S-Prenylated Cysteine Analogues Inhibit Receptor-Mediated G Protein Activation in Native Human Granulocyte and Reconstituted Bovine Retinal Rod Outer Segment Membranes†

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ABSTRACT: We have previously shown that the *S*-prenylated cysteine analogue *N*-acetyl-*S*-*trans,trans*-farnesyl-L-cysteine (L-AFC) inhibits basal and formyl peptide receptor-stimulated binding of guanosine 5′-*O*-(3-thiotriphosphate) (GTP[S]) to and hydrolysis of GTP by membranes of HL-60 granulocytes and have presented evidence suggesting that this inhibition was not caused by reduced protein carboxyl methylation [Scheer, A., & Gierschik, P. (1993) *FEBS Lett. 319*, 110−114]. We now report a detailed analysis of the structural properties of *S*-prenylated cysteine analogues required for this inhibition and demonstrate that *S*-prenylcysteines also suppress basal and receptor-stimulated GTP[S] binding to human peripheral neutrophil and HL-60 granulocyte membranes when stimulated by formyl peptide and complement C5a, respectively. *S*-Prenylcysteines did not affect pertussis toxin-mediated [^{32}P]ADP-ribosylation of G_i proteins. The inhibitory effect of L-AFC was reversible and was not mimicked by farnesylic acid. L-AFC also interfered with GTP[S] binding to retinal transducin when stimulated by light-activated rhodopsin in a reconstituted system. This inhibitory effect was fully reversed upon increasing the concentration of either the G protein $\beta\gamma$ dimer or the activated receptor. On the basis of these results, we suggest that *S*-prenylated cysteine analogues like L-AFC inhibit receptor-mediated G protein activation by specifically and reversibly interfering with the interaction of activated receptors with G proteins, most likely with their $\beta\gamma$ dimers, rather than by inhibiting $\alpha{\cdot}\beta\gamma$ heterotrimer formation.

Signal-transducing heterotrimeric guanine nucleotide-binding proteins (G proteins)[1] couple a great variety of receptors for extracellular signaling molecules or sensory stimuli to plasma membrane-bound or intracellular effector moieties such as adenylyl cyclase, ion channels, or phospholipase C (Birnbaumer et al., 1990; Hepler & Gilman, 1992). Activated receptors interact with heterotrimeric ($\alpha{\cdot}\beta\gamma$) G proteins to catalyze the exchange of GTP for GDP bound to their α subunits. GTP-liganded G proteins are thought to dissociate into GTP-bound α subunits and free $\beta\gamma$ dimers. Both activated α subunits and free $\beta\gamma$ dimers are capable of effector regulation (Birnbaumer, 1992; Conklin & Bourne, 1993; Iñiguez-Lluhi et al., 1993). G protein deactivation results from hydrolysis of GTP bound to the α subunit by its intrinsic GTPase activity, followed by the reassociation of the now GDP-bound α subunit with the $\beta\gamma$ dimer.

G protein γ subunits belong to a familiy of proteins and peptides which are posttranslationally modified at their carboxyl termini by isoprenylation, proteolysis, and methyl esterification [reviewed in Casey (1992), Clarke (1992), Schafer and Rine (1992), Yamane and Fung (1993), and Hrycyna and (Clarke, 1993)]. This family also includes a large number of low-molecular-mass GTP-binding proteins, nuclear lamins, fungal mating factors, rhodopsin kinase, and the large subunits of retinal cGMP phosphodiesterase. G protein γ subunits are first either farnesylated (γ_1) or geranylgeranylated (other γ subunits) at a cysteine residue present at position −4 from the carboxyl terminus. Following *S*-isoprenylation, a membrane-bound proteases(s) cleaves the three terminal amino acids, and the resultant terminal carboxyl group is methyl esterified by a membraneous methyltransferase, also referred to as class III isoprenylcysteine protein carboxyl methyltransferase [see Hrycyna and Clarke (1993) for review].

The functional role(s) of these posttranslational modifications is currently unresolved (Clarke, 1992; Cox & Der, 1992; Marshall, 1993; Hrycyna & Clarke, 1993). On the one hand, it seems clear that they increase the hydrophobicity of the proteins, and it is conceivable that this increase helps to anchor the modified proteins into the plasma membrane lipid

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[1] Abbreviations: G protein, signal-transducing heterotrimeric guanine nucleotide-binding protein; GTP[S], guanosine 5′*O*-(3-thiotriphosphate); fMet-Leu-Phe, *N*-formylmethionylleucylphenylalanine; L-AFC, *N*-acetyl-*S*-*trans,trans*-farnesyl-L-cysteine; D-AFC, *N*-acetyl-*S*-*trans,trans*-farnesyl-D-cysteine; AFCMe, *N*-acetyl-*S*-*trans,trans*-farnesyl-L-cysteine methyl ester; FTP, *S*-(*trans,trans*-farnesyl-3-thio)propionic acid; FTA, *S*-(*trans,trans*-farnesyl-2-thio)acetic acid; F2TP, *S*-(*trans,trans*-farnesyl-(*R,S*)-2-thio)propionic acid; AGC, *N*-acetyl-*S*-*trans*-geranyl-L-cysteine; AGGC, *N*-acetyl-*S*-*all-trans*-geranylgeranyl-L-cysteine; GGTP, *S*-(*all-trans*-geranylgeranyl-3-thio)propionic acid; FOH, *trans,trans*-farnesol; FCHO, *trans,trans*-farnesal; FCOOH, *trans,trans*-farnesylic acid; U-ROS*-M, urea-washed retinal rod outer segment membranes containing light-activated rhodopsin; D-ROS*-S, detergent-solubilized transducin-depleted retinal rod outer segment membranes containing light-activated rhodopsin; CHAPS, 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; IC_{50}, median inhibitory concentration.

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bilayer. Thus, $\beta\gamma$ dimers carrying a serine instead of a cysteine at position −4 from the γ subunit carboxyl terminus are resistant to carboxyl-terminal processing and remain cytosolic when expressed in a variety of heterologous systems (Simonds et al., 1991; Muntz et al., 1992; Pronin & Gautam, 1992; Iñiguez-Lluhi et al., 1992; Dietrich et al., 1992, 1994). On the other hand, it seems also possible that the posttranslationally modified proteins interact, via their modified carboxyl termini, with specific "receptor" proteins present in various cellular compartments. There are several examples where such a specific receptor-mediated interaction may take place, including the recognition of the isoprenylated mating pheromone a-factor by its specific transmembrane receptor (Anderegg et al., 1988; Marcus et al., 1991) and the interaction of several low-molecular-mass GTP-binding proteins with their GDP/GTP exchange factors [see Takai et al. (1992) for review], or with their target proteins (Horiuchi et al., 1992; Kuroda et al., 1993; Itoh et al., 1993).

