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Phosphatidylinositol 4,5-Bisphosphate Modifies Tubulin Participation in Phospholipase Cβ_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 Gαq-regulated phospholipase Cβ_1 (PLCβ_1) is activated by tubulin through a direct transfer of GTP from tubulin to Gαq. However, at high tubulin concentrations, inhibition of PLCβ_1 is observed. This report demonstrates that tubulin inhibits PLCβ_1 by binding the PLCβ_1 substrate phosphatidylinositol 4,5-bisphosphate (PIP_2). Tubulin binding of PIP_2 was specific, because PIP_2 but not phosphatidylinositol 3,4,5-trisphosphate, phosphatidylinositol 3-phosphate, phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine, or inositol 1,4,5-trisphosphate inhibited microtubule assembly. PIP_2 did not affect GTP binding or GTP hydrolysis by tubulin. Muscarinic agonists promoted microtubule depolymerization and translocation of tubulin to the plasma membrane. PIP_2 augmented this process in both Sf9 cells, containing a recombinant PLCβ_1 pathway, and SK-N-SH neuroblastoma cells. Colocalization of tubulin and PIP_2 at the plasma membrane was demonstrated with confocal laser immunofluorescence microscopy. Although tubulin bound to both Gαq and PLCβ_1, PIP_2 facilitated the interaction between tubulin and PLCβ_1 but not that between tubulin and Gαq. However, PIP_2 did augment formation of tubulin–Gαq–PLCβ_1 complexes. Subsequent to potentiating PLCβ_1 activation, sustained agonist-independent membrane binding of tubulin at PIP_2- and PLCβ_1-rich sites appeared to inhibit Gαq coupling to PLCβ_1. Furthermore, colchicine increased membrane-associated tubulin and also inhibited PLCβ_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 (PIP_2)-specific phospholipase C (PLC) enzymes transduce signals by generating two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. The β isoforms of these enzymes, PLCβ_{1-3}, are activated by the α subunit of the G-protein Gq (for review, see Rhee and Bae, 1997). Among the G-protein-coupled receptors linked to activation of Gαq 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 Gα subunits and the subsequent regulation of adenylyl cyclase and PLCβ_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 PLCβ_1 activation both in a positive and negative manner (Popova et al., 1997). At low (nanomolar) concentrations, tubulin activates PLCβ_1, whereas at higher concentrations, enzyme inhibition is observed.

Previous studies have indicated that transactivation of Gαq, through a direct GTP transfer from tubulin, is responsible for PLCβ_1 activation by tubulin (Popova et al., 1997; Popova and Rasenick, 2000). However, the mechanism behind the inhibition of PLCβ_1, observed at high dimeric tubulin concentrations, has not been elucidated.

Tubulin binds PIP_2, and this inhibits microtubule polymerization (Popova et al., 1997). Because PIP_2 is the PLC preferred substrate, sequestration of PIP_2 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 PIP_2 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 PIP_2 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 PLCβ_1 *in vitro*, a similar inhibitory mechanism was suggested (Popova et al., 1997).

This study was designed to evaluate the interaction between tubulin and PIP_2 and test how this interaction affects Gαq and PLCβ_1 activation at the membrane. The results reveal that PIP_2 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 Gαq transactivation, receptor-independent binding of tubulin to PIP_2-rich sites on the membrane obstructs PLCβ_1 activation. Thus, it appears that tubulin and PIP_2 interact to effect a dual regulation of PLCβ_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, Gαq, or PLCβ_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), Gαq/11 (number 0945; from D. Manning, University of Pennsylvania, Philadelphia, PA), and PLCβ_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 [^{3}H]L-quinuclidinyl [phenyl-4(n)]benzilate ([^{3}H]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-azidoanilido)-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 × *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 × *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 antibody (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 μl. 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 polyethyleneimide 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 μl 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 Gαq, were run along the samples to identify the bands of tubulin and Gαq. As shown previously (Popova and Rasenick, 2000), carbachol-evoked membrane association of tubulin and Gαq 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, Gαq, or PLCβ_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 ± *g* at 4°C the extracts (0.5 mg/ml membrane protein) were incubated with 1 μM 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 μCi/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 μM colchicine in the same buffer. After triplicate wash with Locke's buffer, 10 μM 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 μM 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 × *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 (Biotechs, Inc.) were transiently transfected with 5 μg 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 (Scananlatics, 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 μM colchicine. After a PBS wash, the cells were treated for 2 min with 1 mM carbachol, 10 μM 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-PIP_2 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× 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 PIP_2 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-PIP_2 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-PIP_2 antiserum was preincubated with PIP_2 (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 Gαq (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 PIP_2 decreases tubulin polymerization

