The cytoskeletal protein, tubulin, has been shown to regulate adenyl cyclase activity through its interaction with the specific G protein \( \alpha \) subunits, \( \text{G}_{\alpha} \) or \( \text{G}_{\alpha_i} \). Tubulin activates these G proteins by transferring GTP and stabilizing the active nucleotide-bound \( \text{G}_{\alpha} \) conformation. To study the possibility of tubulin involvement in \( \text{G}_{\alpha_i} \)-mediated phospholipase \( \text{C}_{\beta_1} \) (PLC\( _{\beta_1} \)) signaling, the \( m_1 \) muscarinic receptor, \( \text{G}_{\alpha_i} \) and PLC\( _{\beta_1} \), were expressed in SF9 cells. A unique ability of tubulin to regulate PLC\( _{\beta_1} \) was observed. Low concentrations of tubulin, with guanine nucleotide bound, activated PLC\( _{\beta_1} \), whereas higher concentrations inhibited the enzyme. Interaction of tubulin with both \( \text{G}_{\alpha_i} \) and PLC\( _{\beta_1} \), accompanied by guanine nucleotide transfer from tubulin to \( \text{G}_{\alpha_i} \), is suggested as a mechanism for the enzyme activation. The PLC\( _{\beta_1} \) substrate, phosphatidylinositol 4,5-bisphosphate, bound to tubulin and prevented microtubule assembly. This observation suggested a mechanism for the inhibition of PLC\( _{\beta_1} \) by tubulin, since high tubulin concentrations might prevent the access of PLC\( _{\beta_1} \) to its substrate. Activation of \( m_1 \) muscarinic receptors by carbachol relaxed this inhibition, probably by increasing the affinity of \( \text{G}_{\alpha_i} \) for tubulin. Involvement of tubulin in the articulation between PLC\( _{\beta_1} \) signaling and microtubule assembly might prove important for the intracellular governing of a broad range of cellular events.

**EXPERIMENTAL PROCEDURES**

**Baculovirus-directed Protein Expression in SF9 Cells, Membrane Preparation, and Western Blotting**—SF9 cells were infected in different combinations with baculoviruses bearing the \( m_1 \) muscarinic receptor, \( \text{G}_{\alpha_i} \), or PLC\( _{\beta_1} \) cDNAs at a ratio of 1:1 and multiplicity of infection of 5. The construction of the recombinant baculoviruses was described earlier (19–21). Cells were harvested after 65 h, sonicated in ice-cold 20 mM Hepes, pH 7.4, 1 mM MgCl\(_2\), 100 mM NaCl, 1 mM DTT, 0.3 mM phenylmethylsulfonyl fluoride. After centrifugation at 500 \( \times \) \( g \), the supernatant was collected and centrifuged at 100,000 \( \times \) \( g \) for 30 min at 4 °C. The membrane pellet was washed, resuspended in the same
buffer, and frozen in aliquots in liquid nitrogen. Protein concentrations were determined by the Bradford dye-binding assay (22), using bovine serum albumin as a standard. The expression of recombinant proteins was verified by immunoblotting. Membrane proteins transferred to nitrocellulose were probed with antisera specific for the m₁ muscarinic receptor (from G. Ludwig, Philadelphia) or PLCβ₂ (anti-holoenzyme) at a dilution of 1:500. Biotinylated goat anti-rabbit IgG and streptavidin-alkaline phosphatase conjugate were used for detection, and a Molecular Dynamics densitometer was used to assess expression levels, which varied by no more than 10% for a given recombinant polypeptide.

Receptor binding studies using [3H][N,N-Boc-QNB was described (9). GTP analogues were of analytical grade. Tubulin-GppNHp, as described (30), and [3H]inositol trisphosphate ([3H]IP₃) in the aqueous phase were determined by the Bradford dye-binding assay (22), using bovine serum albumin was added, and the tubes were sonicated in a Branson water bath sonicator for 15 min at 4°C, followed by an additional 45 min on ice. The samples were run on a 0.7 × 50-cm column of Ultrigel AcA 34 at 4°C. The column was equilibrated with buffer A (flow rate = 20 ml/h), and fractions of 0.5 ml were collected and assayed for protein. The data are given in arbitrary units since lipids quench the Bradford dye-binding assay (32).

Electrotransfection—Samples were taken from tubulin polymerization reactions (2 mg/ml final protein concentration) carried out in buffer A, containing 3 mM MgCl₂, 1 mM GTP, and 30% glycerol, for 30 min at 37°C with constant shaking. Immune complexes were precipitated with pan-muscarinic receptor, and 100 μg of protein of each immunoprecipitate were subjected to SDS-PAGE and autoradiography. The antisera used showed no cross-reactivity to tubulin.