Class III isoprenylcysteine protein carboxyl methyltransferases recognize and methylate even small structural analogues of *S*-prenylated protein carboxyl termini like *N*-acetyl-*S*-*trans,trans*-farnesyl-L-cysteine (L-AFC) (Volker et al., 1990; 1991a,b; Tan et al., 1991a,b; Shi & Rando, 1992; Pérez-Sala et al., 1991; 1992; Gilbert et al., 1992). L-AFC and several structurally related compounds have also been shown to effectively inhibit the carboxyl methylation of proteins carrying a carboxyl-terminal *S*-prenylated cysteine residue (Pérez-Sala et al., 1991; Tan et al., 1991a,b; Huzoor-Akbar et al., 1991; Volker et al., 1991a,b; Shi & Rando, 1992; Pérez-Sala et al., 1992; Philips et al., 1993; Metz et al., 1993) and to block a variety of extracellular signaling molecule-stimulated functions of intact cells (Volker et al., 1991a; Philips et al., 1993; Huzoor-Akbar, 1993; Metz et al., 1993). In contrast, the *S*-prenylated cysteines did not affect the stimulation of cell functions by agents by-passing cell surface receptors, e.g., phorbol esters or ionomycin (Volker et al., 1991a; Philips et al., 1993; Huzoor-Akbar, 1993). These results led to the suggestions (i) that L-AFC and related prenylcysteines inhibit receptor-mediated signal transduction by interfering with transmembrane signalling at a step prior to stimulation of protein kinase C and mobilization of intracellular Ca^{2+} and (ii) that this inhibition was likely to be due to reduced carboxyl methylation of small molecular weight GTP-binding proteins and/or γ subunits of heterotrimeric G proteins (Volker et al., 1991a; Philips et al., 1993; Huzoor-Akbar, 1993; Metz et al., 1993).

We have previously reported that L-AFC inhibits both basal and formyl peptide receptor-stimulated binding of GTP[S] to and hydrolysis of GTP by membranes of myeloid differentiated HL-60 cells (Scheer & Gierschik, 1993). This inhibition was not observed for *N*-acetyl-*S*-*trans*-geranyl-L-cysteine (AGC) or AFCMe, the methyl ester of L-AFC. The fact that the inhibitory effects of L-AFC were observed in well washed membranes together with the observations that exogeneous *S*-adenosyl-L-methionine was apparently not required for and *S*-adenosyl-L-homocysteine did not attenuate L-AFC inhibition led us to suggest that L-AFC inhibits receptor-mediated G protein activation by a mechanism other than inhibition of protein carboxyl methylation. In the present study, we have analyzed the structural properties of *S*-prenylated cysteine analogues required for their inhibitory effects on formyl peptide receptor-mediated G protein activation in human granulocyte membranes. To investigate the molecular mechanisms by which *S*-prenylated cysteines inhibit receptor-mediated G protein activation, we have examined the receptor specificity and reversibility of the inhibitory prenylcysteine effects and have studied the influence of these compounds on $\alpha\beta\gamma$ heterotrimer formation and on receptor−G protein interaction in a reconstituted system consisting of resolved and/or purified signal transduction components.

MATERIALS AND METHODS

Materials. *N*-Acetyl-L-cysteine, 3-mercaptopropionic acid, and *trans,trans*-farnesol were purchased from Fluka, Neu-Ulm, Germany. D-Cysteine, *trans,trans*-farnesyl bromide, and the reagents used for oxidation of *trans,trans*-farnesol were from Aldrich, Steinheim, Germany. (*R,S*)-2-Mercaptopropionic acid and *all-trans*-geranylgeraniol were obtained from Merck, Darmstadt, Germany, and TCI America, Portland, OR, USA, respectively. [^{35}S]GTP[S] and [^{32}P]-NAD^+ were purchased from New England Nuclear, Bad Homburg, Germany. Recombinant human complement C5a and concanavalin A Sepharose 4B was from Sigma, Deisenhofen, Germany, Blue Sepharose CL-6B from Pharmacia Biotech, Freiburg, Germany, and CHAPS from Boehringer, Mannheim, Germany. Pertussis toxin was purchased from List, Campbell, CA, USA. All other materials were from standard vendors or from sources previously described (Scheer & Gierschik, 1993; Gierschik & Camps, 1994).

Syntheses of Analogues. *all-trans*-Geranylgeranyl bromide was synthesized from *all-trans*-geranylgeraniol (Fukuda et al., 1981; Corey et al., 1972). *N*-Acetyl-*S*-*trans,trans*-farnesyl-D-cysteine (D-AFC) was prepared by *N*-acetylation of *trans,trans*-farnesyl-D-cysteine with acetic anhydride (Frankel et al., 1960; Bodansky & Bodansyky, 1984). L-AFC, *S*-(*trans,trans*-farnesyl-3-thio)propionic acid (FTP), *S*-(*trans,trans*-farnesyl-2-thio)acetic acid (FTA), *N*-acetyl-*S*-*trans*-geranyl-L-cysteine (AGC), and *S*-(*all-trans*-geranylgeranyl-3-thio)propionic acid (GGTP) were synthesized according to a previously reported procedure (Tan et al., 1991a). *S*-(*trans,trans*-Farnesyl-(*R,S*)-2-thio)propionic acid (F2TP) was prepared from *trans,trans*-farnesyl bromide and (*R,S*)-2-mercaptopropionic acid to provide F2TP as a colorless oil in 70% yield: ^{1}H NMR ($CDCl_3$, 250 MHz) δ = 5.22 (1 H, t, J = 7.5 Hz), 5.08 (2H, m), 3.39 (2H, m), 3.20 (1H, q, J = 8.2 Hz), 1.92−2.18 (8H, m), 1.68 (6H, s), 1.59 (6H, s), 1.45 (3H, d, J = 8.2 Hz). *N*-Acetyl-*S*-*all-trans*-geranylgeranyl-L-cysteine (AGGC) was synthesized by a modification of a method previously described (Kamiya et al., 1979). L-AFC was converted into the methyl ester by overnight treatment with 0.1 M methanolic HCl at room temperature (Means & Feaney, 1971). Farnesol was oxidized to farnesal using benzotriazole−chromium trioxide complex (Parish et al., 1990). The aldehyde was converted to farnesylic acid using sodium chlorite as oxidizing agent (Bal et al., 1981). All isolated producs produced single spots by TLC. The physical properties and spectroscopic characteristics (^{1}H NMR, ^{13}C NMR, and mass spectroscopy) of all compounds were consistent with those reported in the literature. All compounds were dissolved in dimethyl sulfoxide. The final assay concentration of dimethyl sulfoxide was 0.5% (v/v).

HL-60 Cell Culture and Granulocyte Membrane Preparation. HL-60 cells were grown in suspension culture and induced to differentiate into mature myeloid forms by

cultivation in the precence of 1.25% (v/v) dimethyl sulfoxide as decribed before (Gierschik et al., 1989). Cells were homogenized by nitrogen cavitation, and membranes were prepared as described (Gierschik et al., 1989). Human neutrophil plasma membranes were prepared as reported in Kupper et al. (1992).

[^{35}S]GTP[S] Binding to Granulocyte Membranes. Binding of [^{35}S]GTP[S] was assayed as described (Gierschik et al., 1991). In brief, membranes (3−4 μg of protein) were incubated for 60 min at 30 °C in a mixture (100 μl) containing 50 mM triethanolamine HCl, pH 7.3, 1 mM dithiothreitol, 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl, 1 μM GDP, and 0.2−0.4 nM [^{35}S]GTP[S] (1200 Ci/mmol). The incubation was terminated by rapid filtration through 0.45 μm pore size nitrocellulose membranes (Advanced Microdevices, Ambala Cantt., India). The membranes were washed, and the retained radioactivity was determined by liquid-scintillation counting. Nonspecific binding was defined as the binding not competed for by 50 μM unlabeled GTP[S].

[^{32}P]ADP-Ribosylation of Membrane Proteins by Pertussis Toxin. HL-60 membranes (50 μg of protein) were incubated for the times indicated in the legend of Figure 3 at 37 °C in a volume of 50 μL containing 100 mM Tris-HCl, pH 8.0, 2 mM ATP, 50 nM [^{32}P]NAD^+ (800 Ci/mmol), and 30 μg/mL dithiothreitol-activated pertussis toxin (Gierschik et al., 1987) and the additions specified in the legend of Figure 3.

