On the Mechanism of the Inhibition of Transducin Function by Farnesylcysteine Analogs†

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ABSTRACT: The γ subunits of heterotrimeric G proteins are isoprenylated/methylated on their carboxy termini. The photoreceptor G protein, transducin, is farnesylated/methylated at this position. Since the isoprenyl group is required for G protein function, it is of great interest to determine the mechanism by which the farnesyl group of Tγ interacts with the other transducin subunits and/or the activated photoreceptor, rhodopsin. Farnesylcysteine derivatives (*N*-acetyl-*S*-farnesyl-L-cysteine and farnesylated peptides) have been previously shown to have effects on transducin activity at high concentrations. Here, an extensive survey is done of farnesylcysteine analogs and other lipid molecules, which are tested for their ability to inhibit GTP/GDP exchange in transducin catalyzed by photolyzed rhodopsin. These studies are carried out to determine the nature of the inhibition process. While it does not appear that these molecules exhibit the specificity which would characterize a ligand−receptor type mechanism, the results suggest that these compounds are not acting in a nonspecific detergent-like manner either. The most likely mode of action of farnesylcysteine analogs is that they interfere with the lipid−lipid based association of Tα and T$\beta\gamma$ through the lipid modifications present on each subunit.

Farnesylcysteine (FC)[1] analogs have proven to have a remarkable range of physiological effects on a variety of cell types. For example, these molecules can stimulate or inhibit superoxide formation in neutrophils (Ding et al., 1994; Philips et al., 1993), inhibit thrombin- or prostaglandin-induced platelet aggregation (Huzoor-Akbar et al., 1993; Ma et al., 1994), inhibit capacitive Ca^{2+} influx into cells (Xu et al., 1996), and block the growth of oncogenic *ras* transformed cells (Marom et al., 1995). Farnesylcysteine derivatives were first synthesized to study the mechanism and function of the isoprenylated protein methyltransferase, of which they are substrates or inhibitors (Gilbert et al., 1992; Tan et al., 1991). This latter enzyme is involved in the isoprenylation pathway of proteins terminating at their carboxyl groups with CAAX (Clarke, 1992; Omer & Gibbs, 1994; Rando, 1996; Casey, 1994; Sinensky & Lutz, 1992). Included in this group of proteins are the heterotrimeric G proteins (Neer, 1995; Rens-Domiano & Hamm, 1995) and the low molecular weight GTP binding proteins (Takai et al., 1992), including *ras*. Since these proteins are important components in signal transduction pathways, it is natural to wonder if FC analogs modulate cellular function by inhibiting the methyltransferase. The answer is no because FC analogs which do not bind to methyltransferase are still physiologically active (Ding et al., 1994; Ma et al., 1994). Moreover, there is no correlation between the inhibition of methyltransferase by FC analogs and their effects on physiological function (Ding et al., 1994; Ma et al., 1994).

One clear mechanism through which these analogs might operate is to interfere with G protein function. All signal transducing G proteins are isoprenylated/methylated at their carboxyl termini. These proteins are either geranylgeranylated/methylated or farnesylated/methylated (the heterotrimers on their γ subunits) (Fukada et al., 1990; Fung et al., 1990; Lai et al., 1990; Mumby et al., 1990; Sanford et al., 1991; Yamane et al., 1990). It is quite possible that FC derivatives interfere with some function of these proteins which is mediated through their isoprenylated termini. The G protein heterotrimer retinal transducin (T$\alpha\beta\gamma$) is an ideal system to study with respect to the function of FC analogs because transducin's interactions with activated rhodopsin have been so well characterized kinetically and mechanistically (Stryer, 1991). Moreover, the X-ray structure of the heterotrimer has recently been reported (Lambright et al., 1996). It is also well-known that transducin is farnesylated/methylated on its γ subunit (Fukada et al., 1990; Lai et al., 1990). Farnesylation is essential for the activity of this G protein (Cheng et al., 1995; Ohguro et al., 1991). In addition, *N*-acetyl-*S*-farnesyl-L-cysteine (L-AFC) and peptide conjugates have been reported to antagonize transducin function (Kisselev et al., 1995, 1994; Matsuda et al., 1994; Scheer & Gierschik, 1995). In order to determine the nature of the interactions leading to the modulation of transducin function, an extensive series of FC analogs and other lipids are studied with respect to their abilities to effect the activated rhodopsin/transducin system. Many of the compounds studied affect these interactions at concentrations between 20 and 200 μM. Many of these analogs exhibit biphasic behavior, activating

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[1] Abbreviations: L-AFC, *N*-acetyl-*S*-*trans*,*trans*-farnesyl-L-cysteine; D-AFC, *N*-acetyl-*S*-*trans*,*trans*-farnesyl-D-cysteine; AFHC, *N*-acetyl-*S*-*trans*,*trans*-farnesyl-L-homocysteine; FC, farnesylcysteine; fMLP, *N*-formyl-Met-Leu-Phe; FTA, *trans*,*trans*-farnesylthioacetic acid; FTP, 3-*trans*,*trans*-farnesylthiopropionic acid; FTS, *trans*,*trans*-farnesylthiosalicylic acid (or 2-*trans*,*trans*-farnesylthiobenzoic acid); 3-FTB, 3-*trans*,*trans*-farnesylthiobenzoic acid; 4-FTB, 4-*trans*,*trans*-farnesylthiobenzoic acid; FTSM, *trans*,*trans* -farnesylthiosalicyclic acid methyl ester; G protein, GTP binding protein; PFC, *N*-pivaloyl-*S*-*trans*,*trans*-farnesyl-L-cysteine; ROS, rod outer segments; T, transducin; UROS, urea-treated rod outer segments.

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GTP-γ-S exchange at lower analog concentrations and inhibiting it at higher concentrations. While highly specific ligand−protein interactions appear not to be involved here, it is possible that these analogs interfere with the lipid−lipid association of Tα and T$\beta\gamma$ mediated through the lipid chains which extend from Tα and Tγ.

