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Farnesylcysteine analogues inhibit chemotactic peptide receptor-mediated G-protein activation in human HL-60 granulocyte membranes

Alexander Scheer and Peter Gierschik

Molecular Pharmacology Division, German Cancer Research Center, Heidelberg, Germany

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Analogues of *S*-prenylated cysteine like *N*-acetyl-*S-trans,trans*-farnesyl-L-cysteine (AFC) have previously been shown to inhibit the carboxyl methylation of proteins carrying a C-terminal *S*-prenylated cysteine residue and to block the endotoxin-activated serum-elicited chemotactic response of mouse macrophages. Here, we show that AFC inhibits both basal and formyl peptide receptor-stimulated binding of guanosine 5′-*O*-(3-thiotriphosphate) (GTP[S]) to and hydrolysis of GTP by membranes of myeloid differentiated HL-60 granulocytes. Receptor-stimulated GTP[S] binding and GTP hydrolysis are more sensitive to AFC inhibition than basal G-protein functions. Inhibition of formyl peptide receptor-mediated G-protein activation is also observed for *S-trans,trans*-farnesyl-3-thiopropionic acid, but not for *N*-acetyl-*S-trans*-geranyl-L-cysteine, *N*-acetyl-L-cysteine, or the methyl ester of AFC, suggesting that the farnesyl moiety and the carboxyl group, but not the peptide bond of AFC are required for inhibition. The observations that exogeneous *S*-adenosyl-L-methionine is apparently not required for and *S*-adenosyl-L-homocysteine does not attenuate the inhibitory action of AFC raise the distinct possibility that AFC inhibits receptor-mediated G-protein interaction by a mechanism other than inhibition of protein carboxyl methylation.

G-protein; Signal transduction; Protein *O*-Methyltransferase; Prenylcysteine; Chemotaxis; HL-60 cell

1. INTRODUCTION

Signal-transducing, heterotrimeric guanine nucleotide binding proteins (G-proteins) are involved in coupling an enormous variety of cell surface receptors to second messenger generating effector enzymes or ion channels [1,2]. G-Proteins are composed of three subunits (α, β, γ), which are members of rapidly growing gene families [2]. To date, at least 20 distinct α subunits, four β subunits and six γ subunits are known to exist in mammalian cells [2].

The γ subunits of heterotrimeric G proteins belong to a family of proteins which are posttranslationally modified at their carboxyl-terminus by isoprenylation, proteolytic cleavage, and methyl esterification (see [3] for review). They are first farnesylated or geranylgeranylated at a cysteine in position −4 from the carboxyl terminus [3,4]. Following *S*-isosprenylation, a membrane-bound protease(s) cleaves the three terminal amino acids [5,6] and the resultant terminal carboxyl group is methyl esterified by a membraneous methyltransferase [7]. It has been suggested that the methylation reaction is the only reaction in this pathway which is reversible and subject to regulation [8,9]. The functional roles of these posttranslational γ subunit modifications remain unresolved. On the one hand, evidence has been presented suggesting that these modifications are important for membrane attachment of the $\beta\gamma$ dimer [10]. On the other hand, the fact that other similarly modified proteins are localized to specific cell membranes or remain soluble has prompted the hypothesis that the C-terminal modifications cause the association of these proteins with specific 'receptor' proteins present in these membranes or in the cytosol [3].

The γ subunit methyltransferase recognizes and methylates even small structural analogues of the *S*-prenylated γ subunit C-terminus, such as *N*-acetyl-*S*-farnesyl-L-cysteine (AFC) [8,11–13]. Recent evidence suggests that AFC competitively inhibits the carboxyl methylation of *S*-prenylated $p21^{ras}$ and several small molecular mass GTP-binding proteins, including human platelet Rap1 [14] and human neutrophil Rap1 and Rap2 [12]. Most interestingly, treatment of intact mouse macrophages with AFC in vivo caused a marked inhibition of their chemotactic response toward endotoxin-activated serum, but not to phorbol ester [12], suggesting that AFC interfered with a step in macrophage signal transduction prior to protein kinase C activation.