Purification of Retinal Transducin. The purification of α_t and $\beta\gamma_t$ from bovine rod outer segment membranes was done as described in (Gierschik & Camps, 1994). Briefly, heterotrimeric transducin was eluted from rod outer segment membranes with hypotonic buffer containing 100 μM GTP and separated into α and $\beta\gamma$ subunits by chromatography on Blue Sepharose CL-6B. All procedures were done at 4 °C under normal laboratory light. The purity of the transducin subunits was at least 95%, as judged by analysis of silver-stained SDS−polyacrylamide gels.

Preparation of Urea-Washed Rod Outer Segment Membranes. Rod outer segment membranes were stripped from peripherally bound membrane proteins by treatment with 5 M urea as described in Yamazaki et al. (1982). In brief, the rod outer segment membrane pellet remaining after extraction with GTP was washed two times by resuspension to 4 mg of protein/mL with buffer containing 100 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 5 M urea, homogenization through a 1 × 120 mm needle, and centrifugation in a Beckman type 55.2 Ti rotor (r_{av} = 73.5 mm) at 45 000 rpm for 30 min. The pellet was resuspended to 4 mg of protein/mL in the same buffer, homogenized, and incubated for 2 h with constant stirring, followed by centrifugation in a Beckman type 55.2 Ti rotor at 45 000 rpm for 30 min. The pellet was then resuspended to 4 mg of protein/mL in buffer containing 10 mM triethanolamine HCl, pH 7.4, homogenized, washed three times in the same buffer as outlined above, and then dialyzed extensively against the same buffer. All procedures were performed at 4 °C under normal laboratory light.

Solubilization of Rhodopsin. Light-activated rhodopsin was solubilized from rod outer segment membranes as described in Litman (1982) and Phillips et al. (1989). Specifically, the rod outer segment membrane pellet remaining after extraction with GTP was resuspended to 2.4 mg of protein/mL in buffer containing 50 mM Tris acetate, pH 7.0, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 150 mM NaCl, and then centrifuged in a Beckman type JA-20 rotor (r_{av} = 70 mm) at 17 000 rpm for 30 min. The pellet was resuspended to 1 mg of protein/mL in buffer containing 10 mM CHAPS, 50 mM Tris acetate, pH 7.0, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 150 mM NaCl. The suspension was incubated for 60 min with constant stirring. Insoluble material was then removed by centrifugation in a Beckman type Ti 70.1 rotor (r_{av} = 61.2 mm) at 38 000 rpm for 60 min, followed by filtration through 0.45 μm pore size cellulose acetate filters. In some experiments, soluble rhodopsin was purified and resolved from phospholipids present in the detergent extract by chromatography on concanavalin A Sepharose, which was performed essentially as described in Litman (1982), except that the column (3 mL bed volume) was equilibrated and run in buffer containing 10 mM CHAPS, 50 mM Tris acetate, pH 7.0, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 150 mM NaCl. Bound rhodopsin was eluted after extensive washing by including 0.1 M α-methyl D-mannoside in this buffer. Phospholipids did not bind to this resin as judged by including radiolabeled phospholipids in the sample and monitoring column fractions for radioactivity. All procedures were done at 4 °C under normal laboratory light.

[^{35}S]GTP[S] Binding to Retinal Transducin. Rhodopsin-stimulated binding of [^{35}S]GTP[S] to transducin was assayed as described (Fawzi et al., 1991). Briefly, urea-washed retinal rod outer segment membranes (U-ROS*-M) or detergent extract of transducin-depleted retinal rod outer segment membranes (D-ROS*-S) was incubated at the concentrations indicated in the legends of Figures 7−9 with α_t or $\alpha_t\beta\gamma_t$ for 2 h at at 30 °C in a mixture (40 μL) containing 20 mM MOPS/NaOH, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 3 mM $MgSO_4$, 100 mM NaCl, 2.3 mM sodium cholate, and 1 μM [^{35}S]GTP[S] (1.5 Ci/mmol). When D-ROS*-S was as a source of rhodopsin, the concentration of CHAPS in the binding assay was 2.4 mM. The incubation was terminated by rapid filtration through nitrocellulose membranes as specified above. Nonspecific binding was defined as the binding not competed for by 50 μM unlabeled GTP[S].

Miscellaneous. SDS−PAGE was performed as described in Laemmli (1970). Protein concentrations were determined according to Bradford (1976) using bovine IgG as standard.

RESULTS

Figure 1 shows a comparison of the effects of L-AFC and its C_{20} analogue *N*-acetyl-*S-all-trans*-geranylgeranyl-L-cysteine (AGGC) on basal and fMet-Leu-Phe-stimulated binding of [^{35}S]GTP[S] to membranes of myeloid differentiated HL-60 cells (Gierschik et al., 1991). As reported before (Scheer & Gierschik, 1993), L-AFC inhibited both basal and formyl peptide receptor-stimulated binding of [^{35}S]GTP[S], and receptor-stimulated [^{35}S]GTP[S] binding was more sensitive than basal binding to L-AFC inhibition. Increasing concentrations of AGGC also reduced basal and fMet-Leu-Phe-stimulated [^{35}S]GTP[S] but elicited these inhibitory effects at lower concentrations. Specifically, the concentrations required for half-maximal inhibition of basal and receptor-stimulated binding of [^{35}S]GTP[S] were about 5.5- and 3.5-fold lower, respectively, for AGGC than for L-AFC. Note that AGGC, unlike L-AFC, inhibited basal and receptor stimulated binding at similar concentrations ($IC_{50} \sim 25$ μM).

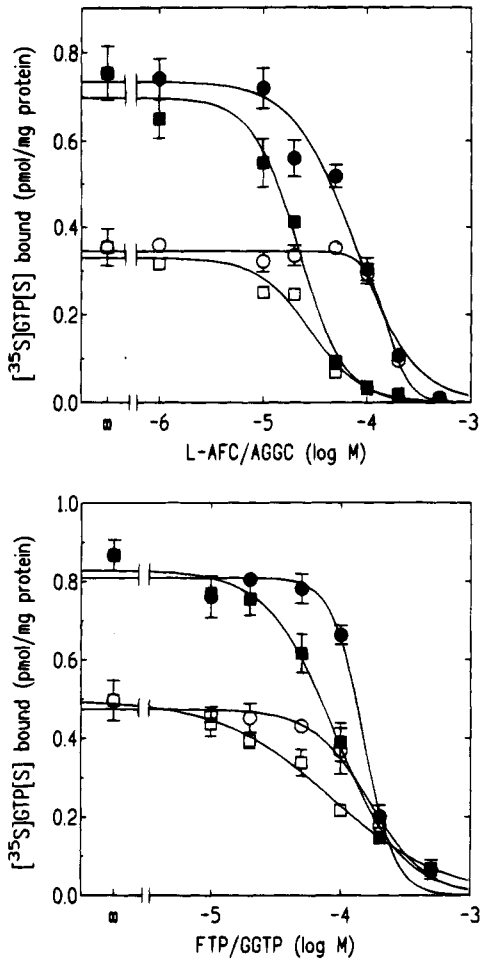

FIGURE 1: Comparison of the effects of *S*-farnesylated and *S*-geranylgeranylated cysteine analogues on basal and fMet-Leu-Phe-stimulated binding of [^{35}S]GTP[S] to HL-60 cell membranes. (A, top) HL-60 cell membranes were incubated in the absence (open symbols) or presence (closed symbols) of 100 nM fMet-Leu-Phe with [^{35}S]GTP[S] and L-AFC (circles) or AGGC (squares) at concentrations indicated at the abscissa. In panel B (bottom), the incubation was performed at increasing concentrations of FTP (circles) or GGTP (squares). The incubation was carried out for 60 min at 30 °C, and samples were then analyzed for [^{35}S]GTP[S] specifically bound to the membranes as described in Materials and Methods.