MATERIALS AND METHODS

Materials

Frozen bovine retinas were obtained from J. A. & W. L. Lawson Co. (Lincoln, NE). GTP, GTP-γ-S, and dithiothreitol (DTT) were from Boehringer Mannheim. GTP-γ-^{35}S (1340 Ci/mmol; 1 Ci = 37 GBq) and [*adenylate*-^{32}P]NAD (2 mCi/mL, 30 Ci/mmol) were from NEN/Dupont. Blue Sepharose CL-6B was from Pharmacia. Filtron-X scintillation fluid was from National Diagnostics. Pertussis toxin was from List Biological Laboratories. Thesit was from Boehringer Mannheim. Thymidine was from Sigma. Oleic acid and palmitic acid were from Fluka. 3-Mercaptobenzoic acid and 4-mercaptobenzoic acid were from Toronto Research Chemicals. *trans,trans*-Farnesyl bromide and methyl thiosalicylate were from Aldrich. Ac-IPEDKNPFKELKGGC was obtained from Bio-Synthesis (Lewisville, TX).

Methods

Synthesis of Farnesylated Analogs. The synthesis of *N*-acetyl-*S*-*trans,trans*-farnesyl-L-cysteine (L-AFC), *trans,trans*-farnesylthioacetic acid (FTA), 3-*trans,trans*-farnesylthiopropionic acid (FTP), *N*-acetyl-*S*-*trans,trans*-farnesyl-L-homocysteine (AFHC) (Tan et al., 1991), *N*-acetyl-*S*-*trans,trans*-farnesyl-D-cysteine (D-AFC) (Gilbert et al., 1992), *N*-acetyl-*S*-*trans,trans*-farnesyl-L-cysteine methyl ester (AFCM), *N*-pivaloyl-*S*-*trans,trans*-farnesyl-L-cysteine (PFC) (Ding et al., 1994), pentapeptide (Ac-LKGGC(*trans,trans*-farnesyl)), and decapeptides (Ac-NPFKELKGGC(*trans,trans*-farnesyl)-OH and Ac-NPFKELKGGC(*trans,trans*-farnesyl)-OCH_3) (Cheng et al., 1995) have been described previously.

3-FTB, 4-FTB, and FTSM were prepared by farnesylation of the corresponding thiol, 3-mercaptobenzoic acid, 4-mercaptobenzoic acid, and methyl thiosalicylate, respectively, with *trans,trans*-farnesyl bromide and guanidine carbonate in acetone, as described previously (Tan et al., 1991). *trans,trans*-Farnesoic acid was prepared by the oxidation of *trans,trans*-farnesol with Jones' reagent (chromium trioxide) in acetone (Parish et al., 1990).

3-trans,trans-Farnesylthiobenzoic acid (3-FTB): The product was purified by preparative thin layer chromatography (5:2 hexane/ethyl acetate containing 2% acetic acid). ^{1}H-NMR (500 MHz, δ, $CDCl_3$) 8.06 (1H, s, Ar-H), 7.91 (1H, d, J = 7.5 Hz, Ar-H), 7.55 (1H, d, J = 7.0 Hz, Ar-H), 7.37 (1H, t, J = 7.7 Hz, Ar-H), 5.31 (1H, t, J = 7.7 Hz, vinyl), 5.07 (2H, m, vinyl), 3.61 (2H, d, J = 7.5 Hz, CH_2S), 2.00−2.08 (6H, m, allylic CH_2), 1.93−1.98 (2H, m, allylic CH_2), 1.67 (3H, s, vinyl Me), 1.63 (3H, s, vinyl Me), 1.59 (3H, s, vinyl Me), 1.58 (3H, s, vinyl Me).

4-trans,trans-Farnesylthiobenzoic acid (4-FTB): The product was purified by preparative thin layer chromatography (5:2 hexane/ethyl acetate containing 2% acetic acid). ^{1}H-NMR (500 MHz, δ, $CDCl_3$) 7.98 (2H, d, J = 8.0 Hz, Ar-H), 7.31 (1H, d, J = 8.0 Hz, Ar-H), 5.32 (1H, t, J = 7.5 Hz, vinyl), 5.07 (2H, m, vinyl), 3.64 (2H, d, J = 8.0 Hz, CH_2S), 2.02−2.10 (6H, m, allylic CH_2), 1.93−1.98 (2H, m, allylic CH_2), 1.71 (3H, s, vinyl Me), 1.68 (3H, s, vinyl Me), 1.60 (3H, s, vinyl Me), 1.59 (3H, s, vinyl Me).

Methyl trans,trans-farnesylthiosalicylate (FTSM): The product was purified by preparative thin layer chromatography (85:15 hexane/ethyl acetate). ^{1}H-NMR (500 MHz, δ, $CDCl_3$) 7.95 (1H, d, J = 8.0 Hz, Ar-H), 7.42 (1H, dd, J = 8.0, 7.5 Hz, Ar-H), 7.31 (1H, d, J = 8.5 Hz, Ar-H), 7.15 (1H, dd, J = 8.0 Hz, 7.0 Hz, Ar-H), 5.34 (1H, t, J = 7.2 Hz, vinyl), 5.09 (2H, m, vinyl), 3.91 (3H, s, methyl), 3.58 (2H, d, J = 7.0 Hz, CH_2S), 2.01−2.11 (6H, m, allylic CH_2), 1.93−1.99 (2H, m, allylic CH_2), 1.72 (3H, s, vinyl Me), 1.68 (3H, s, vinyl Me), 1.59 (6H, s, vinyl Me). FAB-MS, *m*/*z* 395 ([M + Na]$^+$, $C_{23}H_{32}O_2S$ + Na$^+$).

