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Activation and Inhibition of G Proteins by Lipoamines

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ABSTRACT

We have previously shown that alkyl-substituted amino acid derivatives directly activate $G_{i/o}$ proteins. *N*-Dodecyl-N^{α},N^{ϵ}-(bis-*l*-lysinyl)-*l*-lysine amide (FUB132) is a new representative of this class of compounds with increased efficacy. Here, we characterized the molecular mechanism of action of this class of compounds. FUB132 and its predecessor FUB86 were selective receptomimetics for $G_{i/o}$ because they stimulated the guanine nucleotide exchange reaction of purified $G_{i/o}$ as documented by an increased rate of GDP release, GTPγS binding, and GTP hydrolysis. In contrast to the receptomimetic peptide mastoparan, stimulation of G proteins by lipoamines required the presence of neither Gβγ-dimers nor lipids. On the contrary, Gβγ-dimers suppressed the stimulatory effect of FUB132. The stimulation of $G_{i/o}$ by lipoamines and by mastoparan was not additive. A peptide derived from the C terminus of $G\alpha_{o3}$, but not a corresponding $G\alpha_q$-derived peptide, quenched the FUB132-induced activation of $G\alpha_o$. In membranes prepared from human embryonic kidney 293 cells that stably expressed the $G_{i/o}$-coupled human A_1-adenosine receptor, lipoamines impeded high-affinity agonist binding. In contrast, antagonist binding was not affected. We conclude that alkyl-substituted amines target a site, most likely at the C terminus of $G\alpha_{i/o}$-subunits, that is also contacted by receptors. However, because Gβγ-dimers blunt rather than enhance their efficacy, their mechanism of action differs fundamentally from that of a receptor. Thus, despite their receptomimetic effect in vitro, alkyl-substituted amines and related polyamines are poor direct G protein activators in vivo. In the presence of Gβγ, they rather antagonize G protein-coupled receptor signaling.

Heterotrimeric G proteins play a pivotal role in the communication of a given cell with the environment. They are stimulated by cell surface receptors, members of the superfamily of heptahelical receptors [G protein-coupled receptors (GPCR)] that catalyze the exchange of Gα-bound GDP for GTP (Hamm, 1998; Freissmuth et al., 1999). Consequently, the GTP-bound G protein dissociates into two signaling entities: the Gα-subunit and the Gβγ-subunit complex. With the notable exception of $G\beta_5$ CA (A. Babich, A. Shimanets, U. Maier, A. Schulz, I. Stephan, D. Illenberger, K. Spicher, B. Nürnberg, submitted), β- and γ-subunits form tight complexes that cannot be dissociated under nondenaturing conditions. Hence, in vivo, Gβγ-dimers are thought to remain permanently associated and to act as a functional monomer. Gα and Gβγ both modulate cellular effectors. Inactivation occurs by the intrinsic GTPase of Gα; GDP-bound Gα reassociates with Gβγ and this causes mutual inactivation because the effector binding surfaces are inaccessible in the oligomer.

In this cycle of activation and deactivation, specific binding sites on G proteins allow for the sequential, conformation-dependent binding of protein reaction partners. These include receptors that interact with all three subunits (Kisselev et al., 1999), effectors, and regulators of G protein signaling, which bind to the transition state of Gα·GTP and exert a GTPase activating effect. The increased GTP turnover not only accelerates the rate of signal deactivation but also enhances the rate of activation (Berman and Gilman, 1998; Wieland and Chen, 1999). In addition, several modulators have been identified that activate G protein signaling in the absence of a GPCR and that cannot be readily placed into this basal reaction cycle. These include activators of G protein signaling, such as AGS2 (Cismowski et al., 1999; Takesono et al., 1999), PCP2 (Luo and Denker, 1999), and AGS3 (Takesono et al., 1999), which interact with Gα, thereby presumably acting as either a guanine exchange factor or a guanine

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ABBREVIATIONS: GPCR, G-protein-coupled receptor; FUB 132, *N*-dodecyl-N^{α},N^{ϵ}-(bis-*l*-lysinyl)-*l*-lysine amide; GTPγS, guanosine-5′-(3-*O*-thio)triphosphate; HPIA, [(−)N^6-I^3(iodo-4-hydroxyphenyl-isopropyl)adenosine]; DTT, dithiothreitol; HEK, human embryonic kidney; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; dbt-cAMP, N^6,2′-*O*-dibutyryl-adenosine-3′,5′-monophosphate; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PT, pertussis toxin, an exotoxin from *Bordetella pertussis*.

nucleotide dissociation inhibitor, as well as phosducin and phosducin-like molecules that target G$\beta\gamma$ (Lohse et al., 1996).