Inhibition of both basal and fMet-Leu-Phe-stimulated [^{35}S]-GTP[S] binding was also observed for *S*-(*trans,trans*-farnesyl-3-thio)propionic acid (FTP) and its C_{20} analogue *S*-(*all-trans*-geranylgeranyl-3-thio)propionic acid (GGTP) (Figure 1B). FTP was slightly less potent in inhibiting receptor-stimulated [^{35}S]GTP[S] binding than L-AFC, but similarly potent in inhibiting basal activity (cf. Figure 1A). The C_{20} compound GGTP was a more potent inhibitor of both basal and fMet-Leu-Phe-stimulated [^{35}S]GTP[S] binding than its C_{15} analogue FTP. Additional experiments (results not shown) revealed that both *S*-(*trans,trans*-farnesyl-2-thio)-acetic acid (FTA) and *S*-(*trans,trans*-farnesyl-(R,S)-2-thio)-propionic acid (F2TP) also inhibited both basal and fMet-Leu-Phe-stimulated [^{35}S]GTP[S] binding and that the concentrations required to observe inhibition were similar to those observed for L-AFC and FTP (cf. Figure 1A,B). Moreover, when the enantiomer specificity of the effects of L-AFC on the coupling of the formyl peptide receptor to G proteins was examined by comparing the effects of L-AFC and D-AFC on basal and fMet-Leu-Phe-stimulated binding of [^{35}S]GTP[S], there was no difference in the ability of the

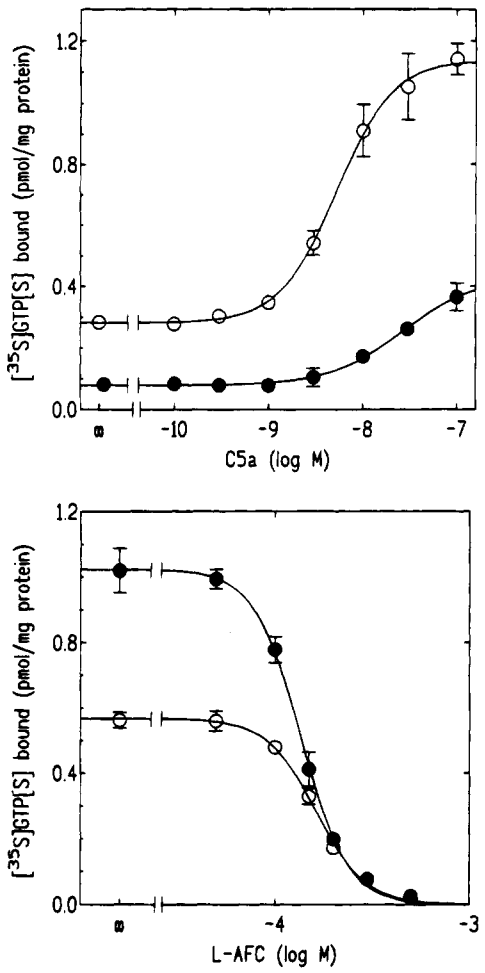

FIGURE 2: Effect of L-AFC on C5a-stimulated binding of [^{35}S]-GTP[S] to HL-60 cell membranes. (A, top) HL-60 cell membranes were incubated in the absence (open symbols) or presence (closed symbols) of 150 μM L-AFC with [^{35}S]GTP[S] and C5a at concentrations indicated at the abscissa. (B, bottom) HL-60 membranes were incubated in the absence (*open symbols*) or presence (*closed symbols*) of 3 nM C5a with [^{35}S]GTP[S] and increasing concentrations of L-AFC.

two enantiomers in inhibiting these two functions (results not shown).

The next experiments were designed to determine whether the inhibitory effects of L-AFC and related compounds were specific for the formyl peptide receptor or observed for other chemoattractant receptors as well. Figure 2A shows the effect of L-AFC (150 μM) on the complement C5a-stimulated binding of [^{35}S]GTP[S] to HL-60 cell membranes. Similar to fMet-Leu-Phe (Gierschik et al., 1991), C5a led to a marked stimulation of [^{35}S]GTP[S] binding in the absence of L-AFC. Half-maximal and maximal (approximately 4-fold) stimulation were observed at about 5 and 30 nM C5a, respectively. Addition of L-AFC (150 μM) led to a substantial (approximately 75%) reduction of [^{35}S]GTP[S] binding. This inhibition was similar in the absence of C5a and in the presence of 100 nM C5a, a concentration which maximally stimulated binding in the absence of L-AFC. Figure 2B shows the effect of increasing concentrations of L-AFC on [^{35}S]GTP[S] binding determined in the absence and presence of 3 nM C5a. Basal and agonist-stimulated binding were approximately equally sensitive to inhibition by L-AFC (IC_{50} $\sim$ 150 μM). In additional experiments (results not shown), we found that L-AFC, D-AFC, and FTP were similarly effective in suppressing [^{35}S]GTP[S] binding in the absence

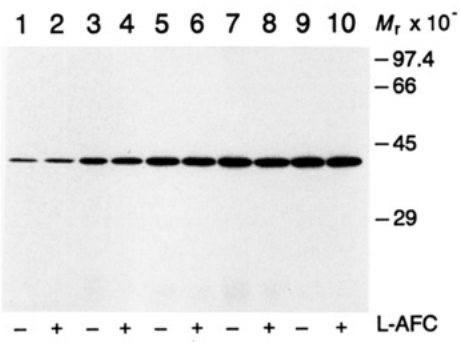

FIGURE 3: [^{32}P]ADP-ribosylation of G_i proteins of HL-60 cell membranes by pertussis toxin. HL-60 membranes were treated as indicated in the absence or presence of 100 μM L-AFC with [^{32}P]-NAD^+ and dithiothreitol-activated pertussis toxin. The incubation was performed for 5 min (lanes 1 and 2), 10 min (lanes 3 and 4), 20 min (lanes 5 and 6), 30 min (lanes 7 and 8), and 60 min (*lanes 9 and 10*). The samples were subjected to SDS−PAGE, and autoradiography of the dried gel was performed. The positions of the molecular mass standards are indicated.

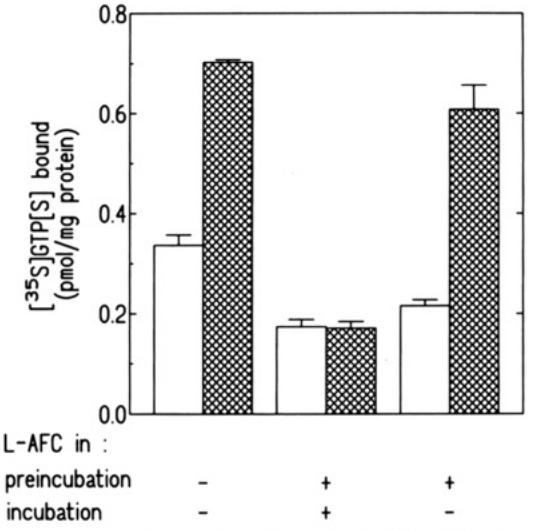

FIGURE 4: Reversibility of the effect of L-AFC on fMet-Leu-Phe-stimulated [^{35}S]GTP[S] binding. HL-60 membranes were pretreated as indicated at the abscissa by incubation for 60 min at 30 °C in the absence or presence of 200 μM L-AFC. See Materials and Methods for experimental details. The pretreated membranes were then sedimented by centrifugation, resuspended in buffer without L-AFC, and then incubated with [^{35}S]GTP[S] in the absence (open bars) or presence (hatched bars) of 100 nM fMet-Leu-Phe. L-AFC (200 μM) was absent or present in this second incubation as indicated at the abscissa.

or presence of C5a (3 nM), whereas AFCMe and L-AGC were without effect (all compounds tested at 100 μM). AGGC and GGTP almost completely suppressed both basal and C5a-stimulated binding when tested at this concentration. Thus, the *S*-prenyl compounds inhibited C5a and formyl peptide receptor-mediated G protein activation with similar specificities [cf. Figure 1 and Scheer and Gierschik (1993)].