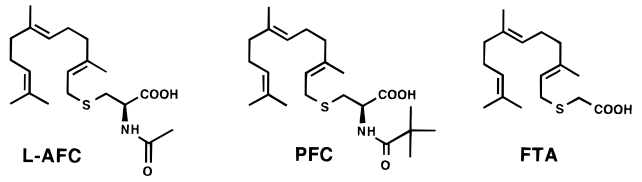
Ac-IPEDKNPFKELKGGC(*trans,trans*-farnesyl) was prepared by farnesylation of the unmodified peptide according to a previously published procedure (Xue et al., 1992). The farnesylated 15 amino acid peptide was purified by HPLC on a C18 column (eluting at 17 min when running a 20 min linear gradient from 55% acetonitrile in water (10 mM TFA) to 5% acetonitrile in water (10 mM TFA)). FAB-MS, *m*/*z* 1920 ([M + H]$^+$, Ac-IPEDKNPFKELKGGC(*trans,trans*-farnesyl) + H$^+$); 1716 ([M + H]$^+$, Ac-IPEDKNPFKELKGGC + H$^+$).

Preparation of Transducin Subunits, Tα and T$\beta\gamma$. Transducin heterotrimer, T$\alpha\beta\gamma$, was isolated and purified from bovine retina as described previously. The subunits were separated by Blue Sepharose chromatography, resulting in Tα and T$\beta\gamma$ preparations (Parish & Rando, 1994).

Preparation of Detergent-Solubilized Rhodopsin and Urea-Treated ROS Membranes. Solubilized rhodopsin in *n*-dodecyl maltoside (Longstaff & Rando, 1985) and urea-treated ROS (UROS) (Fawzi & Northup, 1990) were prepared in the dark as described previously. Solubilized rhodopsin was purified on a concanavalin A−sepharose 4B column as described (Longstaff & Rando, 1985).

Assay of GTP Exchange Activity with Farnesylcysteine Analogs. 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 (10 mM Tris-HCl, pH 7.4/100 mM NaCl/5 mM $MgCl_2$/1 mM DTT/0.1 mM EDTA) (an amount which brings the final assay volume to 100 μL), and Tα (20 μL, 5 μM). Farnesylated derivatives were added to the Tα subunit as DMSO solutions (5 μL). Control assays without farnesylated derivatives contained DMSO (5 μL). T$\beta\gamma$ (7 μM, 15 μL for assays with UROS/5 μL for assays with solubilized rhodopsin) was added to the assay tubes after a 2 min preincubation period. In the dark, solubilized rhodopsin (100 nM, 2 μL) or UROS (50 nM, 10 μL) was added, and the assay tube was incubated at the desired temperature (0 °C for solubilized rhodopsin or 25 °C for UROS). The sample was bleached for 1 min under room light before adding GTP-γ-^{35}S (10 μM, 20 μL, 5000−7000 cpm/pmol). An aliquot (70 μL) of each experiment was removed after 15 min (solubilized rhodopsin) or 30 min (UROS), filtered through a nitrocellulose membrane (Schleicher and Schuell, BA85), and 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). Each membrane was dissolved in Filtron-X scintillation fluid (10 mL) and counted.

Chart 1



ADP-Ribosylation of Tα by Pertussis Toxin. The procedure was carried out essentially as described in Parish (1996). Purified Tα (2.4 μL, 12 nM in 10 mM Tris, pH 7.4/ 0.1 mM EDTA/5 mM $MgCl_2$/1 mM DTT/50 mM NaCl) was preincubated for 30 min at room temperature in the presence of 2.5 μL Thesit (0.1% (w/v) final) and in the absence or presence of the appropriate concentration of the farnesylcysteine analog, dissolved in DMSO. A control sample was incubated in the presence of DMSO alone. Purified Tβγ subunits (1.0 μL, 50 nM in 10 mM Tris, pH 7.4/ 0.1 mM EDTA/5 mM $MgCl_2$/1 mM DTT/100 mM NaCl) were then added, and the reaction was started by the addition of 12.5 μL of radioactive assay buffer, followed by incubation at 37 °C for 15 min. The reaction was stopped by cooling to 0 °C, and 12.5 μL of SDS−PAGE sample buffer was added. An aliquot (20 μL) was analyzed on a 12% SDS−PAGE gel, and after Coomassie blue staining, the labeled Tα subunit was visualized by both autoradiography (20−40 h) and phosphorimaging (3 h exposure, Molecular Dynamics phosphorimager). The intensity of each band was determined by integrating identical areas of the gel obtained from the phosphorimager.

Solubilization of Rod Outer Segments with Farnesylated Derivatives. In each assay tube were added water (205 μL), assay buffer (60 μL of 50 mM Tris-HCl, pH 7.4/500 mM NaCl/25 mM $MgCl_2$/5 mM DTT/0.5 mM EDTA), AFC or FTS (5 μL, DMSO solution at the desired concentration), and UROS (20 μL, 6.8 μM rhodopsin). A control sample contained DMSO, but not AFC or FTS. The samples were left at 25 °C for 10 min and then centrifuged for 30 min (75 000 rpm, 250000*g*, 4 °C, Beckman TL100). The supernatant was removed from the tube, and 20 μL of each sample was analyzed by SDS−PAGE (12.5%). The presence of rhodopsin (40 kDa) was determined by silver staining of each gel.

Miscellaneous Procedures. Protein concentrations were determined using an amido black assay (Schaffner & Weissmann, 1973). Silver staining of SDS−PAGE gels was accomplished with a Daiichi silver stain kit (Integrated Separation Systems).