Previous experiments demonstrated that PIP_2 bound to tubulin and inhibited microtubule assembly (Popova et al., 1997). The specificity of tubulin–PIP_2 interaction was not addressed in that study. Phosphatidylinositol 3,4,5-trisphosphate (PIP_3) as well as the second messenger IP_3 can bind to certain PIP_2-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 PIP_2, several charged and neutral phospholipids as well as IP_3 were included in microtubule polymerization assays. PC-tubulin, purified free of microtubule-associated proteins, was preincubated with the phospholipids tested or IP_3. 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 PIP_2 inhibited tubulin polymerization by $39.9 \pm 3.6\%$ (SEM; $n = 5$) compared with the control not containing this phosphoinositide (Fig. 1*A*). Other closely related anionic phosphoinositides, such as PIP_3, phosphatidylinositol 3-phosphate (PIP), and phosphatidylinositol (PI), as well as the negatively charged inositol phosphate IP_3, 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. 1*A*). Electron microscopy and light scattering of tubulin samples was also done. As was the case with microtubule pellets, PIP_2 but not PIP_3, PC, PE, PI, or IP_3 inhibited microtubule formation. Thus, it is suggested that the regulatory phosphoinositide PIP_2 inhibits microtubule polymerization through a specific interaction with tubulin.

To test whether the effect of PIP_2 on tubulin was concentration-dependent, tubulin polymerization was studied in the absence or presence of PIP_2, PC, or the mixture of both in light-scattering experiments (Fig. 1*B*). When the concentration of PIP_2 was reduced by half (PIP_2 mixed with PC at a molar ratio of 1:1), the inhibition of microtubule formation was also half that seen with PIP_2 alone.

PIP_2 does not affect the binding and hydrolysis of GTP by tubulin

Several possible mechanisms exist through which PIP_2 binding could interfere with tubulin polymerization. One possibility is

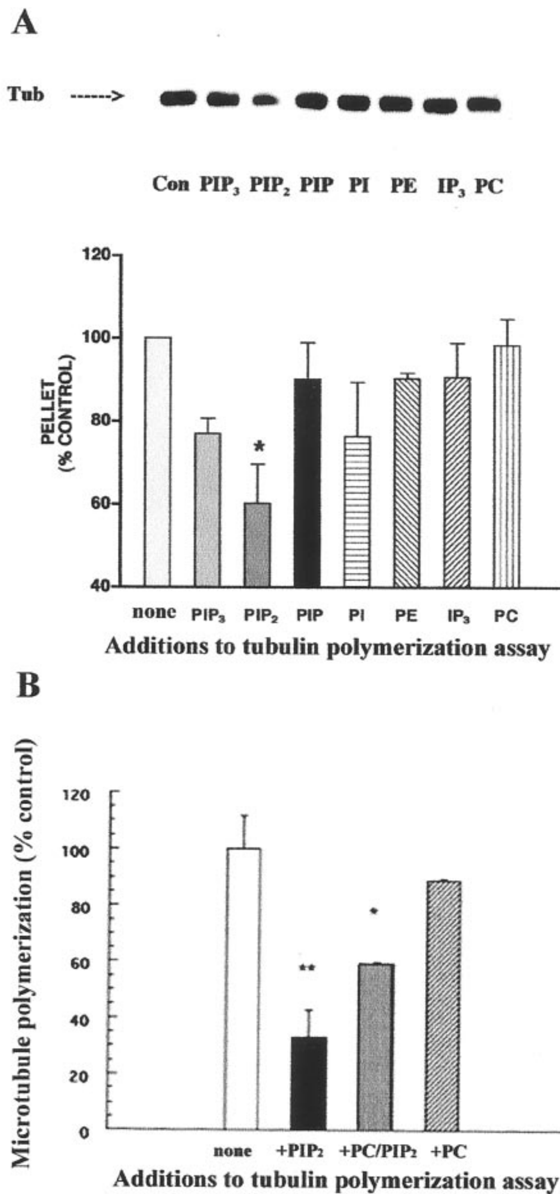

Figure 1. PIP_2 inhibits tubulin polymerization. *A*, Comparison of the effects of various phosphoinositides on microtubule polymerization. Where indicated, PIP_3, PIP_2, PIP, PI, PC, PE, and the inositol phosphate IP_3 (final molar concentration of 75 μM) were preincubated with tubulin (*Tub*) as described 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*, PIP_2 inhibition of microtubule formation is concentration-dependent. PIP_2, PC, mixed vesicles of PIP_2 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 $\pm$ 0.12 (control). *$p < 0.05$; **$p < 0.01$.