The recent finding that L-AFC effectively blocked several cellular functions of intact human neutrophils (Philips et al., 1993) prompted us to determine the effect of L-AFC on basal and fMet-Leu-Phe-stimulated binding of [^{35}S]GTP[S] to purified plasma membranes of these cells (results not shown). Increasing concentrations of L-AFC markedly inhibited both basal and fMet-Leu-Phe-stimulated binding of [^{35}S]GTP[S]. As shown earlier for HL-60 cell membranes, fMet-Leu-Phe-stimulated [^{35}S]GTP[S] binding appeared to be slightly more sensitive than basal binding. Thus, the net increase of binding induced by addition of fMet-Leu-Phe (0.3 μM) was reduced by approximately 60 and 90% at 50 and 100 μM L-AFC, respectively.

The C-terminal γ subunit modification(s) have previously been shown to be required for formation of the $\alpha\beta\gamma$ heterotrimer (Ohguro et al., 1991; Iñiguez-Lluhi et al., 1992), which is known to be required for receptor-mediated G protein activation (Fung, 1983). To examine whether L-AFC inhibited receptor−G protein interaction by interfering with heterotrimer formation, we studied the effect of L-AFC on the pertussis toxin-mediated [^{32}P]ADP-ribosylation of G_i proteins present in HL-60 membranes, which is well known to require the toxin substrate in its heterotrimeric state [see Gierschik (1992) for review]. Figure 3 shows that L-AFC (100 μM) had no effect on the pertussis toxin-mediated G_i protein [^{32}P]ADP-ribosylation under conditions allowing for an increase of the reaction with time, suggesting that inhibition of heterotrimer formation was unlikely to be the cause for the inhibitory effects of L-AFC. In additional experiments (results not shown), we found that L-AFC did not affect pertussis toxin-mediated [^{32}P]ADP-ribosylation of G_i proteins even when tested at concentrations as high as 500 μM.

L-AFC and the other *S*-prenylated analogues characterized in the above experiments are amphiphilic molecules possessing a hydrophobic farnesyl or geranylgeranyl moiety and a hydrophilic carboxyl group. This raises the distinct possibility that these compounds affect receptor−G protein interaction by virtue of a nonspecific, detergent-like property. Several approaches were taken to examine this hypothesis. First, HL-60 cell membranes were incubated for 60 min at 30 °C with buffer containing up to 500 μM L-AFC and then sedimented by centrifugation. The supernatants were concentrated by ultrafiltration and then subjected to determination of protein concentration and immunoblot analysis using antibodies reactive with the G protein subunits α_{i2} or β_1 (Böhm et al., 1991; Gierschik et al., 1985). There was no change in the protein concentration of the supernatants and no evidence for solubilization of α_{i2} or β_1 even at the highest concentration of L-AFC (results not shown). The second approach was to determine the reversibility of the effect of L-AFC on basal and fMet-Leu-Phe-stimulated [^{35}S]GTP[S] binding (Figure 4). When HL-60 cell membranes were preincubated with buffer containing 200 μM L-AFC and then assayed for [^{35}S]GTP[S] binding in the continued presence of 200 μM L-AFC, basal [^{35}S]GTP[S] binding was reduced by about 50% and addition of fMet-Leu-Phe did not increase [^{35}S]GTP[S] binding. In contrast, when L-AFC was omitted from the second incubation, there was partial recovery of basal [^{35}S]GTP[S] binding and a complete recovery of the stimulatory effect of fMet-Leu-Phe. The third approach was to synthesize an analogue of L-AFC which contains the two structural features rendering it an amphiphilic molecule, the farnesyl and the carboxyl moiety, and to examine its effects on [^{35}S]GTP[S] binding to HL-60 cell membranes. We synthesized the oxidized variant of *trans,trans*-farnesol (FOH), the corresponding acid *trans,trans*-farnesylic acid (FCOOH). A comparison of the structures of L-AFC and its analogues used in this or in the preceding communication (Scheer & Gierschik, 1993) is shown in Figure 5. Figure 6 shows that neither FCOOH nor the oxidation intermediate *trans,trans*-farnesal (FCHO) (200 μM) affected basal or fMet-Leu-Phe-stimulated [^{35}S]GTP[S] binding.

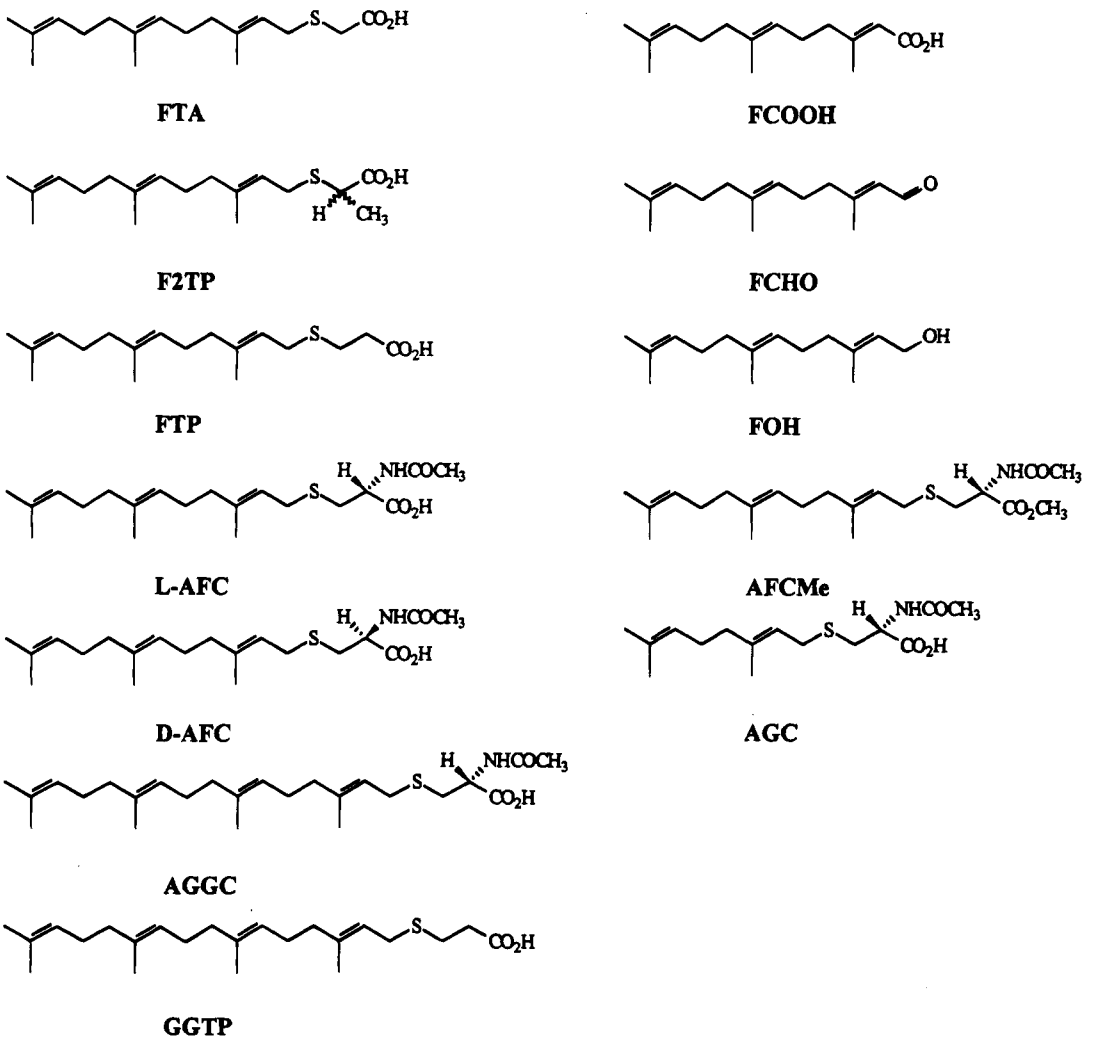

FIGURE 5: Comparison of the structures of *S*-prenylated cysteine analogues. The active compounds FTA, F2TP, FTP, L-AFC, D-AFC, AGGC, and GGTP are shown on the left, the inactive compounds FCOOH, FCHO, FOH, AFCMe, and AGC on the right.

