ~0.1–2.8% (Prinetti et al., 2001), protein/PIP₂ ratios ranging between 1:0.8 and 1:13.5 are estimated. These values are concordant with the tubulin/PIP₂ ratios used in the present study. Lipid-anchored tubulin within detergent-resistant and glycolipid-enriched plasma membrane domains has also been demonstrated (Palestini et al., 2000). Thus, specific binding of tubulin to the minor membrane lipid PIP₂ might facilitate tubulin targeting to such specific membrane locations.

Membrane- or phospholipid-associated tubulin has been reported (Bhattacharyya and Wolff, 1976; Klausner et al., 1981; Kumar et al., 1981; Reaven and Azhar, 1981; Regula et al., 1986; Caron and Berlin, 1987). It appeared that this “membrane” tubulin was similar to the soluble form (Bhattacharyya and Wolff, 1976; Stephens, 1977). The recently discovered microtubule depolymerization and translocation of tubulin from the cytosol to the membrane in response to receptor stimulation showed one mechanism for tubulin targeting to the membrane (Popova and Rasenick, 2000; Ciruela and McIlhinney, 2001; this study). The finding that tubulin is posttranslationally palmitoylated (Caron, 1997; Zambito and Wolff, 1997) supports this observation, be-

cause this reversible and agonist-regulated lipid modification has been shown to facilitate association of $G\alpha$ subunits with membranes (for review, see Casey, 1995; Dunphy and Linder, 1998). However, it has also been suggested that palmitoylation may be insufficient for protein targeting to the detergent-resistant membrane rafts (Melkonian et al., 1999). Additional lipid modifications or binding to additional membrane proteins or lipids may be required (Melkonian et al., 1999). Both myristoylation and palmitoylation of $G\alpha_i$ may be necessary for its association with liposomes and partitioning into rafts (Moffett et al., 2000). Thus, palmitate and the binding of PIP_2 might similarly cooperate to anchor tubulin dimers to specific signaling domains of the plasma membrane.

A number of studies have shown PIP_2 -assisted membrane attachment of regulatory cytosolic proteins. PLC isozymes, phospholipase D, GTPases, guanine nucleotide exchange factors, GTPase-activating proteins, the vesicle-associated GTPase dynamin, and protein kinases interact with PIP_2 at their pleckstrin homology (PH) domains (Musacchio et al., 1993; Shaw, 1993, 1996; Gibson et al., 1994; Hodgkin et al., 1999). The binding of PIP_2 assists the targeting of these proteins to the membrane and facilitates their coupling with membrane-associated signaling molecules. Binding with high affinity to both the activated receptor and phosphoinositides was proposed to provide a multipoint attachment of β -arrestin and arrestin 3 to the plasma membrane (Gaidarov et al., 1999). Tubulin might enjoy a similar attachment.

Thus, in areas proximal to the plasma membrane, PIP_2 could support the receptor-evoked membrane attachment of tubulin-GTP (Popova et al., 1997; Popova and Rasenick, 2000). The subsequent involvement of tubulin in a complex with $G\alpha_q$ and $PLC\beta_1$ might stabilize their active conformation and potentiate $PLC\beta_1$ activation (Fig. 9A). The scenario might be quite different at high local tubulin concentrations. At high tubulin concentrations, the binding of tubulin to PIP_2 -rich sites of the plasma membrane proceeds in a receptor-independent manner, leading to direct association of tubulin with $PLC\beta_1$ and subsequent enzyme inhibition (Fig. 9B). Consistent with this hypothesis is the observation that high concentrations of PIP_2 decrease the interaction of tubulin with $G\alpha_q$ and high concentrations of tubulin decrease the activity of $PLC\beta_1$. Tubulin- PIP_2 - $PLC\beta_1$ complexes should be unable to interact with receptor-activated $G\alpha_q$. This notion is supported by the observation that pretreatment of SK-N-SH cells with the microtubule-depolymerizing agent colchicine decreased $PLC\beta_1$ activation.