Correspondence address. P. Gierschik, Molecular Pharmacology Division, German Cancer Research Center, Im Neuenheimer Feld 280/0425, 6900 Heidelberg, Germany. Fax: (49) 6221-42 3352.

Abbreviations. G-proteins, signal-transducing heterotrimeric guanine nucleotide-binding protein; GTP[S], guanosine 5′-*O*-(3-thiotriphosphate); fMet-Leu-Phe, *N*-formyl-methionyl-leucyl-phenylalanine; AFC, *N*-acetyl-*S-trans,trans*-farnesyl-L-cysteine; AGC, *N*-acetyl-*S-trans*-geranyl-L-cysteine; FTP, *S-trans,trans*-farnesyl-3-thiopropionic acid; AFCMe, *N*-acetyl-*S-trans,trans*-farnesyl-L-cysteine methyl ester.

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We therefore set out to determine the effect of AFC on coupling of chemotactic peptide receptors to G-proteins using myeloid differentiated human HL-60 granulocytes as a model system [15]. While investigating the effects of treating intact HL-60 cells with AFC, we found that AFC interferes with the chemotactic peptide receptor-mediated activation of G-proteins even when added to HL-60 cell membranes. Our results suggest that inhibition of the receptor/G-protein interaction may be a mechanism by which AFC inhibits endotoxin-activated serum-dependent chemotaxis of macrophages [12], but indicate that this inhibition is unlikely to be due to inhibition of isoprenylated protein methyltransferase.

2. MATERIALS AND METHODS

2.1. *Materials*

N-Acetyl-L-cysteine and 3-mercaptopropionic acid were purchased from Fluka. *Trans,trans*-farnesyl bromide and *trans*-geranyl bromide were from Aldrich. [^{35}S]GTP[S] and [γ-^{32}P]GTP were obtained from New England Nuclear. All other materials were from standard vendors or from sources previously described [15].

2.2. *Synthesis of S-prenyl compounds*

AFC, AGC, FTP were prepared through incubation of either *trans,trans*-farnesylbromide or *trans*-geranylbromide with *N*-acetyl-L-cysteine or 3-mercaptopropionic acid according to a previously reported procedure [16]. The methyl ester AFCMe was obtained from the corresponding carboxylic acid by overnight treatment with 0.1 M methanolic-HCl at room temperature [17]. The isolated products produced single spots by TLC. The physical properties and spectroscopic characteristics (^{1}H-NMR, ^{13}C-NMR and mass spectroscopy) of these compounds were consistent with those reported in the literature [13]. The *S*-prenyl-compounds were dissolved in DMSO. The final assay concentration of DMSO was 0.5% (v/v).

2.3. *Cell culture and membrane preparation*

HL-60 cells were grown in suspension culture and induced to differentiate into mature myeloid forms by cultivation in the presence of 1.25% (v/v) dimethyl sulfoxide as described before [18]. Cells were homogenized by nitrogen cavitation and membranes were prepared as described [18].

2.4. *[^{35}S]GTP[S] binding*

Binding of [^{35}S]GTP[S] was assayed as described [15]. In brief, membranes (3–4 μg protein) were incubated 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] (1,200 Ci/mmol). The incubation was terminated by rapid filtration through 0.45 μm pore size nitrocellulose membranes (Advanced Microdevices, India). The membranes were washed and the retained radioactivity was determined by liquid-scintillation counting. Non-specific binding was defined as the binding not competed for by 50 μM unlabelled GTP[S]. Only specific binding is reported.

2.5. *GTPase assay*

Hydrolysis of [γ-^{32}P]GTP was determined in a reaction mixture (100 μl) containing 5 μg membrane protein, 50 mM triethanolamine-HCl, pH 7.3, 1 mM dithiothreitol, 1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 10 μM GDP, and 25 nM [γ-^{32}P]GTP (20 Ci/mmol). The reactions were terminated and the amount of [γ-^{32}P]GTP hydrolyzed was measured as described [18]. High-affinity GTPase activity was calculated by subtracting low-affinity GTPase activity determined at 50 μM GTP from total GTPase activity. Only high-affinity GTPase activity is reported.