Gel Filtration Assay (32)—Homogeneous PIP₂ micelles were prepared by suspending 100 μg of PIP₂ in 0.5 ml of buffer A and sonication on ice as described (32). Tubulin was incubated with PIP₂ micelles in a Branson water bath sonicator for 15 min at 4°C, followed by an additional 45 min on ice. The samples were run on a 0.7 × 50-cm column of Ultrigel AcA 34 at 4°C. The column was equilibrated with buffer A (flow rate = 20 ml/h), and fractions of 0.5 ml were collected and assayed for protein. The data are given in arbitrary units since lipids quench the Bradford dye-binding assay (32).

Materials—[α-32P]GTP was from ICN Biomedicals, Inc. [3H][N,N-Boc-QNB was from American Radiolabeled Chemicals Inc. [3H][N,N-Boc-QNB was from Ameri- sham Corp. GppNHp, GDP, and GDPβS were from Boehringer Mannheim. Carbamyl, atropine sulfate, PIP₂, phosphatidylcholine, and phosphatidylethanolamine were from Sigma. p-Azidoamine was synthesized by Dr. William Dunn (University of Illinois, Chicago). Ultrigel AcA 34 was from Pharmacia Biotech Inc. P6-DG was from Bio-Rad. All other reagents were of analytical grade.

RESULTS AND DISCUSSION

Receptor-independent Regulation of PLCβ₁ by Tubulin—Simultaneous baculovirus-mediated expression of Go₄ and PLCβ₁ was performed in Sf9 cells, and the effects of GppNHp and tubulin with GppNHp bound (tubulin-GppNHp) on Go₄ regulated PLCβ₁ activity were studied in membranes prepared from infected cells (Fig. 1). GppNHp increased the already high basal PLCβ₁ activity in a concentration-dependent and saturable manner (Fig. 1A), confirming the functional coupling between the recombinant Go₄ and PLCβ₁. GppNHp (at 100 μM) did not affect PLCβ₁ activity in membranes from cells expressing only PLCβ₁ (Table I), thus indicating that neither Gβγ nor any Go resident in Sf9 cell membranes is capable of activating the expressed PLCβ₁. Expressed Go₄ did not couple to any endogenous PLC activity. Endogenous PLC activity was also undetectable in the presence or absence of GppNHp. Tubulin-GppNHp had been shown to activate adenylate cyclase in membranes from either COS 1 or C6 glioma cells (16, 17) in a manner similar to GppNHp. Surprisingly, it appeared that in membranes prepared from Sf9 cells expressing Go₄ and PLCβ₁, tubulin-GppNHp inhibited PLCβ₁ under the same conditions that GppNHp stimulated the enzyme (Fig. 1B).

To study this further, extracts of Sf9 cells expressing Go₄...
were reconstituted with purified recombinant PLCβ1 and exogenous phospholipid vesicles and assayed under conditions similar to those used for Sf9 cell membranes (Fig. 2). The effects of tubulin-GppNHp, nucleotide-free tubulin, and GppNHp were compared. Although GppNHp stimulated PLCβ1 activity at concentrations higher than 1 μM, 30 nM tubulin-GppNHp activated the enzyme. However, at higher tubulin-GppNHp concentrations inhibition of PLCβ1 was observed. Nucleotide-free tubulin did not activate but only inhibited PLCβ1 at concentrations higher than 300 nM.

**Tubulin Modulation of m1 Muscarinic Receptor Activation of PLCβ1—** Involvement of tubulin in receptor-triggered PLCβ1 regulation was studied with membranes from Sf9 cells expressing m1 muscarinic receptors, Gαq, and PLCβ1. The m1 muscarinic receptor expression level was estimated by receptor binding studies with [3H]QNB as a ligand. When all three recombinant proteins were expressed, the m1 muscarinic receptor binding capacity (Bmax) was 240 ± 21 fmol/mg membrane protein and Kd = 0.162 ± 0.010 nM (n = 3). The ability to construct a complete, receptor-activated and G protein-mediated system allowed a comparison of the effects of GppNHp, tubulin-GppNHp, and tubulin, denuded of exchangeable nucleotide, on carbachol-stimulated PLCβ1 activity (Fig. 3). Although GppNHp was able to activate PLCβ1 without receptor stimulation, the effect was potentiated by carbachol (Fig. 3A). The response of PLCβ1 to GppNHp (up to 1 μM) under these conditions was concentration-dependent, saturable, and potentiated by carbachol throughout the concentration range (50.5 ± 2.9% at 100 μM GppNHp). However, the effect of tubulin-GppNHp on PLCβ1 activity was again biphasic, stimulatory at the lower (30 nM) and inhibitory at the higher concentrations of tubulin (Fig. 3A). Carbachol potentiated the tubulin-GppNHp-evoked PLCβ1 activation. At 30 nM, tubulin-GppNHp was more efficacious than GppNHp, independent of the addition of carbachol. The effect of carbachol was receptor-mediated since, at tubulin concentrations (30 nM) that stimulated PLCβ1, atropine inhibited carbachol-induced PLCβ1 activity by about 80% (Fig. 3B). However, the m1 muscarinic receptor stimulation was able to overcome the inhibitory effect of higher tubulin concentrations, resulting in an effective PLCβ1 activation. Note
that the highest tubulin concentrations used were below those where tubulin-GppNHp forms polymers. To clarify the mechanism of this dual regulation of PLC$_{\text{b1}}$ by tubulin, the effect of nucleotide-free tubulin on enzyme activity was studied (Fig. 3C). Tubulin (without nucleotide) inhibited PLC$_{\text{b1}}$ activity in a concentration-dependent manner. No enzyme activation was observed at any tested tubulin concentration. Furthermore, unlike tubulin-GppNHp, nucleotide-free tubulin did not potentiate the PLC$_{\text{b1}}$ activation induced by carbachol. Tubulin-GDP or tubulin-GDP/$\beta$S (at 30 nM) also failed to potentiate carbachol-triggered PLC$_{\text{b1}}$ activation or to affect the basal enzyme activity. Thus, it appears that tubulin activates PLC$_{\text{b1}}$ only when GTP or GTP analog occupies the exchangeable GTP-binding site.²