RESULTS

Experiments were performed to investigate the effects of FC derivatives on the photoactivated rhodopsin (R*) catalyzed exchange of GTP-γ-S for GDP in transducin. These experiments were carried out both with purified rhodopsin which was solubilized in detergent (*n*-dodecyl maltoside) and with rod outer segment disk membranes which were treated with urea (UROS) to remove any peripheral membrane proteins. The initial set of experiments were carried out studying the effects of L-AFC, D-AFC, and L-AFC methyl ester (AFCM) on the exchange reaction. The structures of some of the analogs used in these studies are illustrated in Chart 1. As shown in Figure 1, no stereospecificity is

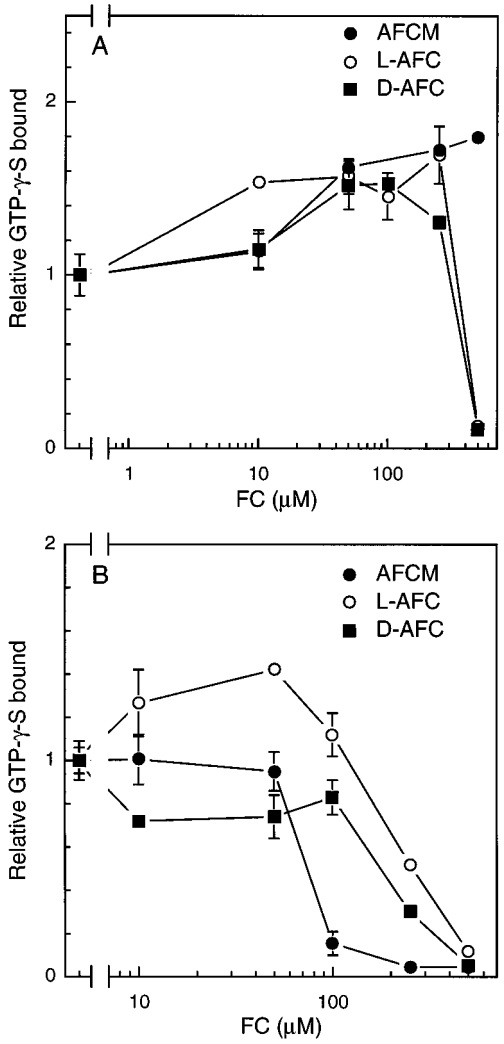


FIGURE 1: GTP-γ-S exchange in the presence of L-AFC, D-AFC, and AFCM. (A) Rhodopsin in urea-treated ROS. (B) Rhodopsin solubilized in *n*-dodecyl maltoside. Assays were performed as described in Materials and Methods. The data represent the mean ± SEM.

observed, as would be expected if typical ligand−protein interactions were involved. For many farnesylcysteine analogs (e.g., L-AFC in Figure 1), a modest increase in GTP-γ-S binding was observed at low analog concentrations, followed by inhibition at higher concentrations. Under the conditions described here with the more physiologically relevant UROS, both L- and D-AFC were only able to inhibit GTP exchange above 250 μM. Further, 500 μM L-AFCM does not inhibit GTP exchange. L-AFCM is of interest because Tβγ is farnesylated/methylated in its active form (Fukada et al., 1990; Lai et al., 1990) and a putative farnesyl-specific docking site should be more potently inhibited by the methylated derivative. In *n*-dodecyl maltoside, L-AFC, D-AFC, and AFCM all inhibit GTP exchange with IC_{50} values of approximately 100−200 μM. While these experiments demonstrate effects of FC derivatives, the inhibition is apparently unrelated to ligand−protein interactions mediated through the methylated farnesylcysteine moiety of Tβγ. It should also be noted that at high concentrations of L-AFC partial dissolution of ROS membranes is noted, as shown in Figure 2. While membrane solubilization does not appear to occur substantially at 200 μM L-AFC, some amount of rhodopsin is solubilized, suggesting that this compound may

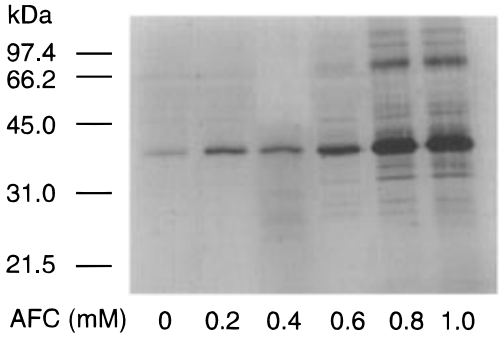


FIGURE 2: Treatment of urea-treated ROS with L-AFC. Silver-stained SDS−PAGE (12.5%) of supernatants after incubation of ROS with the indicated concentration of L-AFC.

have membrane perturbing effects even at lower concentrations. The detergent-like character of L-AFC effects complete dissolution of UROS at approximately 700 μM.

It could be argued that in order to mimic the carboxyl terminus of T$\beta\gamma$, the FC analog should contain some of the peptide structure of T$\beta\gamma$. FC analogs containing the carboxyl terminal five, ten, and fifteen amino acids of Tγ were prepared and studied as inhibitors of transducin function. While these analogs at 50 μM do not inhibit GTP exchange with UROS, they are able to act as inhibitors when assayed with solubilized rhodopsin (Figure 3). When tested with solubilized rhodopsin, the increase in chain length from one amino acid (L-AFC) to fifteen amino acids does result in a gradual decrease in GTP-γ-S binding when these molecules were tested at 50 μM. Unfarnesylated peptides were ineffective as inhibitors in the presence of either solubilized rhodopsin or UROS (Figure 3).

Several other FC analogs were also studied as putative inhibitors of GTP-γ-S exchange. PFC and AFHC proved to be about as active as L-AFC itself, as shown in Figure 4. The peptide bond portion of the AFC could also be removed with little loss in activity, because both FTA and FTP are also active as inhibitors of transducin function at approximately 100−200 μM (Figure 5). Indeed, unsaturated fatty acids, such as farnesoic acid and oleic acid (IC_{50} approximately 50 μM), proved to be more active than L-AFC, while palmitic acid is only slightly less potent at inhibiting GTP exchange with UROS (Figure 6A). With solubilized rhodopsin, farnesoic and palmitic acid had approximately the same effect on GTP-γ-S uptake.

Recently, FTS (Chart 2) has been reported to be quite active as an FC analog (Marom et al., 1995). It was of some interest to test this compound in the rhodopsin/transducin assay described here. FTS is indeed more potent than the other compounds described above as an inhibitor of transducin function (IC_{50} approximately 25 μM) by 2- to 10-fold. This does not appear to be due to an increased membrane perturbing effect since FTS is no more potent than L-AFC at solubilizing rhodopsin from ROS membranes (Figure 7). To further assess the specificity of FTS action, the two regioisomers of FTS, 3-FTB and 4-FTB (Chart 2), were synthesized and studied. FTS, 3-FTB, and 4-FTB were all roughly equipotent, while the methyl ester of FTS (FTSM) was less potent in the assays described here (Figure 8).