3. RESULTS

Fig. 1A shows the effect of AFC on the fMet-Leu-Phe-stimulated binding of [^{35}S]GTP[S] to membranes of myeloid differentiated HL-60 cells. As reported before [15], fMet-Leu-Phe markedly stimulated the binding of [^{35}S]GTP[S] in the absence of AFC. Half-maximal and maximal (approximately 4.5-fold) stimulation were observed at approximately 0.3 μM and 3 μM fMet-Leu-Phe, respectively. AFC (100 μM) led to a substantial (approximately 25-fold) decrease in the potency of fMet-Leu-Phe to stimulate [^{35}S]GTP[S] binding. Specifically, half-maximal stimulation was observed at approximately 7.5 μM fMet-Leu-Phe in the presence of AFC. Although saturation of the effect of fMet-Leu-Phe on [^{35}S]GTP[S] binding was not observed even at the highest concentration of the peptide (30 μM), extrapolation of the binding isotherm suggests that maximal stimulation would require $\geq$100 μM fMet-Leu-Phe.

The effect of increasing concentrations of AFC on basal and fMet-Leu-Phe-stimulated [^{35}S]GTP[S] binding is shown in Fig. 1B. Two aspects of this experiment are significant. First, at concentrations of AFC $\leq$100 μM, basal [^{35}S]GTP[S] binding remained largely unaltered, whereas fMet-Leu-Phe-stimulated binding was markedly reduced. Thus, the net increase in [^{35}S]GTP[S] binding induced by addition of 0.1 μM fMet-Leu-Phe was reduced by approximately 60% at 50 μM AFC and was completely eliminated at approximately 100 μM AFC. Second, AFC markedly inhibited [^{35}S]GTP[S] binding both in the presence and in the absence of the chemotactic peptide at concentrations $\geq$100 μM and led to a complete suppression of specific [^{35}S]GTP[S] binding at approximately 500 μM.

The specificity of the effect of AFC on receptor-stimulated [^{35}S]GTP[S] binding was examined using three *S*-prenylated AFC-analogs (100 μM) (Fig. 2). AFC led to an only slight decrease in basal [^{35}S]GTP[S] binding, but fully suppressed the net increase of binding due to addition of fMet-Leu-Phe (0.1 μM). In contrast, AFCMe, the methyl ester of AFC, did not affect basal or fMet-Leu-Phe-stimulated [^{35}S]GTP[S] binding. Additional experiments revealed that AFCMe was also without effect when tested over a wide range of concentrations up to 500 μM (results not shown). AGC, an analogue carrying a C_{10} geranyl instead of a C_{15} farnesyl moiety, did not interfere with [^{35}S]GTP[S] binding. Interestingly, FTP, an AFC analogue lacking the acetyl amide moiety, reduced basal and fMet-Leu-Phe-stimulated [^{35}S]GTP[S] binding by approximately 60% and 70%, respectively, which resulted in a reduction by approximately 85% in the net response caused by fMet-Leu-Phe. Additional experiments revealed that *N*-acetyl-L-cysteine (50 μM–500 μM) had no effect on basal or fMet-Leu-Phe-stimulated [^{35}S]GTP[S] binding (results not shown). Thus, the farnesyl moiety and the carboxyl group, but not the peptide bond of AFC are