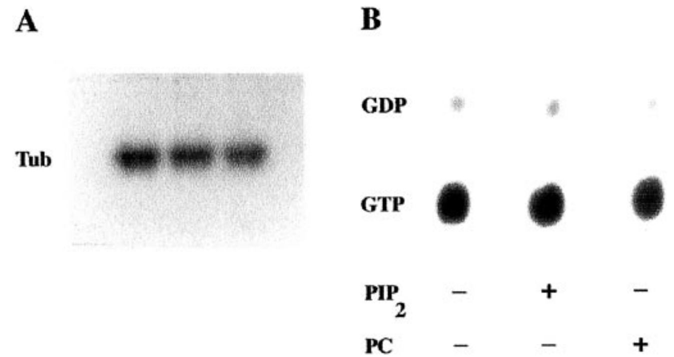

Figure 2. PIP_2 does not alter binding and hydrolysis of GTP by tubulin. *A*, Effects of PIP_2 on [^{32}P]AAGTP binding to tubulin (*Tub*). Phosphocellulose-purified tubulin, stripped of nucleotide, was incubated with [^{32}P]AAGTP in the absence or presence of phosphoinositides. *First lane*, No addition to the binding reaction; *second lane*, PIP_2 added at the start of the binding reaction; *third lane*, PIP_2 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 PIP_2 on GTPase activity of tubulin. Tubulin–[^{32}P]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 PIP_2 affects the binding of GTP to tubulin, because PIP_2 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 PIP_2. Because tubulin must bind GTP to assemble, PIP_2 could block the process by activating tubulin GTPase.

PIP_2 did not modify the amount of [^{32}P]AAGTP or [^{32}P]GTP bound to tubulin, estimated at 0.49 $\pm$ 0.08 mol bound/mol of tubulin (Fig. 2*A*). This was independent of whether PIP_2 was added to tubulin before or after the course of the guanine nucleotide binding reaction. Densitometry revealed relative absorbance values of 100.0 $\pm$ 14.6 for the tubulin–[^{32}P]AAGTP band obtained when PIP_2 was not present in the binding reaction, as well as 94.41 $\pm$ 10.2 and 103.3 $\pm$ 15.1 for the bands obtained when PIP_2 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 (Carlier and Pantaloni, 1981). If PIP_2 activated the GTPase of tubulin dimers, polymerization would be blocked, because those dimers would be binding GDP. However, PIP_2 did not promote hydrolysis of GTP by tubulin. The amount of GTP bound per mole of tubulin remained at 0.55 $\pm$ 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*).

PIP_2 promotes association of tubulin with the membrane but does not promote Gαq transactivation by tubulin

PIP_2 is normally membrane-associated. Although it is not clear how tubulin associates with membranes (a subject of some controversy), it is possible that PIP_2 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 PLCβ_1 (Popova et al., 1997) and membranes from SK-N-SH neuroblastoma cells, which normally

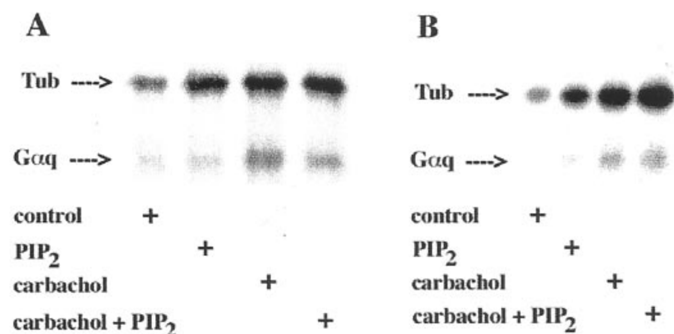

Figure 3. PIP_2 binding increases membrane-associated tubulin (*Tub*). *A*, Membranes from Sf9 cells expressing m1 muscarinic receptors, Gαq, and $PLC\beta_1$ (20 μg of membrane protein) were incubated with 300 nM tubulin–[^{32}P]AAGTP with or without 100 μM carbachol, 30 μM PIP_2, or both for 5 min 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–[^{32}P]AAGTP or carbachol, PIP_2, 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 $PLC\beta_1$ (Fisher and Heacock, 1988).

