Functional Significance of $\beta\gamma$-Subunit Carboxymethylation for the Activation of Phospholipase C and Phosphoinositide 3-Kinase†

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ABSTRACT: The γ subunits of heterotrimeric G proteins are isoprenylated and methylated at their carboxyl-terminal cysteine residues. Since methylation is the only reversible reaction in the isoprenylation pathway, it could be a site of regulation of G protein activity. $\beta\gamma$ subunits have been shown to activate a number of effectors involved in signal transduction pathways. The methyl group of retinal transducin (T) can be hydrolyzed by an immobilized form of pig liver esterase, allowing for a direct determination of the activities of methylated and demethylated $T_{\beta\gamma}$. The abilities of methylated and demethylated $T_{\beta\gamma}$ to stimulate G protein regulated phosphatidylinositol-specific phospholipase C (PIPLC) and phosphoinositide 3-kinase (PI3K) were determined. It is reported here that there is a strong dependence on methylation for activating both PIPLC and PI3K. Demethylated $T_{\beta\gamma}$ is at least 10-fold less active than its methylated counterpart. Therefore, methylation may play an important role in the regulation of these effectors and of signal transduction processes in general.

It has become increasingly evident that heterotrimeric G protein[1] $\beta\gamma$ subunits play substantial roles in signal transduction, beyond their abilities to interact with G protein α subunits (Sternweis, 1994; Clapham & Neer, 1993). For example, $\beta\gamma$ subunits have been shown to regulate a variety of effectors, including K^+ channels (Wickman et al., 1994; Reuveny et al., 1994), adenylyl cyclase (Tang & Gilman, 1991), β-adrenergic receptor kinase (βARK) (Koch et al., 1993; Kameyama et al., 1993), phosphatidylinositol-specific phospholipase C (PIPLC) (Camps et al., 1992; Boyer et al., 1992; Smrcka & Sternweis, 1993), and phosphoinositide 3-kinase (PI3K) (Stephens et al., 1994; Thomason et al., 1994). In a well-studied example, it has been proposed that $\beta\gamma$ subunits function as a facilitator of membrane docking for βARK (Koch et al., 1993). Since all $\beta\gamma$ subunits studied thus far are isoprenylated and methylated on the carboxyl-terminal cysteine residue of their γ subunits (Lai et al., 1990; Fukada et al., 1990; Yamane et al., 1990), it is reasonable to question whether these modifications are germane to the facilitating roles of the various $\beta\gamma$ subunits.

A great deal of evidence has accumulated demonstrating that hydrophobic posttranslational modifications by isoprenylation and methylation enhance the membrane association of the modified protein. Isoprenylation has been shown to be required for the functioning of *ras* (Hancock et al., 1989), transducin (Fukada et al., 1990), and rhodopsin kinase (Inglese et al., 1992). G protein γ subunits that lack a site for isoprenoid modification fail to associate with the membrane when expressed in COS cells (Simonds et al., 1991; Muntz et al., 1992). In vitro reconstitution studies indicate that isoprenylation of γ is essential for $\beta\gamma$ interaction with adenylyl cyclase (Iñiguez-Lluhi et al., 1992) and PIPLC (Dietrich et al., 1994).

The carboxymethylation reaction is potentially of great interest, because it is the only step in the isoprenylation pathway which is reversible and, hence, subject to regulation (Tan & Rando, 1992). In a number of systems, carboxymethylation has been shown to play a functional role. These include fungal mating factors (Anderegg et al., 1988), bacterial chemotaxis (Kort et al., 1975), and nuclear lamins (Chelsky et al., 1989). In this paper we explore the functional role(s) of heterotrimeric G protein carboxymethylation.

A test of the functional role(s) of G protein methylation is possible, because the demethylated $\beta\gamma$ subunit of the visual system heterotrimeric protein has been prepared (Parish & Rando, 1994; Fukada et al., 1994). We have accomplished this demethylation using immobilized pig liver esterase to hydrolyze freshly isolated methylated transducin. While demethylated transducin $\beta\gamma$ is equipotent with its methylated counterpart with respect to the catalyzed uptake of GTP-γ-S by T_α initiated by photolyzed rhodopsin in detergent, it is about one-half as active as its methylated counterpart in disk membranes (Parish & Rando, 1994). A similar decrease in activity of demethylated $\beta\gamma$ as compared to the methylated form is also found using synthetic lipid-based vesicles as the membrane (Fukada et al., 1994).

In this paper, methylated and demethylated transducin $\beta\gamma$ are compared with respect to their abilities to activate PIPLC

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[1] Abbreviations: T, retinal transducin; $T_{\beta\gamma}$-OCH_3, carboxymethylated transducin; $T_{\beta\gamma}$-OH, demethylated transducin; R*, photoactivated rhodopsin; ROS, rod outer segments; iPLE, immobilized pig liver esterase; GTP-γ-S, guanosine 5′-(γ-thio)triphosphate; PIPLC, phosphatidylinositol-specific phospholipase C; PI3K, phosphoinositide 3-kinase; $G_{\beta\gamma}$, bovine brain $\beta\gamma$ subunits; DTT, dithiothreitol; G protein, GTP binding protein; BSA, bovine serum albumin; PI-4-P, phosphatidylinositol 4-phosphate; PI-3-P, phosphatidylinositol 3-phosphate; TLCK, tosyllysyl chloromethyl ketone; TPCK, tosylphenylalanyl chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

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and PI3K, two key enzymes in signal transduction (Sternweis & Smrcka, 1992; Kapellar & Cantley, 1994) which are known to be activated by $\beta\gamma$ subunits. Here it is found that there is a strong dependence on methylation for activation of these enzymes. This represents the first instance where heterotrimeric G protein methylation has been shown to play a large role in a signal transduction process.