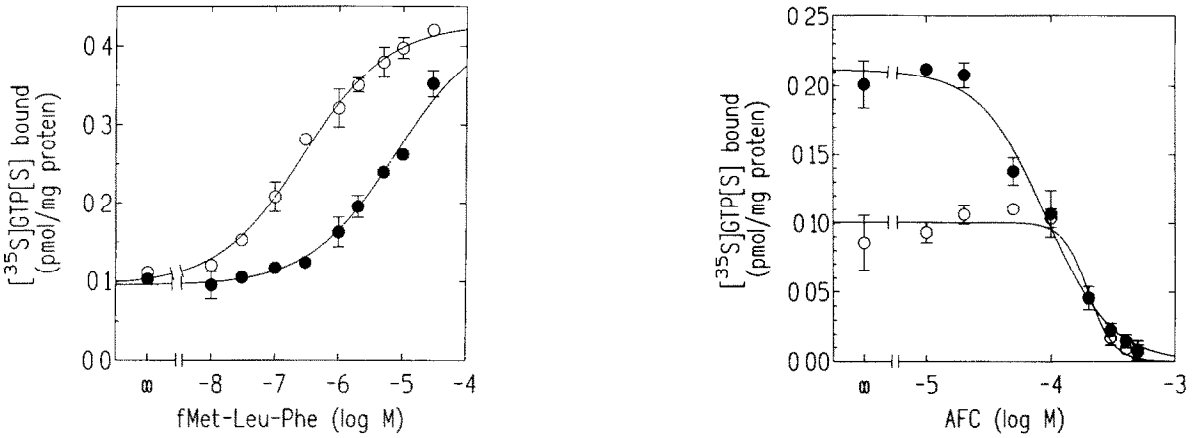

Fig. 1. Effect of AFC on fMet-Leu-Phe-stimulated binding of [^{35}S]GTP[S] to HL-60 cell membranes. (A) HL-60 cell membranes were incubated in the absence (open symbols) or presence (closed symbols) of 100 μM AFC with [^{35}S]GTP[S] and fMet-Leu-Phe at concentrations indicated at the abscissa. The incubation was carried out for 60 min at 30°C. The samples were analyzed for bound [^{35}S]GTP[S] as described in section 2. (B) HL-60 cell membranes were incubated for 60 min in the absence (open symbols) or presence (closed symbols) of 100 nM fMet-Leu-Phe with [^{35}S]GTP[S] and AFC at concentrations indicated at the abscissa.

required for inhibition of receptor/G-protein interaction.

Next, we investigated the effect of AFC on high-affinity GTPase activity of HL-60 membranes. Fig. 3A illustrates that fMet-Leu-Phe led to an about 2-fold stimulation of GTP hydrolysis in the absence of AFC. Half-maximal and maximal stimulation were observed at approximately 2 μM and 10 μM fMet-Leu-Phe, respectively. AFC (100 μM) reduced GTPase activity by about 50% in the absence of fMet-Leu-Phe and by approximately 65% in the presence of a maximally stimulating concentration of the chemotactic agonist (30 μM). Thus, 100 μM AFC caused a reduction by approximately 75% in the net fMet-Leu-Phe-dependent increase in GTP hydrolysis. Fig. 3B shows the inhibition of basal and fMet-Leu-Phe-stimulated GTPase activity by increasing concentrations of AFC. Both basal and fMet-Leu-Phe-stimulated GTP hydrolysis were reduced by 50% at approximately 100 μM AFC and were fully suppressed when AFC was present at concentrations $\geq$400 μM.

4. DISCUSSION

In the late 1970s, the well established regulatory role of protein carboxyl methylation in bacterial chemotaxis (reviewed in [3]) prompted investigators to determine the importance of this posttranslational modification in regulating the chemotaxis of eukaryocytes. Although early studies of this type suggested a close association of protein carboxyl methylation and leukocyte activation [19,20], subsequent experimentation revealed that some of the effects initially observed were highly variable and that experiments based on the use of general methylation inhibitors were more difficult to interpret than anticipated [21,22]. Interest in the potential role of carboxyl methylation in regulating leukocyte activation revived only recently when it became clear that the γ subunits of signal-transducing G-proteins are methyl esterified at their C-termini [3] and that farnesylcysteine analogues like AFC inhibit both carboxyl methylation of *S*-prenylated proteins and leukocyte chemotaxis [12].