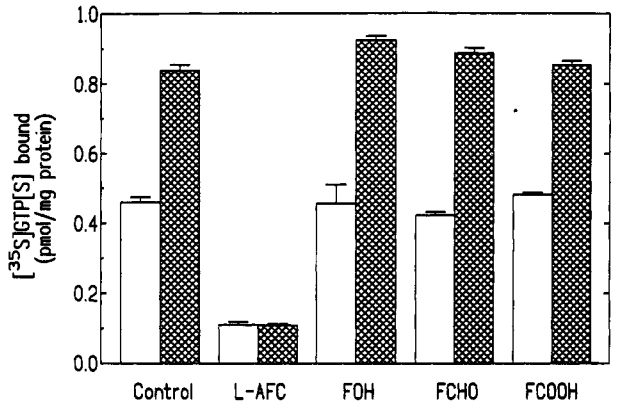

FIGURE 6: Comparison of the effects of L-AFC, farnesol (FOH), farnesal (FCHO), and farnesylic acid (FCOOH) on binding of [^{35}S]-GTP[S] to HL-60 cell membranes. HL-60 cell membranes were incubated for 60 min in the absence (open bars) or presence (hatched bars) of 0.1 μM fMet-Leu-Phe with [^{35}S]GTP[S] as described under Material and Methods. The incubation was performed in the absence (Control) or presence of 200 μM of the farnesyl compounds specified at the abscissa.

A reconstituted system consisting of light-activated rhodopsin devoid of G proteins and purified subunits of retinal transducin, α_t and $\beta\gamma_t$, was used to examine the hypothesis that L-AFC specifically interfered with receptor−G protein interaction in more detail. This system has previously been used by many investigators as a model system to study the molecular mechanisms of receptor−G protein interaction [see Kühn (1984), Cerione (1991), and Hargrave et al. (1993) for review]. Figure 7 shows the effect of L-AFC on the stimulation of [^{35}S]GTP[S] binding to purified heterotrimeric transducin by increasing concentrations of light-activated rod outer segment membrane stripped of transducin by treatment with 5 M urea (U-ROS*-M). In the absence of L-AFC, U-ROS*-M stimulated [^{35}S]GTP[S] binding to transducin half-maximally at 0.12 and 0.48 μg of U-ROS*-M protein per assay. L-AFC (250 μM) led to a distinct decrease in the potency of U-ROS*-M to stimulate [^{35}S]GTP[S] binding. Specifically, half-maximal and maximal stimulation were observed at 0.38 and 1.6 of μg U-ROS*-M protein per assay, respectively, under these conditions. Note that the same maximal binding was observed at the highest concentration of U-ROS*-M.

Transducin $\beta\gamma$ dimers have previously been shown to promote the binding of [^{35}S]GTP[S] to purified α_t, presumably by facilitating the receptor-α subunit interaction (Fawzi et al., 1991; Phillips et al., 1992). To examine whether L-AFC specifically interfered with this $\beta\gamma$ dimer function, U-ROS*-M-stimulated binding of [^{35}S]GTP[S] to α_t was examined at increasing concentrations of $\beta\gamma_t$ in the absence and presence of L-AFC (Figure 8). In the absence of L-AFC, $\beta\gamma_t$ led to a marked (approximately 13-fold) stimulation of

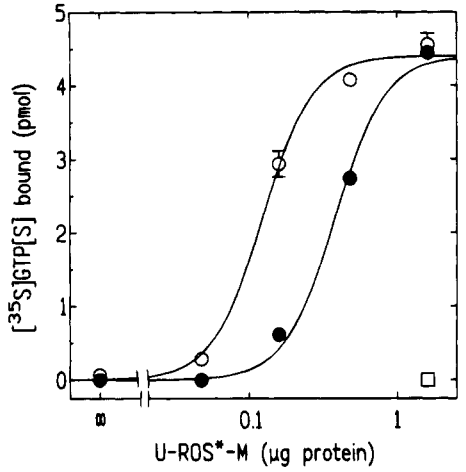

FIGURE 7: Inhibition of rhodopsin-stimulated [^{35}S]GTP[S] binding to heterotrimeric transducin by L-AFC. Purified subunits of heterotrimeric transducin (α_t and $\beta\gamma_t$, 0.5 μM each) were incubated for 120 min in a volume of 40 μL at 30 °C in the absence (open circles) or presence (closed circles) of 250 μM L-AFC with [^{35}S]-GTP[S] (1 μM) and increasing concentrations of bleached, urea-treated rod outer segment membranes (U-ROS*-M). See Materials and Methods for experimental details. The open square indicates the amount of [^{35}S]GTP[S] bound in the absence of transducin at the highest concentration of U-ROS*-M. The amounts of [^{35}S]-GTP[S] bound/assay and U-ROS*-M protein/assay are shown on the abscissa and ordinate, respectively.

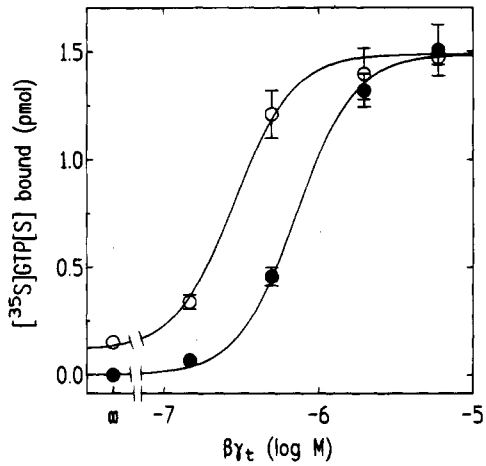

FIGURE 8: Reversal of L-AFC inhibition of rhodopsin-stimulated [^{35}S]GTP[S] binding to α_t by $\beta\gamma_t$. Purified α_t (0.5 μM) was incubated in the absence (open symbols) or presence (closed symbols) of 200 μM L-AFC with [^{35}S]GTP[S], 0.16 μg U-ROS*-M protein/assay, and increasing concentrations of purified $\beta\gamma_t$.

[^{35}S]GTP[S] binding. Half-maximal and maximal stimulation were observed at approximately 0.25 and 1.8 μM $\beta\gamma_t$, respectively. Note that there was a low degree of [^{35}S]GTP-[S] binding in the presence of U-ROS*-M and α_t but absence of $\beta\gamma_t$ (approximately 7.5% of maximal binding). The origin of this activity is currently unknown. It is possible, however, that it is due to the presence of contamining $\beta\gamma_t$ in the α_t and/or U-ROS*-M preparation used here. Addition of L-AFC (200 μM) abolished basal [^{35}S]GTP[S] binding and led to a clear reduction of the potency of $\beta\gamma_t$ to stimulate [^{35}S]GTP-[S] binding to α_t. Thus, half-maximal and maximal stimulation was observed at approximately 0.75 and 5.5 μM $\beta\gamma_t$, respectively, under these conditions. Note that $\beta\gamma_t$ stimulated [^{35}S]GTP[S] binding to α_t to the same maximal extent, so that L-AFC inhibition of [^{35}S]GTP[S] binding was fully reversed at high concentrations of $\beta\gamma_t$.