MATERIALS AND METHODS

Materials

Frozen bovine retinas were obtained from J. A. & W. L. Lawson Co. (Lincoln, NE). Pig liver esterase acrylic beads, hexylagarose, bovine brain phosphatidylinositol 4,5-bisphosphate (PIP_2), and phosphatidylethanolamine (PE) were from Sigma. Bovine liver phosphatidylinositol (PI) and PE were from Avanti Polar Lipids (Alabaster, AL). GTP and GTP-γ-S were from Boehringer Mannheim. GTP-γ-^{35}S (1340 Ci/mmol; 1 Ci = 37 GBq), [*inositol*-2-^{3}H(N)]PIP_2, and [γ-^{32}P]-ATP were from NEN/Dupont. Blue Sepharose CL-6B was from Pharmacia. Filtron-X scintillation fluid was from National Diagnostics.

Methods

Preparation of Detergent-Solubilized Rhodopsin. Solubilized rhodopsin in dodecyl maltoside was prepared in the dark as described previously (Longstaff & Rando, 1985). The rhodopsin samples were stored at −70 °C in 10 mM PIPES, pH 6.5/6 mM dodecyl maltoside/100 mM NaCl.

Preparation of Transducin, $T_{\alpha\beta\gamma}$. The preparation of transducin was based on previously published procedures (Wessling-Resnick & Johnson, 1987; Parish & Rando, 1994). Transducin was extracted completely from ROS membranes by two treatments with 50 μM GTP in 10 mM Tris-HCl, pH 7.4/1 mM DTT/0.1 mM EDTA (total volume 75 mL), for 15 min each. Further purification of transducin was accomplished by hexylagarose chromatography, using a linear gradient of 20−200 mM NaCl in 10 mM MOPS, pH 7.4/1 mM DTT/1 mM EDTA.

Separation of T_α and $T_{\beta\gamma}$-OCH_3. The transducin heterotrimer was separated into its purified T_α and $T_{\beta\gamma}$-OCH_3 subunits by Blue Sepharose chromatography on the basis of previously published procedures (Wessling-Resnick & Johnson, 1987; Fukada et al., 1989; Parish & Rando, 1994). The subunit concentrations were typically 3−5 μM for both T_α and $T_{\beta\gamma}$-OCH_3. T_α was stored in aliquots at −70 °C until use, while $T_{\beta\gamma}$-OCH_3 was kept at 4 °C.

Hydrolysis of $T_{\beta\gamma}$ Subunits. Several samples of $T_{\beta\gamma}$-OCH_3 (600 μL, 3.3 μM) were treated with pig liver esterase conjugated to acrylic beads (iPLE, 25 μL settled volume per tube) and nutated at 4 °C. iPLE has been shown to hydrolyze the carboxyl-terminal methyl ester of the γ subunit of $T_{\beta\gamma}$ (Parish & Rando, 1994). This process leaves the farnesyl moiety unaffected. After 80 h, the supernatants were removed after centrifugation (14 000 rpm, 5 min), and each sample of iPLE was washed with buffer A (10 mM Tris-HCl, pH 7.4/5 mM $MgCl_2$/1 mM DTT/0.1 mM EDTA/100 mM NaCl) (400 μL) for 2 h. The combined supernatants containing $T_{\beta\gamma}$-OH were concentrated (Centricon 10) to 15 μM. Untreated $T_{\beta\gamma}$-OCH_3 samples were concentrated simultaneously (Centricon 10) to 22 μM.

HPLC Analysis of T_γ. Analysis of the γ subunit of transducin (5 μL) was accomplished by reverse-phase HPLC using a C18 column (Dynamax 300A, Rainin) running a linear gradient at 0.75 mL/min. After 10 min at 5% acetonitrile in H_2O with 10 mM TFA, a 40-min gradient was run to 95% acetonitrile in H_2O with 10 mM TFA. At that point, the eluant was held at 95% acetonitrile for an additional 10 min. All transducin samples were injected with added guanidinium chloride at a final concentration of greater than 3 M. The γ subunit eluted as shown in Figure 1, while the β subunit remained bound to the column and did not elute. Unlabeled minor absorbance peaks are present in a background injection. UV absorbance was monitored at 205 nm. The relative concentrations of the $T_{\beta\gamma}$ samples as determined by HPLC analysis were identical to those obtained by an amido black assay.

Assay of GTP Exchange Activity. In a test tube were combined 20 μL of assay buffer (50 mM Tris-HCl, pH 7.4/500 mM NaCl/25 mM $MgCl_2$/5 mM DTT/0.5 mM EDTA), buffer A (an amount that will bring the total assay volume to 100 μL), and T_α and/or $T_{\beta\gamma}$ subunits. In the dark, detergent-solubilized rhodopsin (final concentrations: rhodopsin = 2 nM, dodecyl maltoside = 120 μM) was added, and the tube was incubated at 0 °C. The sample was bleached for 1 min under ordinary room light before GTP-γ-^{35}S was added (2 μM final concentration, 5000−7000 cpm/pmol). An aliquot (70 μL) of each experiment was removed 15 min after GTP-γ-^{35}S addition. Each aliquot was filtered through a nitrocellulose membrane (Schleicher and Schuell, BA85) and immediately washed three times with ice-cold 10 mM Tris-HCl, pH 7.4/100 mM NaCl/5 mM $MgCl_2$/0.1 mM EDTA (4 mL). All assays were done in duplicate. Each membrane was dissolved in Filtron-X scintillation fluid (10 mL) and counted.