These regulatory mechanisms presuppose an agonist-modulated change in localized tubulin dimer concentration. Initially, hormone- or neurotransmitter-mediated activation of PLC would increase local Ca^{2+} concentrations, which, in turn, would cause microtubule depolymerization in this region of the cell. The resulting increase in tubulin dimer (Weisenberg, 1972; Serrano et al., 1986) might then provide a feedback inhibition of $PLC\beta_1$. Rapid increase in membrane-associated tubulin after carbachol treatment of cells has been demonstrated (Fig. 7; Popova and Rasenick, 2000). Furthermore, G_{α_s} and G_{α_i} have been shown to bind tubulin and activate GTPase. This destroys the GTP cap on microtubules (Roychowdhury et al., 1999) and perhaps increases local tubulin dimer concentration in response to agonist activation of G-protein-coupled receptors. The increased association of tubulin-GDP with the membrane and subsequent inhibition of $PLC\beta_1$ after colchicine treatment are consistent with such hypotheses (Fig. 8).

The site(s) on tubulin for specific binding of PIP_2 is not yet

Figure 9. Mechanism of tubulin regulation of $PLC\beta_1$ activity. *A*, Initial association of tubulin with the membrane: activation of $G\alpha_q$ and $PLC\beta_1$. It is hypothesized that m1 muscarinic receptor stimulation triggers association of tubulin with the plasma membrane, resulting in subsequent regulation of $PLC\beta_1$ signaling. The binding of tubulin to PIP_2 at the membrane supports the membrane association of tubulin and, perhaps, the formation of the active tubulin- $G\alpha_q$ - $PLC\beta_1$ complex. *B*, Increased association of tubulin with membranes inhibits $PLC\beta_1$. At high local concentrations of tubulin, receptor-independent interaction of tubulin with $PLC\beta_1$ through PIP_2 renders the enzyme inaccessible for receptor-activated $G\alpha_q$, leading to $PLC\beta_1$ inhibition. The physiological relevance of dual regulation of $PLC\beta_1$ by tubulin is supported by the observation that $PLC\beta_1$ activation increases intracellular Ca^{2+} concentration, which in turn causes microtubule depolymerization. Feedback inhibition of $PLC\beta_1$ at elevated concentrations of tubulin dimers is suggested. m1 AchR, m1 Muscarinic acetylcholine receptor.

identified. PH domains on a number of signaling molecules, including G-protein-coupled receptor kinases (GRKs), have been implicated in interacting with PIP_2 and G-protein $\beta\gamma$ subunits (Musacchio et al., 1993; Shaw, 1993, 1996; Gibson et al., 1994), but not all of them bind these ligands (Davis and Bennett, 1994). Furthermore, the G-protein-coupled receptor kinase GRK5 does not possess a PH domain and does not bind $G\beta\gamma$ (Pitcher et al., 1996). However, GRK5 contains regions rich in basic amino acids within both its N and C termini (Pitcher et al., 1996), and these regions might represent lipid-binding domains (Kunapuli et al., 1994; Casey, 1995; Pronin et al., 1998). Although tubulin does not have a typical PH domain, it contains regions rich in basic amino acids that might be involved in the binding of PIP_2 . However, because other negatively charged phospholipids fail to affect tubulin polymerization, the interaction of PIP_2 with tubulin appears to be specific and not solely electrostatic.

The findings described in this paper demonstrate that the specific interaction of tubulin with the integral membrane lipid and $PLC\beta_1$ substrate PIP_2 defines its membrane association and involvement in $G\alpha_q$ -mediated signaling. This reversible association might represent a highly localized phenomenon, whereby tubulin could temporarily attach to specific membrane domains

for the purpose of directing G-protein-mediated signaling. This type of focal signaling, requiring local changes in calcium and microtubules, represents a continuum between G-protein signaling and the cytoskeleton.

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