The ability of these compounds to inhibit the T$\beta\gamma$-stimulated ADP-ribosylation of Tα is summarized in Table 1. This assay is a measure of Tα−T$\beta\gamma$ interaction, as the presence of the $\beta\gamma$ subunit is required for ADP-ribosylation

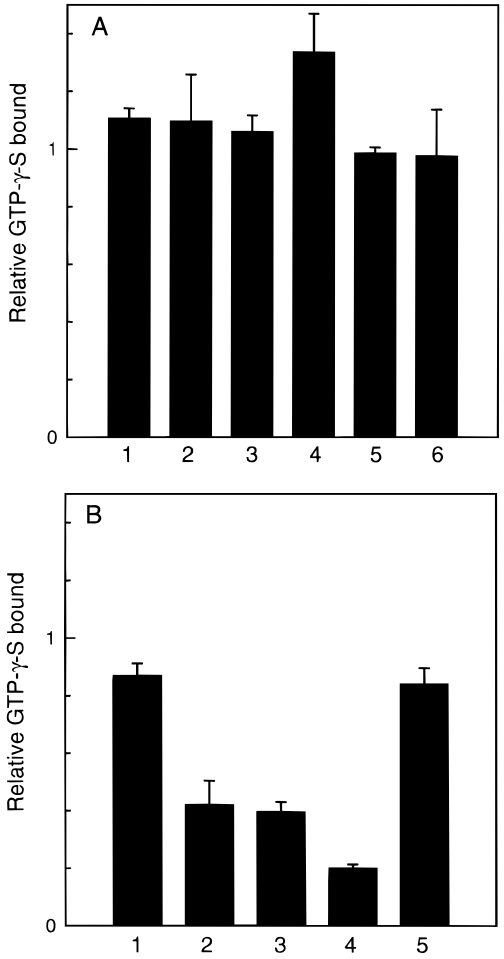
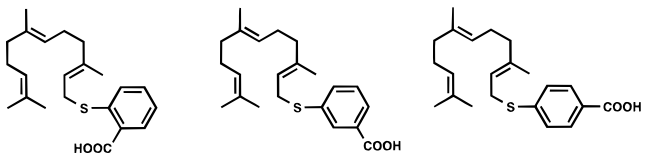


FIGURE 3: GTP-γ-S exchange in the presence of peptides corresponding to the carboxyl terminus of transducin. Each experiment contained 50 μM of the indicated compound. (A) Rhodopsin in urea-treated ROS. (B) Rhodopsin solubilized in *n*-dodecyl maltoside. Lane 1, Ac-LKGGC(*trans*,*trans*-farnesyl); lane 2, Ac-NPFKELKGGC(*trans*,*trans*-farnesyl); lane 3, Ac-NPFKELKGGC-(*trans*,*trans*-farnesyl)-OCH_3; lane 4, Ac-IPEDKNPFKELKGGC-(*trans*,*trans*-farnesyl); lane 5, Ac-NPFKELKGGC; lane 6, Ac-IPEDKNPFKELKGGC. Assays were performed as described in Materials and Methods. The data represent the mean ± SEM.

Chart 2



of the α subunit (Watkins et al., 1985). It can be seen that FTS, FTP, farnesoic acid, and FTA all inhibit ADP-ribosylation to a similar degree (Table 1). In additional experiments, it was observed that FTS and its regioisomers (3-FTB and 4-FTB) exhibited no clear difference in their ability to mediate inhibition of T$\beta\gamma$-induced ADP-ribosylation of Tα (data not shown).

DISCUSSION

G proteins are isoprenylated by farnesylation or geranylgeranylation at their carboxyl terminal cysteine residues (Rando, 1996; Casey, 1994; Sinensky & Lutz, 1992). Mono-isoprenylated proteins are also carboxymethylated at this

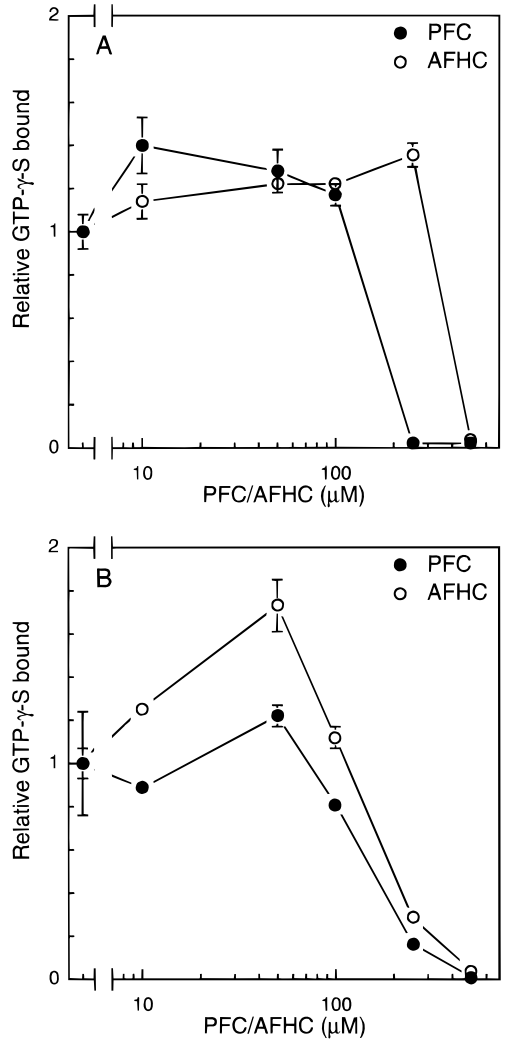


FIGURE 4: GTP-γ-S exchange in the presence of PFC and AFHC. (A) Rhodopsin in urea-treated ROS. (B) Rhodopsin solubilized in *n*-dodecyl maltoside. Assays were performed as described in Materials and Methods. The data represent the mean ± SEM.