Assay of PIPLC Activity. PIPLC β3 activity was assayed as described previously (Smrcka & Sternweis, 1993), with minor modifications. Activity was assayed in sonicated micelles of 50 μM phosphatidylinositol 4,5-bisphosphate, 200 μM bovine brain PE, and [*inositol*-2-^{3}H(N)]PIP_2 (4000 cpm/assay) in a solution containing 50 mM NaHepes, pH 7.5/0.17 mM EDTA/3 mM EGTA/1 mM DTT/17 mM NaCl/67 mM KCl/0.83 mM $MgCl_2$/1 mg/mL BSA (Sigma)/0.05% octyl β-D-glucopyranoside, and 2.7 mM $CaCl_2$, to give 1 μM free Ca^{2+}. Assays were initiated by the addition of $CaCl_2$ and transfer from 4 to 30 °C. Reactions were terminated after 15 min by transfer from 30 to 4 °C and by the addition of 200 μL of ice-cold 10% trichloroacetic acid and 100 μL of 10 mg/mL BSA. Assay mixtures were centrifuged to remove precipitated protein and intact PIP_2, and the supernatant was analyzed by liquid scintillation counting.

Preparation of PIPLC. Phospholipase C β3 was purified from rat brain essentially as described previously (Smrcka & Sternweis, 1993). The protein concentration was estimated by comparative silver staining with a preparation of phospholipase C β1, whose concentration was determined by amido black as described below.

Assay of PI3K Activity. Phosphoinositide 3-kinase was assayed using sonicated micelles containing 600 μM bovine liver PE and 300 μM bovine liver PI in a solution containing 40 mM NaHepes, pH 7.4/2 mM EGTA/1 mM DTT/0.2 mM EDTA/120 mM NaCl/5 mM $MgCl_2$/1 mM β-glycerophosphate/50 μM sodium orthovanadate/1 mg/mL BSA. Cholate (0.1%) was present in the assays used for partial purification

of the PI3K but was omitted in the experiments that compared methylated and demethylated $\beta\gamma$. Reactions were initiated by the addition of 10 μM ATP with [γ-^{32}P]ATP at 5 μCi/assay and by transfer from 4 to 30 °C. Reactions were terminated by addition of a 2:1 methanol:chloroform solution. A stable two-phase system is generated by addition of 100 μL of 2.4 M HCl with 5 mM tetrabutylammonium hydrogen sulfate followed by 440 μL of $CHCl_3$. Samples were centrifuged, and the lower organic phase containing the lipids was extracted with a solution containing 48% methanol/3% chloroform/0.5 M HCl/1 mM EDTA/10 mM tetrabutylammonium hydrogen sulfate. The lower organic phase was dried, suspended in 2:1 methanol:chloroform, and spotted onto TLC plates which were developed with methanol: chloroform:ammonia:water (10:7:1.5:2.5). Plates were analyzed by using an Ambis gas ionization detector, and lipids migrating at the same position as a PI-4-P standard were counted. PI-4-P and PI-3-P are not resolved in this system. It was assumed that the lipid produced in the presence of $\beta\gamma$, but not in its absence, migrating at this position was PI-3-P, for two reasons. (1) It has been previously established that the $\beta\gamma$-stimulated activity identified in the cytosol of these cells was a PI3K activity (Stephens et al., 1994). (2) $\beta\gamma$-stimulated activity at this position was completely inhibited by wortmannin, a specific inhibitor of PI3K (Okada et al., 1994) (data not shown). Immediately after partial purification, as described below, no product migrated at the PI-3-P/PI-4-P position in the absence of $\beta\gamma$ subunits. For experiments comparing methylated and demethylated $\beta\gamma$, radioactivity in the samples was determined using the Cerenkov counting method, prior to confirming the identity of the lipid product by TLC. Since greater than 95% of the label migrated in the PI-3-P position, the numbers determined by Cerenkov counting were used after determining a counting efficiency of 50%.

Partial Purification of PI3K. U937 cells were grown in 10-L spinner flasks in RPMI 1640 with 1% fetal calf serum (UBI), 5 units/mL penicillin, 5 μg/mL streptomycin, and 1× lipid concentrate (Gibco) at 37 °C to a density of 10^6 cells/mL. Cells were harvested by centrifugation at 1000g for 20 min, suspended in phosphate-buffered saline (pH 7.4) containing 10 μM PMSF, and collected again by centrifugation for 20 min. The cells were suspended in 75 mL of a solution of 50 mM Tris-HCl, pH 7.5/2 mM EGTA/1 mM EDTA/1 mM DTT/350 mM sucrose, plus a protease inhibitor cocktail (2 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor, 10 μM PMSF, 1 μg/mL pepstatin A, 20 μg/mL TPCK, and 20 μg/mL TLCK) that was included in all of the buffers used throughout the preparation. The cells were disrupted in a Parr cell disruption bomb by rapid decompression after equilibration at 4 °C for 45 min with N_2 at 500 psi. Unbroken cells and intact nuclei were removed by centrifugation at 700g for 20 min at 4 °C. The membranes were removed by centrifugation at 100 000g for 1 h at 4 °C. The cytosolic supernatant was frozen in liquid N_2 and stored at −70 °C until use.