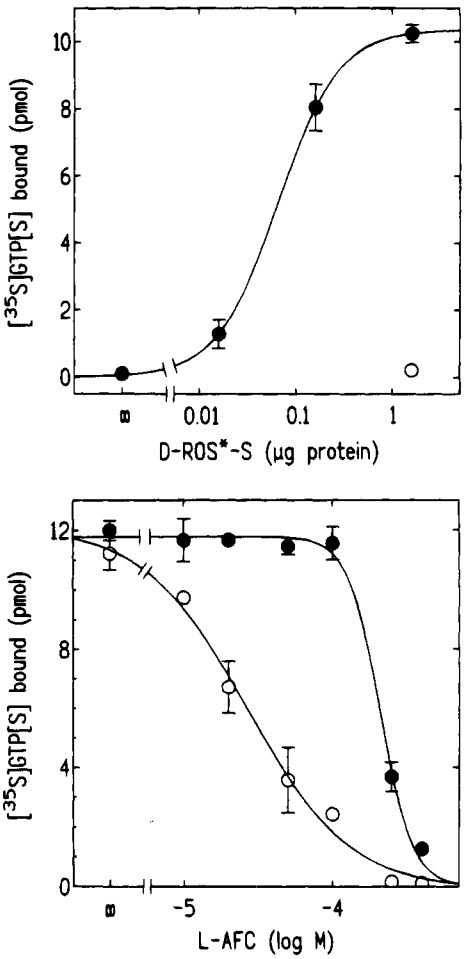

FIGURE 9: Effect of L-AFC on stimulation of [^{35}S]GTP[S] binding to heterotrimeric transducin by solubilized rhodopsin. (A, top) Purified subunits of heterotrimeric transducin (α_t and $\beta\gamma_t$, 0.5 μM each) were incubated with [^{35}S]GTP[S] (1 μM) and increasing concentrations of detergent soluble extract of bleached, transducin-depleted rod outer segment membranes (D-ROS*-S) (closed circles). The open circle indicates the amount of [^{35}S]GTP[S] bound in the absence of transducin at the highest concentration of D-ROS*-S. In panel B (bottom), the incubation was performed at increasing concentrations of L-AFC in the presence of 0.3 μg of D-ROS*-S protein/assay (open circles) or 1.6 μg of D-ROS*-S protein/assay (closed circles).

The next experiments were performed to examine whether an intact lipid bilayer and/or phospholipid environment was required to observe L-AFC inhibition of rhodopsin-mediated [^{35}S]GTP[S] binding to heterotrimeric transducin. As rhodopsin is well known to interact with transducin in the absence of lipid vesicles (Kühn, 1984; König et al., 1989; Phillips & Cerione, 1992; Phillips et al., 1992), light-activated rhodopsin was solubilized from transducin-depleted rod outer segment membranes, and the effect of L-AFC was examined on the binding of [^{35}S]GTP[S] to soluble transducin stimulated by the soluble receptor in the absence of the native membrane lipid bilayer. Figure 9A shows that addition of detergent-solubilized, light-activated rhodopsin (D-ROS*-S) to $\alpha_t\beta\gamma_t$ led to a marked stimulation of [^{35}S]GTP[S] binding and that this activity was absolutely dependent on the presence of $\alpha_t\beta\gamma_t$. The effect of increasing concentrations of L-AFC on D-ROS*-S-stimulated [^{35}S]GTP[S] binding was then determined at 0.3 and 1.6 μg of D-ROS*-S protein/assay, i.e., at concentrations which almost maximally and maximally, respectively, stimulated this activity (Figure 9B; cf. Figure 9A). In both cases, L-AFC concentration-dependently in-

hibited [^{35}S]GTP[S] binding and completely abolished this activity at approximately 500 μM. Most interestingly, however, the concentration of L-AFC necessary to elicit half-maximal inhibition of [^{35}S]GTP[S] binding was markedly dependent on the concentration of light-activated rhodopsin present in the assay. Thus, the IC_{50} of L-AFC increased from approximately 30 μM at the lower receptor concentration to approximately 210 μM at the higher receptor concentration. Additional experiments (not shown) revealed that L-AFC failed to inhibit receptor-stimulated [^{35}S]GTP[S] binding when tested up to 500 μM in the presence of an even higher concentration of D-ROS*-S (16 of μg D-ROS*-S protein/assay) and showed that L-AFC inhibited [^{35}S]GTP[S] binding to transducin stimulated by a lower concentration of rhodopsin purified and resolved from phospholipids by concanavalin A Sepharose chromatography, indicating that phospholipids were not required to observe the inhibitory L-AFC effect.

DISCUSSION

In this paper, we present a detailed analysis of the structural properties of *S*-prenylated cysteine analogues required for their inhibitory effects on receptor-mediated G protein activation in human granulocyte membranes. In addition to L-AFC and FTP, which we have previously shown to inhibit basal and formyl peptide receptor-mediated GTP[S] binding to and GTP hydrolysis by HL-60 cell membranes (Scheer & Gierschik, 1993), AGGC and GGTP, the C_{20} analogues of L-AFC and FTP, respectively, FTA, an analogue of FTP lacking one methylene group between the sulfur atom and the carboxyl group, and F2TP, a position isomer of FTP (cf. Figure 5), were found to be strong inhibitors of receptor-mediated G protein activation in these membranes. Taken together, these results suggest (i) that the isoprenyl moiety (C_{15} or C_{20}) and the free carboxyl group, but not the peptide bond of *S*-prenylated cysteine analogues, are required for their inhibitory effects, and (ii) that the length (C_1 or C_2) of the aliphatic chain between the sulfur atom and the carboxyl group and its position isomerism do not influence the inhibitory activity of these compounds. The effects of L-AFC and D-AFC on receptor-stimulated GTP[S] binding were indistinguishable, suggesting that the effects of these compounds are not enantiomer-specific.

L-AFC and AGGC have previously been shown to block a variety of extracellular signaling molecule-stimulated cell functions. Examples include the activation of mouse macrophage chemotaxis by endotoxin-activated serum (Volker et al., 1991a), the stimulation of human neutrophil O_2^- generation, homotypic aggregation, and primary granule secretion by fMet-Leu-Phe (Philips et al., 1993), the activation of insulin release from rat pancreatic islets by glucose and the amino acid metabolite α-oxo-4-methylpentanoate (Metz et al., 1993), and the stimulation of human platelet aggregation and serotonin secretion by agents such as ADP, collagen, thrombin, and U46619 (a stable analogue of prostaglandin H_2) (Huzoor-Akbar et al., 1993). The fact that L-AFC and AGGC are powerful inhibitors of *S*-prenylated protein carboxyl methylation *in vitro* (Pérez-Sala et al., 1991; Tan et al., 1991a,b; Huzoor-Akbar et al., 1991; Volker et al., 1991a,b; Shi & Rando, 1992; Pérez-Sala et al., 1992; Philips et al., 1993; Metz et al., 1993), together with the actual demonstration in some of the above studies that methylation of low-molecular-mass GTP-binding proteins was inhibited when intact cells were exposed to L-AFC (Volker et al., 1991a; Philips et al., 1993; Metz et al., 1993), prompted authors to suggest that inhibition of protein carboxyl methylation was the basis for the inhibition of intact cell functions by L-AFC and AGGC (Volker et al., 1991a; Philips et al., 1993; Huzoor-Akbar et al., 1993; Metz et al., 1993).