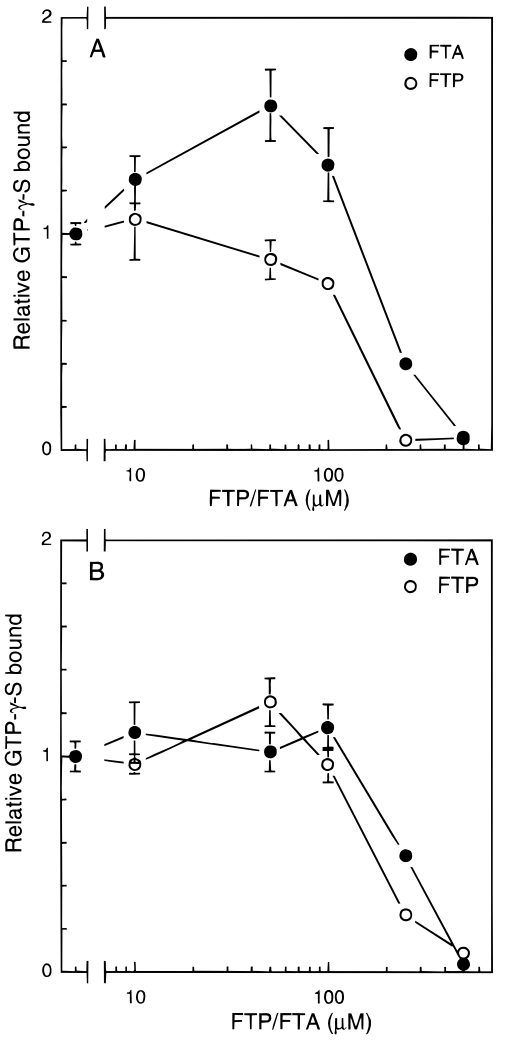


FIGURE 5: GTP-γ-S exchange in the presence of FTA and FTP. (A) Rhodopsin in urea-treated ROS. (B) Rhodopsin solubilized in *n*-dodecyl maltoside. Assays were performed as described in Materials and Methods. The data represent the mean ± SEM.

Table 1: Inhibiton of T$\beta\gamma$-Stimulated Tα-ADP Ribosylation by FTS and Its Analogs[a]

analog at 100 μM	% inhibition of ADP-ribosylation
FTS	47.35 ± 1.55
FTP	52.00 ± 5.40
farnesoic acid	42.09 ± 10.9
FTA	37.8

[a] ADP-ribosylation assays were performed as descibed in Materials and Methods. Each analog was tested at 100 μM, and the final DMSO concentration was 1.25%. Percent inhibition was calcuated by dividing the intensity of the labeled band obtained in the presence of the various compounds by that obtained in the presence of DMSO alone (control). The data represent the mean ± SEM (the result for FTA was obtained by a single experiment, but inhibition was seen using multiple concentrations of this compound).

cysteine residue (Clarke, 1992). Most mono-isoprenylated G proteins are geranylgeranylated, with the notable exceptions of the heterotrimeric G protein retinal transducin and the low molecular weight GTP binding protein *ras*, both of which are farnesylated (Fukada et al., 1990; Lai et al., 1990). Farnesylated cysteine analogs, which were originally synthesized to study the mechanism of the isoprenylated protein methyltransferase (Gilbert et al., 1992; Tan et al., 1991), have proved to possess a myriad of physiological effects on a range of cell types (Ding et al., 1994; Huzoor-Akbar et al., 1993; Ma et al., 1994; Philips et al., 1993; Scheer & Gierschik, 1993, 1995). As mentioned earlier, these effects are not related to methyltransferase blockade because FC analogs incapable of interacting with the methyltransferase are still physiologically active (Ma et al., 1994).

One interesting effect of these molecules is on superoxide generation in neutrophils (Ding et al., 1994; Philips et al., 1993). FC analogs are able to induce superoxide formation or inhibit superoxide formation induced by fMLP (Ding et al., 1994). Small changes in the structures of the FC analogs lead to a switch from agonist to antagonist activity, suggesting a specific mode of interaction of FC analogs with a cellular receptor(s). However, the physiological effects elicited are also non-stereospecific, with D-AFC being as active, if not more active, than its enantiomer (Ding et al., 1994; Ma et al., 1994). These results suggest a quasi-specific mode of action of these analogs which may not be related to typical drug−receptor interactions.

It is, of course, of interest to consider what the targets might be for FC analogs. G proteins are obvious targets because they are isoprenylated/methylated at their carboxyl termini. It is possible that FC analogs could interfere with interactions mediated between the farnesylated/methylated

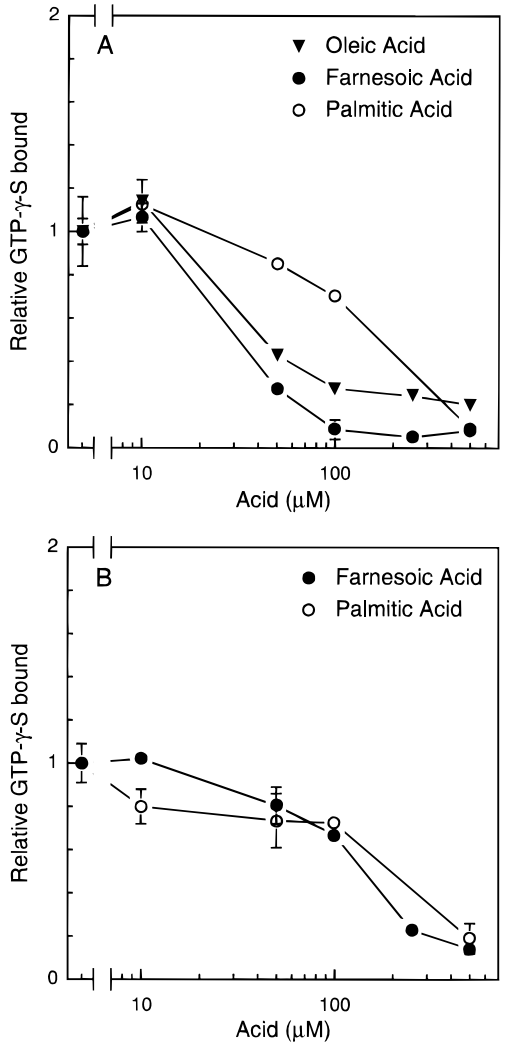


FIGURE 6: GTP-γ-S exchange in the presence of farnesoic, oleic and palmitic acid. (A) Rhodopsin in urea-treated ROS. (B) Rhodopsin solubilized in *n*-dodecyl maltoside. Assays were performed as described in Materials and Methods. The data represent the mean ± SEM.