In membranes from infected Sf9 or SK-N-SH cells, both carbachol and PIP_2 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–[^{32}P]AAGTP with the Sf9 cell membranes was 99.6 ± 22.4% (SD; $n = 3$) in the presence of carbachol and 96.1 ± 17.7% ($n = 3$) in the presence of PIP_2. When both of them were present, association of tubulin with the membrane was 115.1% ± 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 [^{32}P]AAGTP from tubulin to Gαq. Although Gαq transactivation by tubulin increased by 124.0 ± 23.0% (SD; $n = 3$) after muscarinic receptor stimulation (Fig. 3*A*), it was not affected by PIP_2. Atropine inhibited the membrane association of tubulin evoked by carbachol, but it failed to suppress the PIP_2-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 PIP_2 on tubulin regulation of Gαq. PIP_2 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–[^{32}P]AAGTP gave similar results. The effects of PIP_2 on tubulin–[^{32}P]AAGTP membrane association were also concentration-dependent (Fig. 4*B*). However, as shown in Figure 4*C*, over a range of tubulin–[^{32}P]AAGTP concentrations, transactivation of Gαq by tubulin was independent of PIP_2. In fact, a decrease in the carbachol-evoked [^{32}P]AAGTP transfer from tubulin to Gαq was observed at PIP_2 concentrations of >40 μM (Fig. 4*D*). Thus, although PIP_2 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 PIP_2 binding to tubulin interfered with both tubulin polymerization properties and the ability to transactivate Gαq.

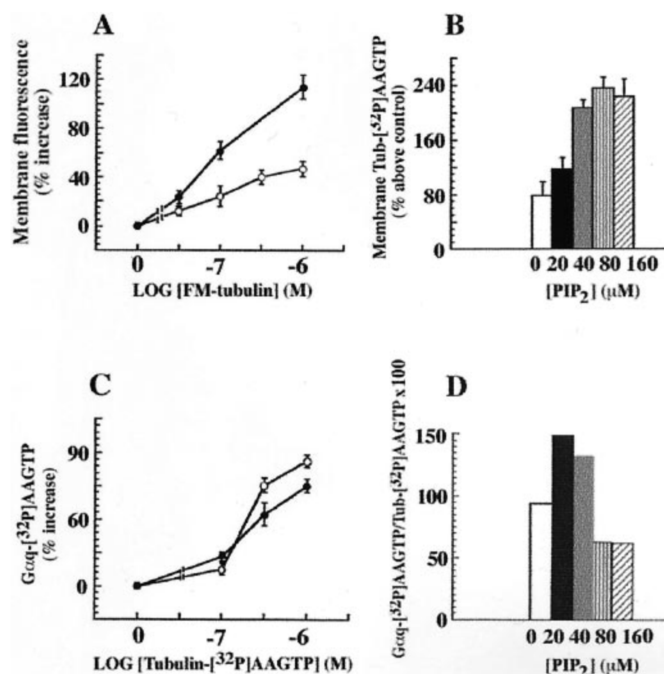

Figure 4. PIP_2 does not increase Gαq transactivation by tubulin. *A*, The effects of carbachol and PIP_2 on the membrane association of tubulin are additive. Recruitment of tubulin to the membrane was studied using increasing concentrations of FM-tubulin. Membranes from Sf9 cells expressing m1 muscarinic receptors, Gαq, and $PLC\beta_1$ were incubated with carbachol and FM-tubulin (at the indicated concentrations), with or without PIP_2. 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 PIP_2. *B*, PIP_2-assisted recruitment of tubulin–[^{32}P]AAGTP to the membrane is concentration-dependent. Membranes from Sf9 cells containing m1 muscarinic receptors, Gαq, and $PLC\beta_1$ were incubated with 1 mM carbachol, 1 μM tubulin–[^{32}P]AAGTP, and increasing concentrations of PIP_2, as described in Figure 1. After SDS-PAGE (50 μg of membrane protein in each *lane*) ^{32}P-labeled protein bands were measured by phosphorimage 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 PIP_2. The experiments were done as described in *A*, except that tubulin–[^{32}P]AAGTP was used. Proteins were resolved by SDS-PAGE (50 μg of membrane protein in each lane), and the radioactivity of the Gαq bands ([^{32}P]AAGTP was transferred from tubulin) was measured by phosphorimage 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 PIP_2. *D*, The increased membrane association caused by PIP_2 was not linked to Gαq transactivation. The percent ratios of [^{32}P]AAGTP-labeled Gαq and tubulin–[^{32}P]AAGTP at the various PIP_2 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 PIP_2.

PIP_2 increases the association of tubulin with $PLC\beta_1$

Because PIP_2 is the natural substrate for $PLC\beta_1$, the relevance of $PLC\beta_1$ to the process of PIP_2-mediated association of tubulin with the membrane was tested. In the absence of $PLC\beta_1$, PIP_2 had no effect on the association of tubulin with the Sf9 cell membranes (Fig. 5*A*). Thus, it appeared that $PLC\beta_1$ was involved in the PIP_2-promoted membrane association of tubulin.