The cytosol (70 mL at 5 mg/mL) was applied to a 40-mL column of Q Sepharose (Pharmacia) equilibrated with buffer B (50 mM Tris-HCl, pH 7.5/2 mM EGTA/0.2 mM EDTA/1 mM DTT/50 mM NaCl/10% sucrose/1% betaine/0.05% Tween 20 and protease inhibitors). The column was washed with 100 mL of buffer B; this was followed by elution with a gradient from 50 to 400 mM NaCl in buffer B. Fractions were assayed in the presence and absence of 300 nM bovine brain $\beta\gamma$ ($G_{\beta\gamma}$), purified according to Sternweis and Robishaw (1984). Fractions containing the highest activity in the presence of $\beta\gamma$ were pooled, avoiding fractions that contained activity in the absence of $\beta\gamma$. These fractions were concentrated to 6 mL by pressure filtration though an Amicon PM 30 filter and applied to a 200-mL column of Ultrogel AcA 34 (LKB) size exclusion resin equilibrated with buffer C (buffer B, substituting 50 mM Mes, pH 6.5, for Tris-HCl, and 100 mM instead of 50 mM NaCl). The protein was eluted in the same buffer, assayed ± $\beta\gamma$ as described, and peak $\beta\gamma$ stimulated activity was pooled. These fractions were applied to a 5-mL HiTrap SP (Pharmacia) cation-exchange column equilibrated with buffer C and attached to a Pharmacia FPLC system. The column was washed with 10 mL of buffer C and eluted with a gradient from 100 mM to 1 M NaCl in buffer C. Peak $\beta\gamma$ stimulated fractions were pooled and frozen in aliquots at −70 °C. This procedure yielded PI3K activity that was free of other isozymes of PI3K, as judged by a complete dependence on $\beta\gamma$ for activity immediately after purification. With storage at −70 °C over the course of a few months, the activity in the absence of $\beta\gamma$ increased with time, for unknown reasons. The extent of this increase in background activity over a 3-month period can be seen in Figure 4 where no $\beta\gamma$ has been added to the assay.

Analysis of $\beta\gamma$/Micelle Interaction. The association of methylated and nonmethylated $\beta\gamma$ subunits to phospholipid/detergent micelles was assessed by gel filtration chromatography following the method of Bigay et al. (1994). A Superose 12 HR 10/30 column was equilibrated with buffer D (20 mM Tris-HCl, pH 7.5/120 mM NaCl/1 mM $MgCl_2$/5 mM β-mercaptoethanol/100 μM PMSF), with or without lipid/detergent micelles, at a flow rate of 0.5 mL/min. For experiments with lipid/detergent micelles, bovine liver PE (120 μmol) and bovine liver PI (60 μmol) were combined as a $CHCl_3$ solution and dried to a residue with a stream of argon. After the combined lipids were dried in vacuo for 15 h, the PE/PI mixture was sonicated with 0.2% sodium cholate into buffer D (200 mL). These concentrations of PE and PI correspond to those present in the assay of PI3K activity, as described above. Without added detergent, the lipids, at these concentrations, do not completely sonicate into buffer. $T_{\beta\gamma}$-OCH_3 (4 μM, 100 μL) and $T_{\beta\gamma}$-OH (2 μM, 100 μL) were injected onto the Superose 12 column and 500-μL fractions were collected. For injections where the buffer contained the lipid/cholate micelles, the $T_{\beta\gamma}$ samples were preequilibrated for 30 min at 4 °C with buffer D containing PE/PI/cholate micelles (100 μL). The elution of $T_{\beta\gamma}$ subunits was detected by silver staining gels run with aliquots (20 μL) of each fraction. As determined by HPLC analysis, this $T_{\beta\gamma}$-OH sample contained less than 5% $T_{\beta\gamma}$-OCH_3, while the $T_{\beta\gamma}$-OCH_3 sample contained approximately 10% $T_{\beta\gamma}$-OH. Molecular weight standards (BSA, 67 000; ovalbumin, 45 000; chymotrypsinogen A, 25 000; ribonuclease A, 14 000) were injected without lipid/cholate micelles.

Miscellaneous Procedures. Protein concentrations were determined by an amido black assay using BSA as a standard (Schaffner & Weissmann, 1973).

RESULTS

Effects of Methylation on $T_{\beta\gamma}$ Function. Transducin $\beta\gamma$ was prepared and demethylated using immobilized pig liver