At first glance, inhibition of γ subunit methylation appears to be a likely explanation for the marked inhibitory effects of AFC on basal and formyl peptide receptor-stimulated [^{35}S]GTP[S] binding and [γ-^{32}P]GTP hydrolysis in HL-60 membranes reported here. However, the fact that this inhibition is observed in well washed membrane preparations, which should be devoid of the methyl transferase co-substrate *S*-adenosyl-L-methionine, argues against such a mechanism. Furthermore, the

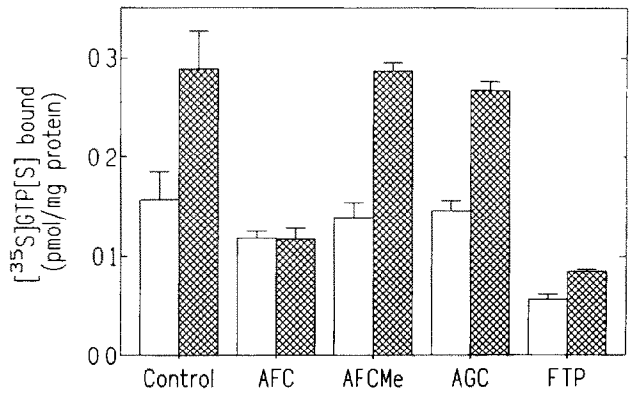

Fig. 2. Effect of AFC-related *S*-prenyl compounds on basal and fMet-Leu-Phe-stimulated [^{35}S]GTP[S] binding to HL-60 cell membranes. HL-60 cell membranes were incubated for 60 min in the absence (open bars) or presence (hatched bars) of 0.1 μM fMet-Leu-Phe with [^{35}S]GTP[S] as described in section 2. The incubation was performed in the absence (Control) or presence of 100 μM of the *S*-prenyl compounds specified at the abscissa.

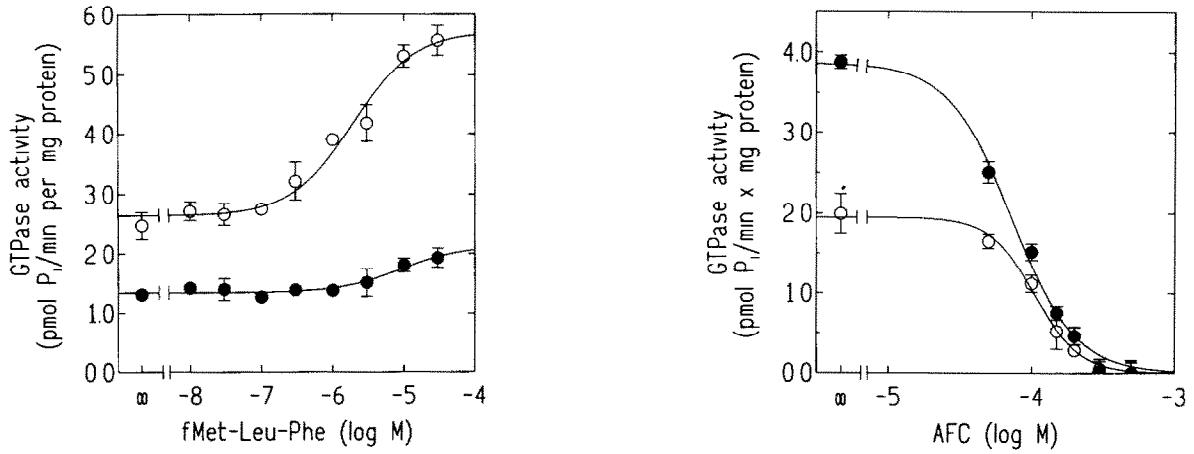

Fig. 3. Effect of AFC on fMet-Leu-Phe-stimulated GTP hydrolysis by HL-60 cell membranes. (A) High-affinity GTPase activity was determined by incubating HL-60 cell membranes for 15 min at 30°C in the absence (open symbols) or presence (closed symbols) of 100 μM AFC with increasing concentrations of fMet-Leu-Phe. See section 2 for experimental details. (B) High-affinity GTPase activity of HL-60 cell membranes was determined in the absence (open symbols) or presence (closed symbols) of 0.1 μM fMet-Leu-Phe at concentrations of AFC indicated at the abscissa.