It appears unlikely, however, that inhibition of carboxyl methylation is involved in the effects of *S*-prenylated cysteine analogues on receptor-mediated G protein activation reported here. Thus, as we have already pointed out earlier (Scheer & Gierschik, 1993), the inhibitory effects described here were observed in well washed membrane preparations, which should be devoid of the methyl transferase cosubstrate *S*-adenosyl-L-methionine. Furthermore, addition of exogenous *S*-adenosyl-L-methionine to the membranes did not enhance the inhibitory effect of L-AFC, and addition of the methyl transferase inhibitor *S*-adenosyl-L-homocysteine (1−100 μM) did not affect basal and fMet-Leu-Phe-stimulated [^{35}S]GTP[S] binding. Finally, both fMet-Leu-Phe stimulation of [^{35}S]GTP[S] binding and inhibition of this function by L-AFC (100 μM) were observed without delay, arguing against the notion that inhibition of an enzymatic reaction was the basis of the inhibitory effects of L-AFC. Of interest in this respect are recent results obtained by Rando and colleagues, who reported the synthesis of novel farnesylcysteine analogues, which are neither substrates nor inhibitors of isoprenylated protein methyltransferase but are nevertheless active inhibitors of thrombin—or endoperoxide analog U46619—stimulated platelet aggregation (Ma et al., 1994). Moreover, several lines of evidence were recently provided by the same authors to suggest that inhibition of protein carboxyl methylation was unlikely to be the basis of L-AFC inhibition of fMet-Leu-Phe-stimulated human neutrophil O_2^- generation (Ding et al., 1994). The fact that L-AFC inhibition of formyl peptide receptor-stimulated [^{35}S]GTP[S] binding was observed in plasma membranes from human neutrophils, i.e., one of the cell types studied in the above intact cell systems (Philips et al., 1993), not only confirms the earlier suggestion that *S*-prenylated cysteine analogues inhibit stimulated cell functions by interfering with signal transduction at a step prior to stimulation of protein kinase C and mobilization of intracellular Ca^{2+} (Volker et al., 1991a; Huzoor-Akbar, 1993; Philips et al., 1993) but also suggests that suppression of receptor-mediated G protein activation may be one of the mechanisms by which L-AFC inhibits stimulated intact cell functions.

L-AFC and related compounds are unlikely to act as specific formyl peptide receptor antagonists, because the inhibitory effects of *S*-prenylated cysteine analogues on receptor-mediated G protein activation were not restricted to the formyl peptide receptor but were also observed with similar sensitivity and selectivity when GTP[S] binding was stimulated by complement C5a (Figure 2A and 2B). The fact that formyl peptides and C5a are structurally unrelated, together with our finding that *S*-prenylated cysteine analogues also inhibit the rhodopsin-stimulated binding of GTP[S] to retinal transducin (Figures 7−9), argues against the hypothesis that L-AFC and related compounds inhibit receptor-mediated G protein activation by directly interacting with the ligand binding sites of those receptors.

Since isoprenylation of the G protein γ subunit carboxyl terminus has previously been shown to be required for the formation of the $\alpha\beta\gamma$ heterotrimer (Ohguro et al., 1990;

Iñiguez-Lluhi et al., 1992), we speculated that L-AFC and related compounds might act as competitive inhibitors of the $\alpha\beta\gamma$ subunit interaction and thereby inhibit the formation of the agonist–receptor–G protein complex. However, L-AFC did not appear to interfere with pertussis toxin-mediated [^{32}P]ADP-ribosylation of the G_i proteins present in HL-60 membranes, suggesting that inhibition of heterotrimer formation was unlikely to be the cause for the inhibitory effects of prenylcysteines. On the other hand, it is conceivable that $\beta\gamma$ subunits interact directly with receptors and that this interaction gives rises to the inhibitory effects of prenylcysteines observed here. Thus, several groups have presented evidence favoring the concept that $\beta\gamma$ subunits directly interact with receptors and that this interaction is operative even in the absence of α subunits (Halpern et al., 1987; Im et al., 1988; Kelleher et al., 1988; Kurstjens et al., 1991; Heithier et al., 1992; Phillips & Cerione, 1992). Recent results obtained by analyzing the interaction of G protein-coupled receptor kinases (GRKs) with the corresponding receptors are consistent with the notion that G protein-coupled receptors contain a "docking site" for carboxyl-terminally isoprenylated proteins, i.e., rhodopsin kinase or $\beta\gamma$ dimers [see Lefkowitz (1993) for review]. These findings raise the possibility that *S*-prenylated cysteine analogues interfere with receptor-stimulated [^{35}S]GTP[S] binding by specifically interacting with this "docking site" and interfering with the binding of the $\beta\gamma$ dimer to the receptor. Our observation in a reconstituted system that L-AFC inhibition of rhodopsin-stimulated binding of [^{35}S]-GTP[S] to the α subunit of retinal transducin was fully reversed by increasing the concentration of either the $\beta\gamma$ dimer (Figure 8) or the activated receptor (Figure 9B) provides direct experimental support to this concept.

It is unlikely that *S*-prenylated cysteine analogues affect receptor-mediated [^{35}S]GTP[S] binding on the basis of a putative unspecific, detergent-like property of these amphiphilic compounds. This notion is not only supported by the previous observations that L-AFC ($\leq 100\ \mu$M) (i) did not affect motility, morphology, viability, and phorbol ester-induced chemotactic responses of intact mouse macrophages (Volker et al., 1991a), (ii) did not alter the incorporation of [^{35}S]methionine into cultured transformed fibroblast and had little effects on the proliferation of these cells (Volker et al., 1991a), (iii) did not cause leakage of lactate dehydrogenase from neutrophils or inhibition of phorbol ester-induced O_2^- generation by these cells (Philips et al., 1993), (iv) did not alter basal intact platelet cAMP, ionomycin-, phorbol ester-, or bacterial phospholipase C-induced platelet aggregation, or ionomycin-stimulated increase in platelet $[Ca^{2+}]_i$ (Huzoor-Akbar et al., 1993), and (v) had only minimal effects on K^+-induced secretion from rat pancreatic islets (Metz et al., 1993). Several lines of direct experimental evidence are provided in this manuscript arguing against the possibility of nonspecific prenylcysteine action. First, there was no evidence of prenylcysteine-mediated solubilization of membrane proteins even at high concentrations of L-AFC (500 μM). Second, the effect of L-AFC on fMet-Leu-Phe-stimulated [^{35}S]GTP[S] binding was fully reversible. Third, and probably most importantly, FCOOH a structural analogue of L-AFC carrying both the hydrophobic farnesyl and the hydrophilic carboxyl moiety (cf. Figure 6) failed to affect basal or fMet-Leu-Phe-stimulated [^{35}S]GTP[S] binding. Finally, L-AFC inhibition of receptor–G protein interaction was also evident in a reconstituted system consisting of purified proteins in the absence of a membrane lipid bilayer and was observed even after removal of phospholipids.

On the basis of these results, it is tempting to speculate that *S*-prenylated cysteine analogues interfere with receptor-mediated G protein activation by specifically interacting with one or more of the protein components involved in transmembrane signaling. Interaction with G protein α subunits seems unlikely at present, but more experimentation is required to formally exclude this possibility. We expect that synthesis and use of radiolabeled analogues of L-AFC suitable for affinity labeling of proteins will allow to examine whether any of the known signaling components carries a recognition unit for *S*-prenylated cysteine analogues.

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