This was tested by coimmunoprecipitation. Tubulin coimmu-

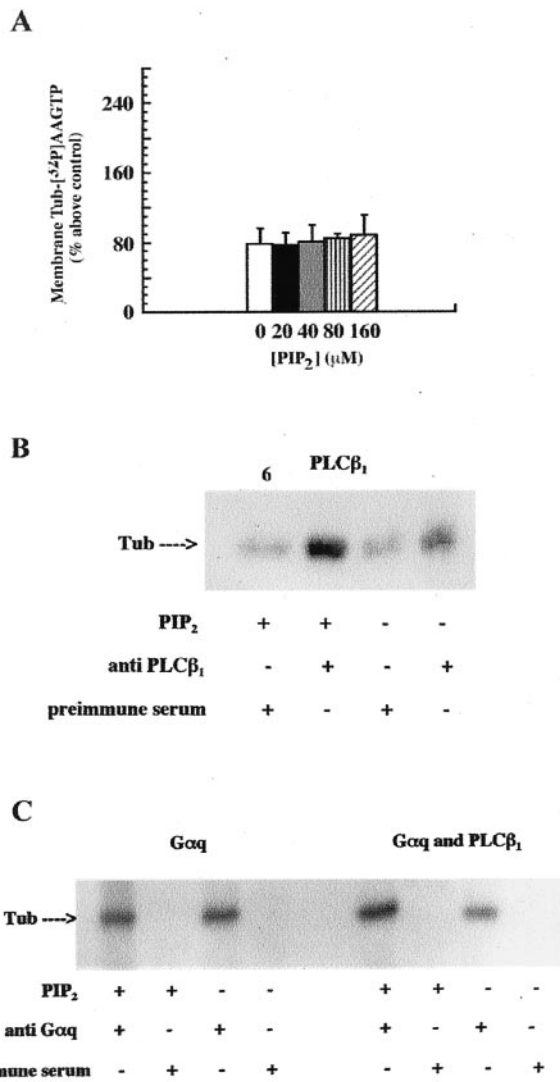

Figure 5. PIP_2 is involved in the association of tubulin with PLCβ1. *A*, PIP_2 does not potentiate the membrane association of tubulin when $PLC\beta_1$ is not present. Membranes from Sf9 cells containing m1 muscarinic receptors and Gαq but not $PLC\beta_1$ were incubated with tubulin–[^{32}P]AAGTP and the indicated concentrations of PIP_2 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*, PIP_2 increases coimmunoprecipitation of $PLC\beta_1$ with tubulin (*Tub*). Membrane preparations of Sf9 cells containing $PLC\beta_1$ were extracted with 1% sodium cholate. Where indicated, tubulin–[^{32}P]AAGTP was preincubated with PIP_2 as described in Materials and Methods. After UV irradiation, each sample was incubated overnight with anti-$PLC\beta_1$ 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*, PIP_2 increased coimmunoprecipitation of Gαq and tubulin when $PLC\beta_1$ was present. Membrane preparations of Sf9 cells, expressing either Gαq or Gαq and $PLC\beta_1$, 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 $PLC\beta_1$ 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, $PLC\beta_1$ (Popova et al., 1997). However, the mechanism whereby PIP_2 affects these interactions has not been evaluated. Extracts from Sf9 membranes, containing $PLC\beta_1$, Gαq, or both, were tested (Fig. 5*B,C*). PIP_2 increased coimmunoprecipitation of tubulin–[^{32}P]AAGTP with $PLC\beta_1$ by approximately twofold [204 ± 11.0% (SD)], suggesting stabilization of tubulin–$PLC\beta_1$ interaction (Fig. 5*B*). PIP_2 did not alter the coimmunoprecipitation of tubulin and Gαq (Fig. 5*C*, *left*). However, when Gαq and $PLC\beta_1$ were both present on the membrane, PIP_2 increased Gαq–tubulin coimmunoprecipitation by twofold [216 ± 10.0% (SD); Fig. 5*C*, *right*]. These results suggested that PIP_2 might promote the formation of tubulin–Gαq–$PLC\beta_1$ complexes.