methyl transferase inhibitor *S*-adenosyl-L-homocysteine [23] (1 μM–100 μM) had no effect on basal and fMet-Leu-Phe-stimulated [^{35}S]GTP[S] binding in this membrane preparation and AFC (100 μM) blocked the effect of fMet-Leu-Phe on [^{35}S]GTP[S] binding without delay (results not shown). Thus, the possibility has to be considered that AFC affects receptor/G-protein coupling by a mechanism distinct from inhibition of γ subunit carboxyl methylation.

Since AFC is an amphiphilic molecule possessing a hydrophobic farnesyl moiety and a hydrophilic carboxyl group, the inhibition of receptor/G-protein coupling by AFC could conceivably be due to a non-specific, detergent-like property of this compound. We do, however, not feel that this is a likely possibility, at least not at concentrations up to 100 μM, since these concentrations of AFC have previously been reported to have no effect on motility, morphology, viability, and the phorbol ester-induced chemotactic response of intact mouse macrophages [12]. Furthermore, the compound did not alter the incorporation of [^{35}S]methionine into cultured transformed fibroblasts and had little effect on the proliferation of these cells [12].

It thus appears likely that AFC interferes the activation of G-proteins by the agonist-activated formyl peptide receptor in a more specific fashion. The fact that AFC inhibits the activation of G-proteins by at least two other G-protein-coupled receptors (Scheer and Gierschik, in preparation) argues against the hypothesis that AFC inhibits the interaction of formyl peptides with their receptors. Of interest, a retinal $\beta\gamma$ subunit lacking the carboxyl terminal isoprenylated cysteine has previously been shown to support the pertussis toxin-mediated ADP-ribosylation of transducin α subunit only poorly [24,25]. These results indicated that the C-terminal γ subunit modification may be essential for the formation of the $\alpha\beta\gamma$ heterotrimer, which is known to be required for receptor-mediated G-protein activation [26]. It is thus conceivable that AFC acts primarily as a competitive inhibitor of the $\alpha \cdot \beta\gamma$ subunit interaction. Our finding that AFC also inhibited high-affinity binding of the chemotactic agonist fMet-Leu-[^{3}H]Phe to HL-60 cell membranes (results not shown) is consistent with this notion, since high affinity agonist binding to G-protein-coupled receptors is known to depend on the interaction of the holomeric G-protein with the receptor [27].

On the other hand, very recent results obtained by analyzing the interaction of rhodopsin kinase and the β-adrenergic receptor kinase with the corresponding receptors are consistent with the notion that these receptors contain a 'docking site' for C-terminally isoprenylated proteins [28,29]. These findings raise the intriguing possibility that AFC interferes with the receptor/G-protein interaction by specifically blocking the docking of G-protein $\beta\gamma$ subunits to receptors. At first glance, our finding that AFC also inhibited mastoparan-stimulated [^{35}S]GTP[S] binding to HL-60 membranes (results not shown), appears to argue against such a mechanism, since mastoparan is believed to activate heterotrimeric G-proteins by directly interacting with the carboxyl-terminus of their α subunits [30], which are not isoprenylated [31,32]. However, recent evidence suggests that mastoparan also interacts with the low molecular mass GTP-binding proteins *rho/rac* [33]. Very interestingly, isoprenylation is a prerequisite for this interaction [34], suggesting that the isoprenylated carboxyl-termini of low molecular mass GTP-binding proteins – and possibly of G-protein γ subunits – may interact with mastoparan as well.

In any case, experiments examining the interaction of purified receptors and purified G-protein subunits in a

reconstituted system will be required to decide whether AFC interacts with receptors, G-proteins or both. Notwithstanding the outcome of these studies, we speculate that AFC might not only turn out to be a novel type of inhibitor of the receptor/G-protein or G-protein subunit interaction, but might also represent the prototype of a new class of drugs acting at these early steps of transmembrane signalling.

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