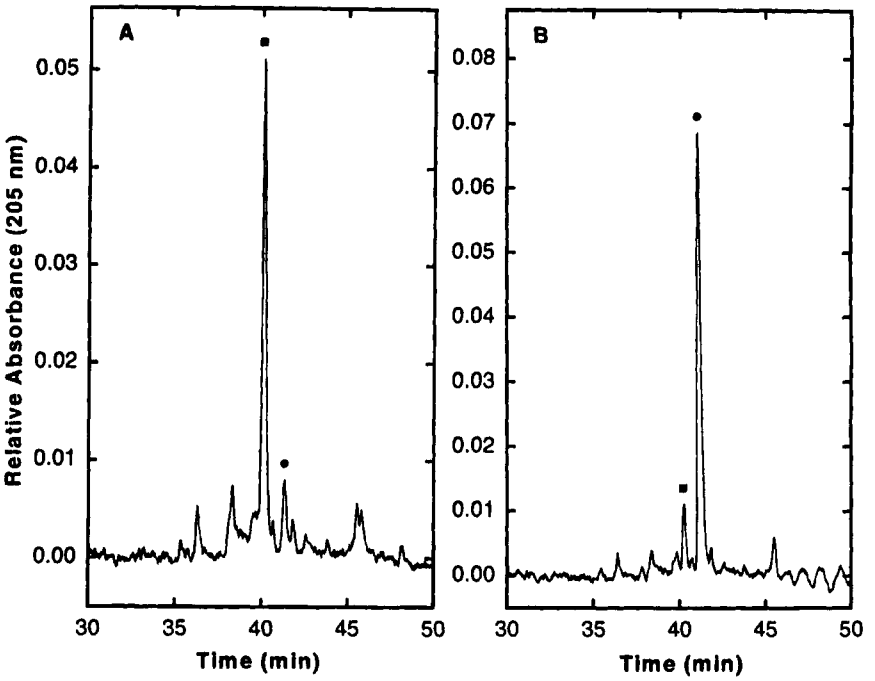

FIGURE 1: HPLC analysis of $T_{\beta\gamma}$-OH and $T_{\beta\gamma}$-OCH_3: (A) hydrolyzed $T_{\beta\gamma}$, 5 μL; (b) untreated $T_{\beta\gamma}$, 5 μL. Symbols: T_γ-OCH_3 (●); T_γ-OH (■). Each $T_{\beta\gamma}$ sample was diluted to 50 μL with buffer A. After the addition of guanidinium chloride (6 M, 75 μL), the sample was analyzed as described in Materials and Methods. The β subunit does not elute from the column.

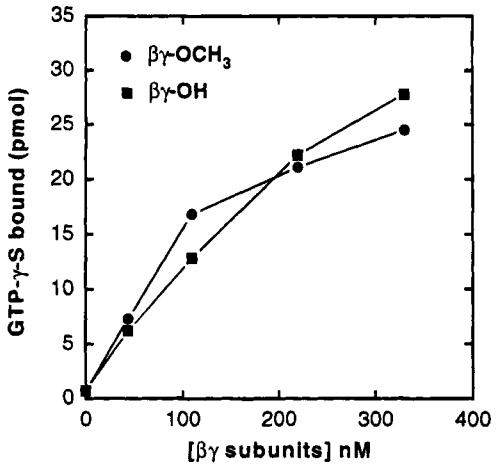

FIGURE 2: GTP-γ-S exchange assays. The indicated concentrations of $T_{\beta\gamma}$ were assayed for the binding of GTP-γ-S to T_α after 15 min (see Materials and Methods). Each experiment contained T_α (0.6 μM), detergent-solubilized rhodopsin (2 nM), and GTP-γ-S (2 μM) and was performed in duplicate at 0 °C.

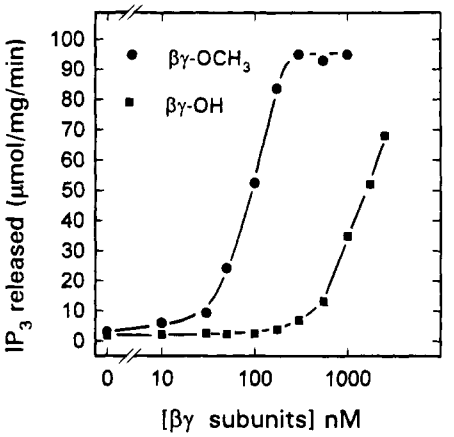

FIGURE 3: Activation of PIPLC β3 by $T_{\beta\gamma}$-OH and $T_{\beta\gamma}$-OCH_3. Assays were conducted as described in Materials and Methods. Each assay contained 0.5 ng of purified PIPLC β3 from rat brain that had been stored at −70 °C.

esterase (iPLE) as previously described (Parish & Rando, 1994). The extent of methylated $\beta\gamma$ hydrolysis can be quantitatively determined by HPLC. In the present case, approximately 90% hydrolysis occurred (Figure 1). It is impractical to achieve quantitative hydrolysis, because of the lower recovery of active $\beta\gamma$ obtained when increasing amounts of iPLE are used. When the demethylated $T_{\beta\gamma}$ was compared to its methylated counterpart in a photolyzed rhodopsin system in detergent, the results shown in Figure 2 were obtained. Activation of rhodopsin is required to bring about the exchange of GTP for GDP on the transducin heterotrimer, resulting in subunit dissociation. In the absence of $T_{\beta\gamma}$, very little GTP-γ-S exchange on T_α is observed under the assay conditions described here. As expected, the two $\beta\gamma$s were equipotent with respect to their abilities to enhance GTP-γ-S exchange on T_α (Parish & Rando, 1994). It is assumed that the β subunit is unaffected by the hydrolysis conditions since it is not methylated and the hydrolyzed material is fully active in the GTP-γ-S binding experiments.