Carbachol stimulation causes redistribution and colocalization of intracellular tubulin with PIP_2 at the plasma membrane

If tubulin–PIP_2 interaction modulates a related membrane signaling event, we would expect to see colocalization of tubulin and PIP_2 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 PIP_2 in carbachol-treated and untreated SK-N-SH cells. A monoclonal antibody shown to bind specifically to endogenous PIP_2 and to inhibit the intracellular breakdown of this phosphoinositide was used (Fukami et al., 1988). This antibody blocked the PIP_2-mediated increase in tubulin binding to isolated SK-N-SH membranes. Because Lipofectin treatment compromised membrane PIP_2, 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-PIP_2 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 PIP_2 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 PIP_2 in regions along the plasma membrane. Tubulin and PIP_2 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 $PLC\beta_1$ 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-

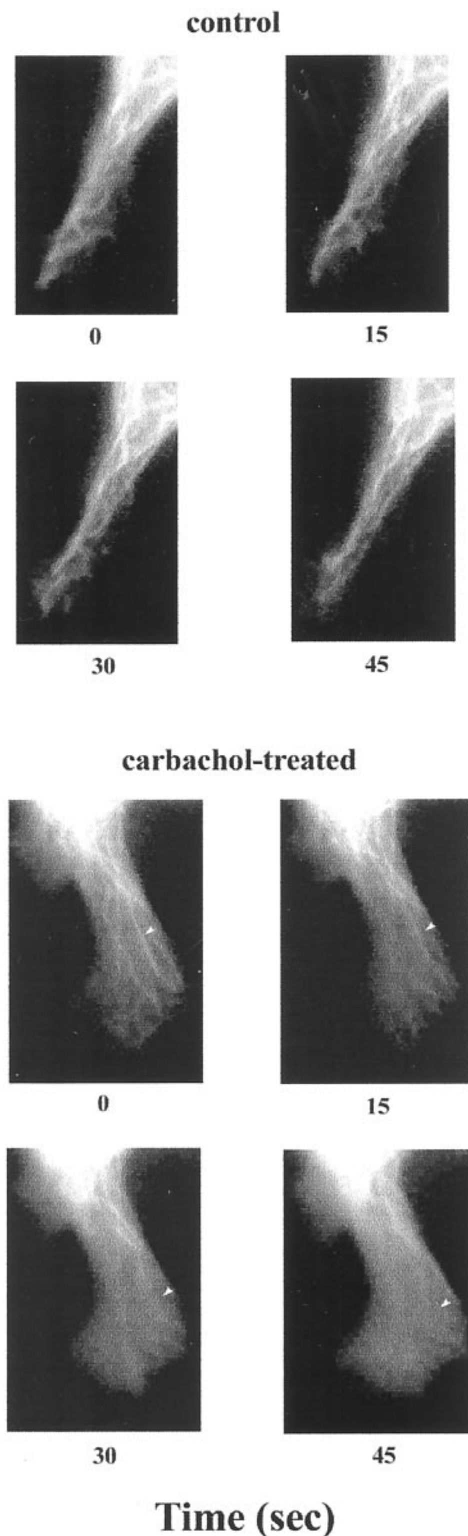

Figure 6. Microtubule depolymerization and redistribution of tubulin in response to carbachol stimulation in GFP–tubulin-expressing SK-N-SH cells. SK-N-SH cells were transfected with EGFP–tubulin cDNA as described. Twenty four hours after transfection, cells treated with either vehicle (control) or 100 μM 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.