Thus, several specific binding sites exist on the Gα subunit that may be exploited for the design of synthetic stimulatory or inhibitory compounds. In both experimental pharmacology and clinical pharmacotherapy, G protein-dependent signaling pathways are activated or inhibited by employing appropriate receptor agonists or antagonists, respectively. Several arguments indicate that G proteins can per se also be targeted by drugs and that this approach may, at least conceptually, offer advantages (Freissmuth et al., 1999; Höller et al., 1999). Numerous low-molecular weight compounds have been identified that bind directly to G proteins (Mousli et al., 1990; Nürnberg et al., 1999). Mechanistic aspects have been studied in detail for the receptomimetic peptide mastoparan (Higashijima et al., 1988), receptor-derived peptides (Taylor and Neubig, 1994), and suramin analogs, which act as subtype-selective G protein antagonists (Beindl et al., 1996; Freissmuth et al., 1996; Hohenegger et al., 1998). Previously, we have studied the structure-activity relation of alkyl-substituted amino acid amides and analogs to identify synthetic direct G protein activators that were not peptides (Leschke et al., 1997). By using the lead structure, we identified a more efficacious compound, *N*-dodecyl-N^{α},N^{ϵ}-(bis-*l*-lysinyl)-*l*-lysine amide (FUB 132) in the present study and we explored the mechanism of action of these lipoamines. Our observations show that these compounds activate G protein α-subunits because they promote GDP release. However, mechanistically, because they are blunted by G$\beta\gamma$-dimers, their action differs fundamentally from that of receptors.

Experimental Procedures

Materials. Peptides used (mastoparan, INLKA LAALA KKIL; C-termini of $G\alpha_{o3}$, CDIII ADNLR GCGLY, and $G\alpha_q$, CLQLN LKEYN LV) were supplied by Peter Henklein (Humboldt-Universität zu Berlin). [α-^{32}P]GTP, [γ-^{32}P]GTP, [^{35}S]GTPγS and ^{125}I were purchased from PerkinElmer Life Sciences (Zaventem, Belgium). ^{125}I-HPIA was synthesized according to Linden (1984). *l*-α-Phosphatidylcholine (Type IV-S, purified from soy bean), was from Sigma-Aldrich Chemie Gmbh (Munich, Germany). The synthesis of FUB86 has been described elsewhere (Leschke et al., 1997). In brief, the synthesis of FUB132 was as follows: dodecylamine and *Z*-Lys(*Z*)-OH were dissolved in dimethylformamide. Diisopropylethylamine and 2-(1-*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate were added and the solution was stirred overnight. Thereafter the solution was mixed with excess water; the precipitated product was trapped on a filter and dried. The benzyl-oxycarbonyl (*Z*)-groups were cleaved by hydrobromic acid in acetic acid and the product was precipitated with ether. The product was again dissolved in dimethylformamide; subsequently, *Z*-Lys(*Z*)-OH, diisopropylethylamine, and 2-(1-*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate were added and again the solution was stirred overnight. Upon mixing with excess water, a precipitate formed. The precipitated product was trapped on a filter and dried. The Z-groups were cleaved by hydrobromic acid in acetic acid and the product was precipitated with ether. Re-crystallization in ethanol/ether gave the pure product. A more detailed description of synthesis of FUB132 will be reported elsewhere (R. Storm, E. Breitweg-Lehmann, O. Kudlacek, K. Schimmelpfennig, M. Freissmuth, B. Nürnberg, W. Schunack, in preparation). All other reagents were of highest purity available.

Preparation of Native and Recombinant G Proteins. Heterotrimeric G proteins were purified from bovine brain membranes using cholate as the detergent and conventional columns as the chromatographic support (Nürnberg et al., 1994). Separation of Gα from G$\beta\gamma$ was achieved using Mono Q fast-performance liquid chromatography columns (Amersham Biosciences, Freiburg, Germany) in the presence of aluminum fluoride (Exner et al., 1999). G protein isoforms were identified by their immunoreactivity to specific antisera. Protein preparations were stored at −70°C and aluminum fluoride was removed from buffer before use. Recombinant Gα subunits were expressed in *Escherichia coli* and purified from bacterial lysates ($r\mathrm{G}\alpha_{ss}$ as in Freissmuth and Gilman, 1989; $r\mathrm{G}\alpha_{i1}$ and $r\mathrm{G}\alpha_{o}$ as in Mumby and Linder, 1994).