**Activation of Go$_{\alpha}$ by Tubulin and Recruitment of Tubulin to the Plasma Membrane**—To study directly the ability of tubulinguanine nucleotide to interact with Go$_{\alpha}$, the transfer of the hydrolysis-resistant photoaffinity GTP analog, $[^{32}\text{P}]$AAGTP, from tubulin to Go$_{\alpha}$ was examined in SF9 cells expressing the $m_1$ muscarinic receptor, Go$_{\alpha}$ and PLC$_{\text{b1}}$. Nucleotide transfer from tubulin appears to be responsible for the activation of Go$_{\alpha}$ and Go$_{\alpha_1}$ and this has been observed in permeable cells, membrane preparations, and reconstituted systems (13–17). It has been shown that Go proteins receive $[^{32}\text{P}]$AAGTP from tubulin under conditions when Go is incapable of binding the free nucleotide. This appears to be due to the formation of a complex between tubulin and Go. Under these conditions, tubulin retains the nucleotide without releasing it to the media (15). Carbachol (1 mM) increased (36 ± 7%) the binding of tubulin-$[^{32}\text{P}]$AAGTP to the membrane, and there was commensurate loss (45 ± 8%) of tubulin-$[^{32}\text{P}]$AAGTP from the supernatant (Fig. 4). Transfer of $[^{32}\text{P}]$AAGTP from tubulin to Go$_{\alpha}$ was also potentiated (42 ± 9%) by carbachol. Atropine blocked carbachol-induced binding of tubulin to the membrane as well as the increase in $[^{32}\text{P}]$AAGTP transfer.³ The manner in which the activated $m_1$ muscarinic receptor increases tubulin association with the membrane to regulate PLC$_{\text{b1}}$ is currently under study. The increase in tubulin-GppNHp activation of PLC$_{\text{b1}}$ brought by carbachol may be explained partially by this facilitated association of tubulin with the membrane.

These findings, together with the results in Figs. 2 and 3, support the idea that nucleotide transfer from tubulin to Go$_{\alpha}$ is a potential mechanism for PLC$_{\text{b1}}$ activation. Carbachol had no effect on the membrane association of tubulin, nucleotide transfer to Go$_{\alpha}$ or PLC$_{\text{b1}}$ activity in SF9 cells expressing only Go$_{\alpha}$ and PLC$_{\text{b1}}$. No effect of carbachol, guanine nucleotide, or tubulin-guanine nucleotide was observed in control SF9 cells when measuring PIP$_2$ hydrolysis or photoaffinity labeling.

**Tubulin, Go$_{\alpha}$, and PLC$_{\text{b1}}$ Form a Complex**—To understand better the mode of interaction of tubulin-GppNHp with the members of PLC$_{\text{b1}}$ signaling cascade, coimmunoprecipitation studies with tubulin-$[^{32}\text{P}]$AAGTP were performed. As seen in

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2 No baculovirus encoding G protein $\beta_\gamma$ subunit was included in these experiments. However, since the level of receptor expression is moderate, carbachol- and GTP-dependent activation of PLC$_{\text{b1}}$ suggest that the expressed $m_1$ muscarinic receptors are coupling to expressed Go$_{\alpha}$ and endogenous SF9 G$_{\gamma_1}$ (33).