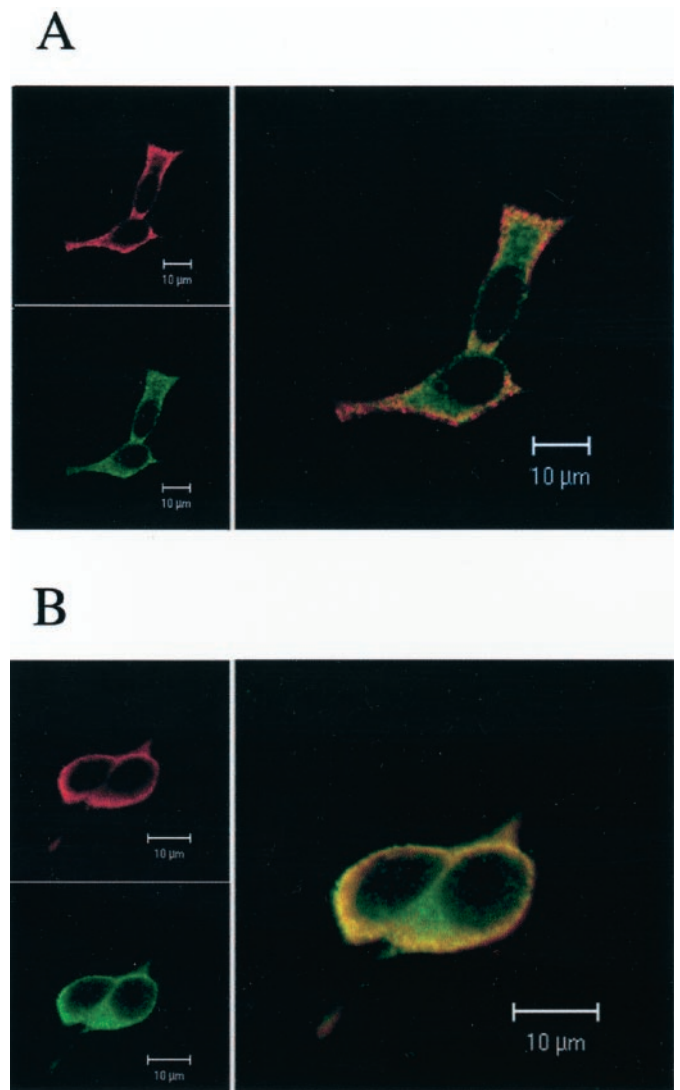

Figure 7. Carbachol stimulation causes microtubule depolymerization and translocation of tubulin to PIP_2-enriched membrane regions of SK-N-SH-neuroblastoma cells. Cells were untreated (*A*) or treated with 1 mM carbachol for 2 min (*B*) before fixation, followed by FITC labeling of tubulin and Texas Red labeling of PIP_2, as described. Carbachol-induced concentration of tubulin in the PIP_2-enriched membrane and submembrane areas of the cells (*B*) is apparent. Tubulin–PIP_2 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 Gαq.

Endogenous phosphoinositide pools of SK-N-SH cells were prelabeled with *myo*-[^{3}H]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 PIP_2 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

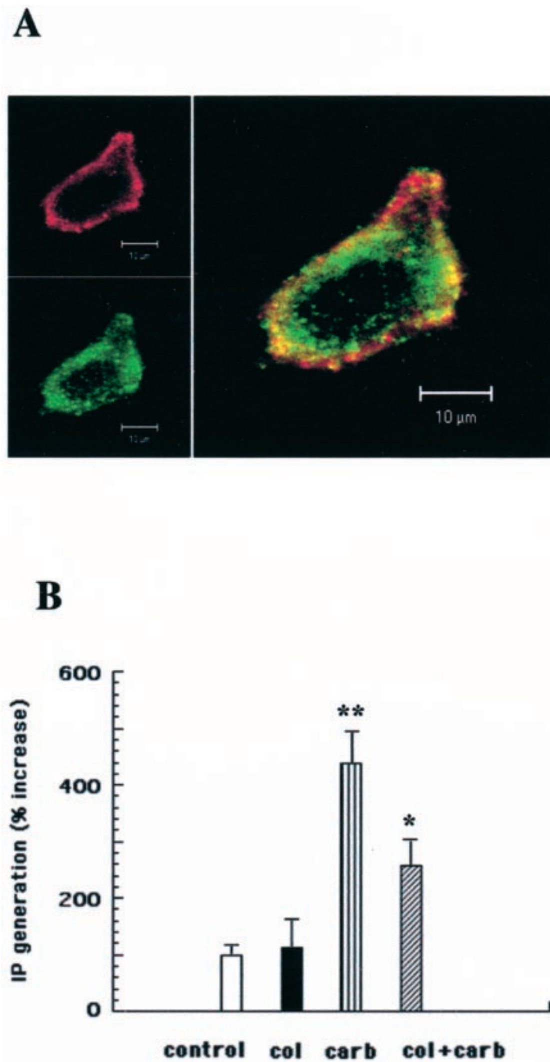

Figure 8. Colchicine-evoked microtubule depolymerization inhibits PLCβ signaling in SK-N-SH neuroblastoma cells. *A*, Confocal immunofluorescence image of an SK-N-SH cell treated for 15 min with 33 μM colchicine as described in Materials and Methods. Microtubule depolymerization as well as colocalization (*yellow*) of tubulin (*green*) with PIP_2 (*red*) is demonstrated. *B*, *Myo*-[^{3}H]inositol-prelabeled SK-N-SH cells were treated for 15 min with 33 μM colchicine (*col*). Carbachol (*carb*; 10 μM) 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 Gαq or $PLC\beta_1$. When Sf9 membranes containing these proteins were pretreated for 15 min with colchicine (10 μM), the membrane association of 1 μM tubulin–[^{32}P]AAGTP, induced by carbachol or PIP_2, was unaltered. Carbachol (100 μM) and guanosine 5′-(β,γ-imido)triphosphate (10 μM) activation of $PLC\beta_1$ were also unaffected when Sf9 membranes were pretreated with colchicine (2.71 ± 0.2 nmol · min^{-1} · mg of protein^{-1} and 2.79 ± 0.6 nmol · min^{-1} · mg of protein^{-1} before and after colchicine, respectively).