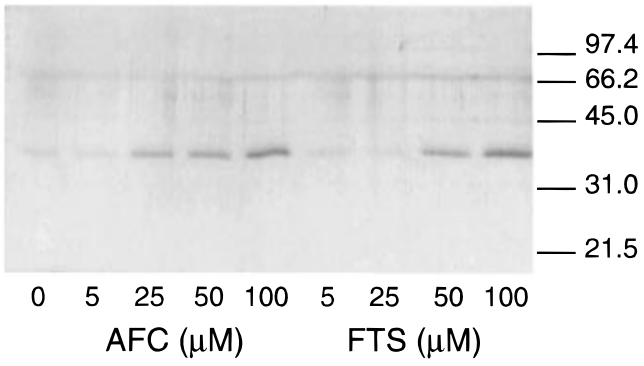


FIGURE 7: Treatment of urea-treated ROS with L-AFC and FTS. Silver-stained SDS−PAGE (12.5%) of supernatants after incubation of ROS with the indicated concentration of L-AFC.

cysteine moiety of a G protein and a receptor. In the case of heterotrimeric G proteins, it is possible that interactions between α and $\beta\gamma$ subunits are mediated via interactions of the farnesylated/methylated cysteine moiety on the $\beta\gamma$ subunit and the α subunit (Bigay et al., 1994). Membrane receptors for the $\beta\gamma$ subunit, which recognize the farnesylated/methylated cysteine moiety, could also be considered.

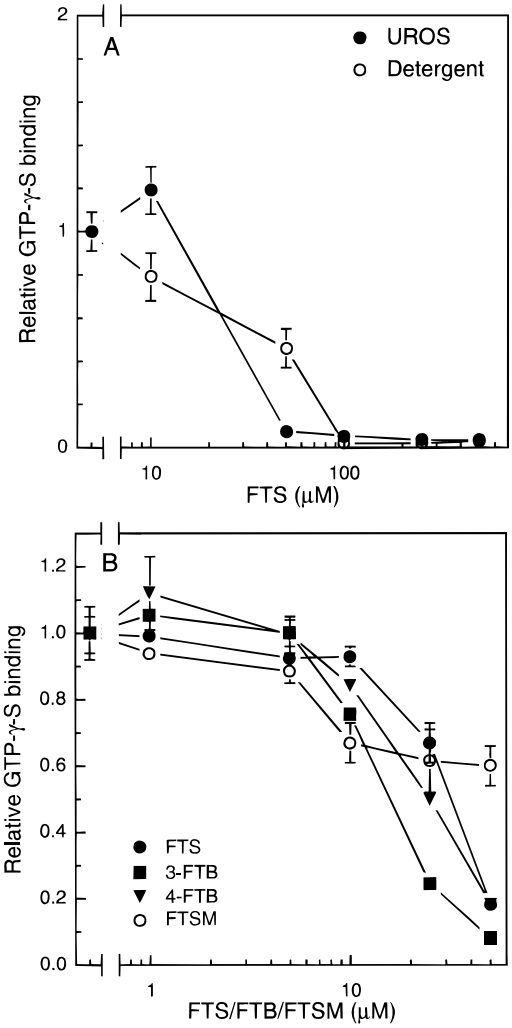


FIGURE 8: GTP-γ-S exchange in the presence of FTS and its isomers. (A) GTP-γ-S exchange in the presence of FTS with detergent-solubilized rhodopsin and urea-treated ROS. (B) GTP-γ-S exchange in the presence of FTS, 3-FTB, 4-FTB, and FTSM with urea-treated ROS. Assays were performed as described in Materials and Methods. The data represent the mean ± SEM.

The latter scenario could also be imagined to occur with low molecular weight GTP binding proteins such as *ras*.

As described earlier, the rhodopsin/transducin system is an ideal one for studying the action of FC analogs because the system is well described and understood. Several studies reported in the literature have already described the effects of FC analogs on rhodopsin/transducin interactions (Kisselev et al., 1995, 1994; Matsuda et al., 1994; Scheer & Gierschik, 1995). Strong inhibitory effects were observed with molecules such as L-AFC, but at concentrations >100 μM (Scheer & Gierschik, 1995). It seemed important to study a range of AFC-like molecules to demonstrate the specificity of action of these analogs and to begin to reveal possible mechanisms for each analog's effect on transducin function. This is the impetus of the current report.

As is clear from Figure 1, L-AFC does indeed inhibit GTP exchange in transducin at approximately 100−200 μM. If a typical ligand−protein interaction were involved here, then we would expect that D-AFC would be relatively inert. This is not what was found. It is interesting to note that D-AFC was also found to be an active inhibitor of GTP-γ-S binding in HL-60 cell membranes (Scheer & Gierschik, 1995). Moreover, L-AFCM proved to be no more effective than its

unmethylated counterpart. Since Tγ is normally methylated (Fukada et al., 1990; Perez-Sala et al., 1991), it would be expected that methylation would only enhance an inhibitory effect of these compounds. However, this difference may be rather small since the unmethylated form of T$\beta\gamma$ is only approximately 2-fold less active than its methylated counterpart (Parish & Rando, 1994). Moreover, it is also important to recognize that L-AFCM is of very limited solubility, possibly preventing access to a putative target.