We sought to determine if the state of methylation of $T_{\beta\gamma}$ is of quantitative significance in the activation of PIPLC and PI3K. In these experiments, a $T_{\beta\gamma}$ sample was used which had been hydrolyzed to approximately 90% completion, as shown in Figure 1. As shown in Figures 3 and 4, the demethylated $T_{\beta\gamma}$ subunits were significantly less potent than methylated $T_{\beta\gamma}$ with respect to activating PIPLC and PI3K. It is difficult to calculate the EC_{50} for demethylated $\beta\gamma$, since saturation was not reached in these experiments. If one assumes that the two proteins activate with the same efficacy, approximate EC_{50} values can be calculated in order to compare the proteins. PI3K is activated with an EC_{50} of approximately 300 nM by methylated $T_{\beta\gamma}$, whereas if activation by demethylated $T_{\beta\gamma}$ were to saturate at the same activity, the EC_{50} would be approximately 2 μM. The differences are even greater for PIPLC activation, with EC_{50}

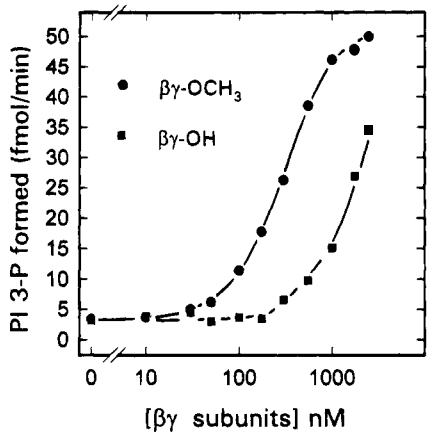

FIGURE 4: Activation of PI3K by $T_{\beta\gamma}$-OH and $T_{\beta\gamma}$-OCH_3. Assays were conducted as described in Materials and Methods. Each assay contained 5 μL of partially purified PI3K that had been stored at −70 °C.

values of 90 nM and 1.6 μM for methylated and demethylated $T_{\beta\gamma}$, respectively.

Since there is about a 10-fold difference in potency between $T_{\beta\gamma}$-OCH_3 and $T_{\beta\gamma}$-OH, and in fact approximately 10% of the $T_{\beta\gamma}$-OH sample is methylated, what activation is recorded could be due to the remaining methylated $T_{\beta\gamma}$. Several experiments utilizing $T_{\beta\gamma}$ samples which contained 5−15% methylated materials gave results similar to those in Figures 3 and 4. Those $T_{\beta\gamma}$-OH samples with less methylated $T_{\beta\gamma}$ appeared to give concomitantly less stimulation. Therefore, it is likely that demethylated $T_{\beta\gamma}$ is essentially inert with respect to the activation of PI3K and PIPLC.

As can be seen when comparing Figures 3 and 4, demethylation appears to have a greater effect on the ability of $T_{\beta\gamma}$ to activate PIPLC than on its ability to activate PI3K. This difference was observed consistently over the course of many trials. The assays of these two enzymes are done under very different conditions, as can be seen when examining the Materials and Methods. The mechanisms of activation of PIPLC and PI3K are unknown and could be different for the two enzymes. Finally, carboxymethylation itself could have a differential effect on the affinities of $T_{\beta\gamma}$ for the two enzymes. Further investigation will be required to determine which of these or other explanations might be responsible for the apparently distinct effects of demethylation on PIPLC and PI3K.

Binding of $T_{\beta\gamma}$ to Micelles. One simple mechanism that would explain the results reported above would involve the lack of binding of demethylated $T_{\beta\gamma}$ to the micelles. In order to investigate the interaction between either methylated or demethylated $T_{\beta\gamma}$ and the phospholipid micelles used in these experiments, a gel filtration chromatography method, previously described by Bigay et al. (1994), was employed. A 2:1 PE:PI mixture was sonicated with sodium cholate (0.2%) using the same phospholipid concentrations used for the assay of PI3K activity. The added cholate was required to solubilize the micelles so that the chromatography could be performed. Despite the fact that cholate is not present when assaying PI3K, we desired some qualitative information regarding the interaction of $T_{\beta\gamma}$-OCH_3 and $T_{\beta\gamma}$-OH with these micelles. Both methylated and demethylated $T_{\beta\gamma}$ began eluting at the proper molecular weight (43 000), but the greater hydrophobicity of $T_{\beta\gamma}$-OCH_3 manifested itself with a large degree of trailing. $T_{\beta\gamma}$-OCH_3 continued to elute from the column well after the point where a 14 000 standard (ribonuclease A) eluted in a separate injection (see Table 1). $T_{\beta\gamma}$-OH also trailed from the 43 000 position, but not to as large an extent as $T_{\beta\gamma}$-OCH_3, finishing its elution by the 14 000 position. In the presence of the PE/PI/cholate micelles, both $T_{\beta\gamma}$-OCH_3 and $T_{\beta\gamma}$-OH eluted sooner than in the absence of lipids, indicating an interaction of both forms with the micelles. Methylated $T_{\beta\gamma}$ was shifted to a greater extent than demethylated $T_{\beta\gamma}$, but the observable effect on the elution of demethylated $T_{\beta\gamma}$ indicates that $T_{\beta\gamma}$-OH does interact with these micelles. These results are consistent with those obtained by Bigay et al. (1994) using phosphatidylcholine/cholate micelles, where $T_{\beta\gamma}$-OCH_3 bound more strongly than $T_{\beta\gamma}$-OH.

Table 1: Elution of $T_{\beta\gamma}$ Subunits from Superose 12 HR 10/30 column[a]

$T_{\beta\gamma}$-OR	±PE/PI/cholate	fractions
R = CH_3	−	28−45
R = H	−	28−38
R = CH_3	+	20−33
R = H	+	24−32

[a] Methylated and demethylated $T_{\beta\gamma}$ subunits were injected onto a Superose 12 HR 10/30 gel filtration column which had been equilibrated with or without PE/PI/cholate micelles. The fractions which contained eluted $T_{\beta\gamma}$ are indicated. The molecular weight standards, in the absence of PE/PI/cholate micelles, eluted in the following fractions: BSA (67 000), fractions 25−27; ovalbumin (43 000), fractions 27−29; chymotrypsinogen A (25 000), fractions 32−35; ribonuclease A (14 000), fractions 36−40.