3 J. S. Popova, J. C. Garrison, S. G. Rhee, and M. M. Rasenick, unpublished observations.
The source of [32P]AAGTP was tubulin. No tubulin-protein to PIP2 micelles inhibits PLC assembly (34) and that the binding of profilin (an actin-binding protein) to PIP2 micelles, as has been observed in the case of profilin (32). To resolve this, polymerization of tubulin in the absence of PIP2 was studied. In the membrane extracts from normal Sf9 cells were tested with anti-Gq or anti-PLCβ1 antisera. Thus, it is suggested that the formation of a complex between Gq, PLCβ1, and tubulin-GppNHp might be responsible for the higher efficacy of tubulin-GppNHp, compared with GppNHp, during the m1 muscarinic receptor stimulation.

**Fig. 4.** Muscarinic receptor-triggered binding of tubulin-[32P]AAGTP to the membrane and the transfer of [32P]AAGTP to Gq. Membranes were prepared from Sf9 cells expressing recombinant m1 muscarinic receptors, Gq, and PLCβ1 and incubated with 1 μM tubulin-[32P]AAGTP in the presence or absence of 1 mM carbachol for 10 min at 23 °C as described under “Experimental Procedures.” The tubes were UV-irradiated, and the membrane pellets and concentrated supernatants were subjected to SDS-PAGE as described. An autoradiogram of one of five independent experiments with similar results is shown.

**Fig. 5.** Both Gq antisera and, to a lesser extent, PLCβ1 antisera coimmunoprecipitated tubulin when membrane extracts of cells infected with viruses for Gq or PLCβ1, respectively, were studied. In the membrane extracts from cells expressing Gq-[32P]AAGTP-labeling of Gq was also observed. The source of [32P]AAGTP was tubulin. No tubulin-[32P]AAGTP coimmunoprecipitation was observed when extracts from normal Sf9 cells were tested with anti-Gq or anti-PLCβ1 antisera. Thus, it is suggested that the formation of a complex between Gq, PLCβ1, and tubulin-GppNHp might be responsible for the higher efficacy of tubulin-GppNHp, compared with GppNHp, during the m1 muscarinic receptor stimulation.

**PIP2 Binding to Tubulin—** In order to clarify the inhibitory action of tubulin on PLCβ1, the interaction between tubulin and the PLCβ1 substrate, PIP2, was studied. It has been shown previously that phosphatidylinositol inhibits microtubule assembly (34) and that the binding of profilin (an actin-binding protein) to PIP2 micelles inhibits PLCγ1-directed PIP2 hydrolysis (35). To test the possibility that tubulin binds PIP2, a gel filtration assay was performed. Two peaks of tubulin were resolved, one represented a small portion of oligomeric tubulin, and the second represented dimeric tubulin (Fig. 6). When the same amount of tubulin was incubated with PIP2 micelles and passed over an Ultrogel AcA 34 column, an increase in the higher molecular weight peak was observed along with a corresponding decrease in the dimeric tubulin peak. When tested under the same conditions, phosphatidylcholine micelles did not shift the mobility of tubulin. Since PIP2 forms micelles of about 93 kDa in aqueous solutions (36), the increase in the higher molecular weight fraction could be attributed to either PIP2-induced tubulin oligomerization or to a binding of tubulin to PIP2 micelles, as has been observed in the case of profilin (32). To resolve this, polymerization of tubulin in the absence (Fig. 7A) or presence (Fig. 7B) of PIP2 micelles was studied by electron microscopy. Tubulin failed to polymerize properly in the presence of PIP2 (Fig. 7B). Microtubules were considerably shorter (1.7 ± 1.0 μm) compared with 10.2 ± 4.8 μm in the absence of PIP2 and 3–4 times thicker (75–100 nm versus 25 nm in the absence of PIP2). These results suggest a direct interaction between tubulin and PIP2 and support the hypothesis that increased concentrations of dimeric tubulin might prevent access of PLCβ1 to its substrate. Since increased activation of PLCβ1 might evoke localized increases in calcium which could increase tubulin dimer concentration, this might represent a feedback mechanism for the regulation of receptor-G protein-activated phospholipase C. It is noteworthy in this regard that phosphorylation of PLCγ1 by EGF receptor tyrosine kinase appears to overcome the inhibitory effect of profilin binding to PIP2, resulting in an effective activation of the enzyme (35). Similarly, m1 muscarinic receptor stimulation might reverse tubulin-evoked inhibition of PLCβ1 by increasing the affinity of tubulin.
late microtubule assembly. These reciprocal events could be involved both in intracellular signaling and the control of spindle morphogenesis and reorganization of microtubule arrays during the cell cycle. Since alterations in the metabolism of phosphoinositides (40, 41) or the expression of Gα subunits (42) or G proteins (43, 44) have been implicated in cellular transformation, this regulatory mechanism might prove valuable in the control of cell proliferation as well.

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