Farnesylated peptides containing the Tγ carboxyl terminal sequence were not effective inhibitors of GTP exchange in transducin when ROS were used as the source of rhodopsin. Interestingly, these same peptides demonstrated an ability to inhibit GTP exchange when rhodopsin was solubilized in *n*-dodecyl maltoside. Indeed, the FC analogs tested here were generally more effective when tested in the less physiologically relevant detergent-solubilized system.

Other amphipathic farnesylated molecules, such as FTA and FTP, were approximately as active as AFC itself as an inhibitor of transducin function. Indeed, a farnesyl moiety was not even required for activity because *N*-acetyl-*S*-(*n*-dodecyl)-L-cysteine was active as well (data not shown). The end point in this kind of analysis is found in the observation that simple fatty acids, such as oleic acid, farnesoic acid, and palmitic acid, are quite active as well. It should also be noted that at concentrations as low as 25−50 μM L-AFC the solubilization of some amount of rhodopsin from ROS is apparent (Figure 7), suggesting that micromolar concentrations of L-AFC might have significant membrane perturbing effects.

Farnesylthiosalicylic acid (FTS) was tested as well. This analog, which is essentially a ring-locked version of the previously studied FTP, has been shown to be significantly more potent at inhibiting *ras* function than other FC analogs (Marom et al., 1995). FTS appears to block the association of *ras* to membranes and inhibits the cell growth of oncogenic *ras* transformed cells, as well as oncogenic transformants of genes upstream of *ras*. Interestingly, FTS does not inhibit the growth of cells containing non-oncogenic *ras*, nor does it block the growth of cells transformed with oncogenes downstream of *ras* (Marom et al., 1995). These and other results are consistent with a specific mode of action of FTS which occurs independently of inhibition of enzymes involved in the isoprenylation pathway. When FTS was studied as an inhibitor in the rhodopsin/transducin system, it proved to be significantly more potent than AFC. However, the meta and para isomers of FTS, 3-FTB and 4-FTB, were equipotent with FTS, while the methyl ester derivative of FTS was less potent. These data indicate that the negative charge of FTS is required for activity, but its position relative to the farnesyl group is not critical. This result was also observed when pertussis toxin-catalyzed ADP-ribosylation of the Tα subunit was examined. In these experiments, FTS and its regioisomers (3-FTB and 4-FTB) were roughly equipotent with regard to inhibition of ADP-ribosylation. Similar to the results obtained with GTP-γ-S binding in detergent, FTS was approximately as potent as FTP, farnesoic acid, or FTA in inhibiting T$\beta\gamma$-stimulated ADP-ribosylation of Tα (Table 1).

How can all of this be explained? It seems clear that a mechanism involving the docking of a farnesyl/methyl group in a specific ligand−receptor interaction is not present in the rhodopsin/transducin system. That is, neither Tα nor photoactivated rhodopsin possesses a binding site which specifically recognizes a farnesyl cysteine moiety. On the other hand, published evidence and the data described here suggest the possibility of more than a simple membrane disruptive effect as the basis of the mechanism of action of FC analogs. From previous studies on the effects of FC analogs on pertussis toxin-mediated ADP-ribosylation of Tα in the presence of T$\beta\gamma$ (Matsuda et al., 1994), it would appear that FC analogs can interfere with intramolecular interactions between the transducin subunits. Similar results were obtained here using FTS, FTP, farnesoic acid, and FTA. These experiments are performed in the absence of membranes. Analagous results in the detergent-based assays performed here indicate that FC analogs can also interfere with GTP exchange. The ability of these compounds to inhibit the T$\beta\gamma$-mediated stimulation of Tα ADP-ribosylation (Table 1) supports the hypothesis that these analogs may interfere with Tα−T$\beta\gamma$ interaction.

Interactions between Tα and T$\beta\gamma$ are strongly enhanced in membranes (Bigay et al., 1994). This could occur by means of lipid−lipid interactions between the hydrophobically post-translationally modified Tα and Tγ subunits. Tα is modified by a heterogeneous mixture of saturated and unsaturated lipids on its amino terminus (Kokame et al., 1992). It is quite possible that FC analogs interfere with these interactions, leading to inhibition of GTP exchange in transducin at higher concentrations of these analogs. The broad range of compounds which can effect transducin activity would seem to indicate that this effect is related to the ability of these compounds to disrupt Tα−T$\beta\gamma$ interactions. The amphipathic nature of these analogs may affect the lipid−lipid association of the heterotrimer or interfere with the ability of the protein to insert into the phospolipid membrane. Oleic acid contains one site of unsaturation, and its ability to inhibit transducin activity may derive from its similarity to the lipid modifications of Tα. Farnesoic acid may act in a similar manner while mimicking the farnesyl group of Tγ. It may be possible to prepare additional farnesylated analogs which more potently affect the transducin heterotrimer association. Interestingly, at lower concentrations of a number of FC analogs, some activation of GTP-γ-S exchange in transducin is generally observed. It has been found that farnesylated peptides can enhance the lifetime of metarhodopsin II, the active form of photactivated rhodopsin (Kisselev et al., 1995, 1994). The physiological significance of this observation remains obscure, because T$\beta\gamma$ does not function in such a manner (Kisselev et al., 1994). These effects may be similar to those observed for a variety of detergents (Baldwin & Hubbell, 1985; König et al., 1989) and alcohols (Mitchell et al., 1996) which also stabilize metarhodopsin II.

While a definitive mechanism of action of FC analogs cannot be enunciated here, it is possible that more specific ligand−receptor interactions occur in other G protein linked signal transducing systems. A more specific mechanism than the one described here might be more relevant in low molecular weight GTP binding proteins such as *ras*. The remarkable and specific effects of FTS on oncogenic *ras* transformed cells might indicate a ligand−receptor mediated event. Of course, it is also quite possible that a quasi-specific mechanism of the kind described here is at the basis of the effects of FTS on *ras*. Finally, it is interesting to note that a variety of signal transducing events appear to be induced

by diverse polyunsaturated fatty acids (Kang & Leaf, 1996). The underlying mechanisms of these effects have not been explained. These mechanisms might also be similar to the type described here.

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