DISCUSSION

In the experiments reported here, it is demonstrated that demethylated $T_{\beta\gamma}$ is at best a weak activator of both PI3K and PIPLC, when compared to its methylated counterpart. It is difficult to be certain that demethylated $T_{\beta\gamma}$ is completely inert, because there was approximately 10% of residual methylated $T_{\beta\gamma}$ remaining in the sample. However, the activity of the demethylated $T_{\beta\gamma}$ samples appears to correspond to that of the remaining methylated material. This would suggest that any activity observed in these experiments is due to the small amount of methylated $T_{\beta\gamma}$ remaining. It is currently not experimentally feasible to obtain large amounts of a sample with >95% demethylated $T_{\beta\gamma}$.

The lack of activity of demethylated $T_{\beta\gamma}$ with respect to the activation of PI3K and PIPLC is in sharp contrast to what has been observed with activated rhodopsin and T_α. Methylated $T_{\beta\gamma}$ has been shown to be approximately twice as potent as its demethylated counterpart when assayed with native disk membranes (Parish & Rando, 1994) or synthetic liposomes (Fukada et al., 1994). As mentioned previously, this difference disappeared when the assays were performed in detergent, suggesting the requirement of an intact membrane to observe the effect (Parish & Rando, 1994). There is little difference seen in the effects of methylated and demethylated $T_{\beta\gamma}$ in promoting GTP exchange on T_α with ROS disk membranes versus rhodopsin reconstituted in liposomes. This argues against a specific isoprenylated/methylated protein receptor in the membrane having a role in transducin binding. Further studies will establish whether mechanisms of this type are cogent and whether the isoprenylated/methylated cysteine residue of $\beta\gamma$ subunits is involved in specific $\beta\gamma$-receptor recognition events.

There are two other mechanisms (other than a receptor-mediated mechanism) worth considering. The first involves enhanced membrane association due to methylation, and the second involves specific $\beta\gamma$-effector interactions mediated by the isoprenylated/methylated cysteine residue. With respect to the first, it has been shown that, in the absence of T_α, demethylated $T_{\beta\gamma}$ associates significantly less strongly with liposomal membranes (Bigay et al., 1994) and with disk membranes (Fukada et al., 1994) than does methylated $T_{\beta\gamma}$. It appears from gel filtration studies in this work (Table 1) and elsewhere (Bigay et al., 1994) that demethylated $T_{\beta\gamma}$ does interact with phospholipid/cholate micelles, however to a lesser extent than $T_{\beta\gamma}$-OCH_3. This appears to exclude a mechanism based solely on the hydrophobic association of $T_{\beta\gamma}$ with the lipid micelles. Since both PI3K and PIPLC are found in both membrane and cytosolic fractions, a mechanism where $\beta\gamma$ assists in membrane translocation needs to be explored. It should be mentioned that all $\beta\gamma$ subunits other than retinal $\beta\gamma$ are geranylgeranylated. The relative contribution of methylation may be diminished in the presence of the more hydrophobic C20 modification. Further experiments are under way to examine this possibility.

While we have no evidence for the second mechanism, it is noteworthy that farnesylated and methylated cysteine derivatives, which were originally designed as isoprenylated protein methyltransferase substrates or inhibitors (Gilbert et al., 1992), have remarkable effects on signal transduction in a variety of cells. It turns out that these effects are unrelated to methyltransferase inhibition, where studied in human neutrophils (Ding et al., 1994), platelets (Ma et al., 1994), and HL-60 cells (Scheer & Gierschik, 1993). A possible mechanism of action for these farnesylcysteine derivatives involves interfering with the recognition between $\beta\gamma$ subunits and their effectors as mediated through carboxyl-terminal isoprenylated/methylated cysteine moieties. Interference of the $\beta\gamma$-mediated activation of PI3K and/or PIPLC in neutrophils by farnesylcysteine analogs would lead to profound effects on signal transduction.

In this paper we have demonstrated that carboxymethylation has an effect on the ability of $\beta\gamma$ subunits to stimulate enzymes important in signal transduction pathways. While carboxymethyl groups of isoprenylated proteins undergo turnover in vitro (Pérez-Sala et al., 1991), it is not known if hydrolysis of $\beta\gamma$ subunits occurs in vivo and if this turnover is responsive to extracellular stimuli. Answering these questions may reveal whether methylation plays a significant role in the regulation of signal transduction in cells.

REFERENCES

Anderegg, R. J., Betz, R., Carr, S. A., Crabb, J. W., & Duntze, W. (1988) *J. Biol. Chem. 263*, 18236−18240.

Bigay, J., Faurobert, E., Franco, M., & Chabre, M. (1994) *Biochemistry 33*, 14081−14090.

Boyer, J. L., Waldo, G. L., & Harden, T. K. (1992) *J. Biol. Chem. 267*, 25451−25456.

Camps, M., Hou, C., Sidiropoulos, D., Stock, J. B., Jakobs, K. H., & Gierschik, P. (1992) *Eur. J. Biochem. 206*, 821−831.