DISCUSSION

The purpose of this study was to determine whether and how PIP_2 contributes to the regulation of $PLC\beta_1$ signaling by tubulin. Although PIP_2 is the preferred substrate for $PLC\beta_1$, 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, $PLC\beta_1$ (Popova et al., 1997). Tubulin binding to Gαq, followed by transactivation of Gαq attributable to the transfer of GTP from tubulin, appears responsible for the activation phase (Popova and Rasenick, 2000). The mechanism by which tubulin inhibits $PLC\beta_1$ had not been revealed, but it appeared to involve PIP_2.

A previous study (Popova et al., 1997) speculated that, at high tubulin concentrations, association of PIP_2 with tubulin might render PIP_2 unavailable to $PLC\beta_1$ decreasing $PLC\beta_1$ activity. However, this previous study left open another possibility, which is that the binding of PIP_2 might also affect the GTP-binding or -hydrolyzing properties of tubulin (Davis et al., 1994) and might render tubulin unable to transactivate Gαq (Popova et al., 1997). Data in Figures 1 and 2 showed that although PIP_2 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 PIP_2 had no direct effect on Gαq transactivation by tubulin, it supported the membrane association of tubulin in both Sf9 cells, which ectopically express recombinant muscarinic receptors, Gαq, and $PLC\beta_1$, and SK-N-SH neuroblastoma cells, which normally contain these proteins (Figs. 3, 4). Colocalization of tubulin and PIP_2 along the plasma membrane of SK-N-SH neuroblastoma cells was also observed (Fig. 7). These results are concordant with the idea that PIP_2-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. PIP_2 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 PIP_2 is ~3% (Prinetti et al., 2001). Because the protein content of these domains is ~0.1–2.8% (Prinetti et al., 2001), protein/PIP_2 ratios ranging between 1:0.8 and 1:13.5 are estimated. These values are concordant with the tubulin/PIP_2 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 PIP_2 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 Gα 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 Gαi 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 Gαq and $PLC\beta_1$ might stabilize their active conformation and potentiate $PLC\beta_1$ activation (Fig. 9*A*). 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. 9*B*). Consistent with this hypothesis is the observation that high concentrations of PIP_2 decrease the interaction of tubulin with Gαq 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 Gαq. 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, Gαs and Gαi 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 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 βγ 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 Gβγ (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.

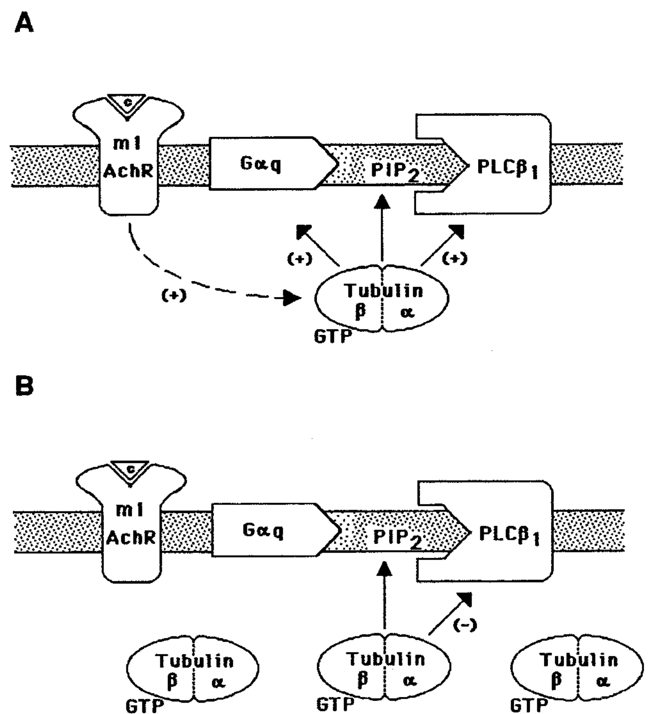

Figure 9. Mechanism of tubulin regulation of $PLC\beta_1$ activity. *A*, Initial association of tubulin with the membrane: activation of Gq and $PLC\beta_1$. It is hypothesized that m1 muscarinic receptor stimulation triggers association of tubulin with the plasma membrane, resulting in subsequent regulation 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–Gαq–$PLC\beta_1$ 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 Gαq, 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, m_1 Muscarinic acetylcholine receptor.

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 Gαq-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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