Chelsky, D., Sobotka, C., & O'Neill, C. L. (1989) *J. Biol. Chem. 264*, 7637−7643.

Clapham, D. E., & Neer, E. J. (1993) *Nature 365*, 403−406.

Dietrich, A., Meister, M., Brazil, D., Camps, M., & Gierschik, P. (1994) *Eur. J. Biochem. 219*, 171−178.

Ding, J., Lu, D. J., Pérez-Sala, D., Ma, Y. T., Maddox, J. F., Gilbert, B. A., Badwey, J. A., & Rando, R. R. (1994) *J. Biol. Chem. 269*, 16837−16844.

Fukada, Y., Ohguro, H., Saito, T., Yoshizawa, T., & Akino, T. (1989) *J. Biol. Chem. 264*, 5937−5943.

Fukada, Y., Takao, T., Ohguro, H., Yoshizawa, T., Akino, T., & Shimonishi, Y. (1990) *Nature 346*, 658−660.

Fukada, Y., Matsuda, T., Kokame, K., Takao, T., Shimonishi, Y., Akino, T., & Yoshizawa, T. (1994) *J. Biol. Chem. 269*, 5163−5170.

Gilbert, B. A., Tan, E. W., Pérez-Sala, D., & Rando, R. R. (1992) *J. Am. Chem. Soc. 114*, 3966−3973.

Hancock, J. F., Magee, A. I., Childs, J. E., & Marshall, C. J. (1989) *Cell 57*, 1167−1177.

Inglese, J., Glickman, J. F., Lorenz, W., Caron, M. G., & Lefkowitz, R. J. (1992) *J. Biol. Chem. 267*, 1422−1425.

Iñiguez-Lluhi, J. A., Simon, M. I., Robishaw, J. D., & Gilman, A. G. (1992) *J. Biol. Chem. 267*, 23409−23417.

Kameyama, K., Haga, K., Haga, T., Kontani, K., Katada, T., & Fukada, Y. (1993) *J. Biol. Chem. 268*, 7753−7758.

Kapeller, R., & Cantley, L. C. (1994) *BioEssays 16*, 565−576.

Koch, W. J., Inglese, J., Stone, W. C., & Lefkowitz, R. J. (1993) *J. Biol. Chem. 268*, 8256−8260.

Kort, E. N., Goy, M. F., Larsen, S. H., & Adler, J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3939−3943.

Lai, R. K., Pérez-Sala, D., Cañada, F. J., & Rando, R. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7673−7677.

Longstaff, C., & Rando, R. R. (1985) *Biochemistry 24*, 8137−8145.

Ma, Y.-T., Shi, Y.-Q., Lim, Y. H., McGrail, S. H., Ware, J. A., & Rando, R. R. (1994) *Biochemistry 33*, 5414−5420.

Muntz, K. H., Sternweis, P. C., Gilman, A. G., & Mumby, S. M. (1992) *J. Biol. Chem. 267*, 23409−23417.

Okada, T., Sakuma, L., Fukui, Y., Hazeki, O., & Ui, M. (1994) *J. Biol. Chem. 269*, 3563−3567.

Parish, C. A., & Rando, R. R. (1994) *Biochemistry 33*, 9986−9991.

Pérez-Sala, D., Tan, E. W., Cañada, F. J., & Rando, R. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3043−3046.

Reuveny, E., Slesinger, P. A., Inglese, J., Morales, J. M., Iñiquez-Lluhi, J. A., Lefkowitz, R. J., Bourne, H. R., Jan, Y. N., & Jan, L. Y. (1994) *Nature 370*, 143−146.

Schaffner, W., & Weissmann, C. (1973) *Anal. Biochem. 56*, 502−514.

Scheer, A., & Gierschik, P. (1993) *FEBS Lett. 319*, 110−114.

Simonds, W. F., Butrynski, J. E., Gautam, N., Unson, C. G., & Spiegel, A. M. (1991) *J. Biol. Chem. 266*, 5363−5366.

Smrcka, A. V., & Sternweis, P. C. (1993) *J. Biol. Chem. 268*, 9667−9774.

Stephens, L., Smrcka, A., Cooke, F. T., Jackson, T. R., Sternweis, P. C., & Hawkins, P. T. (1994) *Cell 77*, 83−93.

Sternweis, P. C. (1994) *Curr. Opin. Cell Biol. 6*, 198−203.

Sternweis, P. C., & Robishaw, J. D. (1984) *J. Biol. Chem. 259*, 13806−13813.

Sternweis, P. C., & Smrcka, A. V. (1992) *Trends Biochem. Sci. 17*, 502−505.

Tan, E. W., & Rando, R. R. (1992) *Biochemistry 31*, 5572−5578.

Tang, W.-J., & Gilman, A. G. (1991) *Science 254*, 1500−1503.

Thomason, P. A., James, S. R., Casey, P. J., & Downes, C. P. (1994) *J. Biol. Chem. 269*, 16525−16528.

Wessling-Resnick, M., & Johnson, G. L. (1987) *J. Biol. Chem. 262*, 3697−3705.

Wickman, K. D., Iñiquez-Lluhi, J. A., Davenport, P. A., Taussig, R., Krapivinsky, G. B., Linder, M. E., Gilman, A. G., & Clapham, D. E. (1994) *Nature 368*, 255−257.

Yamane, H. K., Farnsworth, C. C., Xie, H., Howald, W., Fung, B. K.-K., Clarke, S., Gelb, M. H., & Glomset, J. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5868−5872.

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