GTPase Activity of Purified G Proteins. GTPase activity of purified G proteins was determined basically as described previously (Leschke et al., 1997). In brief, assays were conducted in a final volume of 100 μl containing 0.15 to 0.4 pmol of G proteins reconstituted in phospholipid vesicles (see below) and a reaction mixture containing 2 mM $MgCl_2$, 0.1 mM ATP, 100 nM GTP, 20 mM NaCl, 1 mg/ml of creatine kinase, 5 mM creatine phosphate, 0.1 mM EGTA, 40 mM tetraethyl ammonium, and 10 mM HEPES, pH 7.4, in the absence or presence of G protein modulators. ATP, creatine kinase, creatine phosphate, and EGTA were included to allow a direct comparison between GTPase assays in cell membranes and purified G proteins (Leschke et al., 1997). After incubation for 3 min at 25°C for $G_{i/o}$ and G_o proteins and at 30°C for G_i proteins, the reaction was started by addition of [γ-^{32}P]GTP (10–15 cpm/fmol, final concentration) and continued for 15 min ($G_{i/o}$ and G_o proteins) or 30 min (G_i proteins). Thereafter, the reaction was stopped with 900 μl of ice-cold charcoal solution (5%) in 50 mM sodium phosphate buffer, pH 2.0. Samples were centrifuged for 50 min at 12,000*g*. An aliquot of the supernatant (600 μl) was withdrawn and analyzed by liquid scintillation counting.

GTPγS Binding to Purified G Proteins. Binding of [^{35}S]GTPγS to purified G proteins was determined as described previously (Leschke et al., 1997) with minor modifications. Heterotrimeric G proteins (0.15–0.4 pmol/tube) were reconstituted into phospholipid vesicles (see below) and incubated in a final volume of 100 μl containing 2 mM $MgCl_2$, 0.1 mM ATP, 20 mM NaCl, 1 mg/ml of creatine kinase, 5 mM creatine phosphate, 0.1 mM EGTA, 40 mM tetraethylammonium, and 10 mM HEPES, pH 7.4, with or without G protein modulators. These additions were included to allow for a direct comparison between experiments done in HL-60 membranes (GTPase assays require the presence of ATP and an ATP regenerating system to prevent cleavage of GTP by ectonucleotidases). The presence or absence of these components did not have any appreciable effect on the activation by FUB132 and was therefore omitted in assays done in the absence of phospholipids. The reaction was started by the addition of [^{35}S]GTPγS (20–100 nM, 50–300 cpm/fmol) and stopped after 30 s with 1 ml of ice-cold wash buffer. Gα isoforms (20–40 nM) were incubated in a final volume of 25 to 50 μl of buffer A consisting of 0.01% Lubrol, 1 mM DTT, 1 mM EDTA, and 50 mM HEPES, pH 7.4, containing 2 mM $MgCl_2$ in the presence or absence of phospholipids and of G protein modulators; in some instances, the combination of 0.45 mM $MgSO_4$ and of 100 mM NaCl was employed instead of 2 mM $MgCl_2$. The reaction was started with [^{35}S]GTPγS (100 to 200 nM, 20–60 cpm/fmol) at temperatures indicated and stopped after the times indicated with 1 ml of ice-cold wash buffer.

Release of GDP from Gα. Release of bound GDP from $G\alpha_o$ proteins was measured as described previously (Freissmuth et al., 1996). In brief, purified $G\alpha_o$ subunits (1–2 pmol) were prelabeled in the presence of 1 μM [α-^{32}P]GTP (23 cpm/fmol) in buffer A containing 10 mM $MgSO_4$ for 15 min at 20°C. The catalytic rate of GTP hydrolysis exceeds the rate of GDP release by a factor of >10; therefore, the nucleotide bound at equilibrium is [α-^{32}P]GDP. Dissociation was subsequently initiated by the addition of 100 μM unlabeled GTP in the presence or absence of FUB132. At the indicated times, the reaction was quenched by the addition of a buffer consisting of 20 mM $MgCl_2$, 10 mM NaF, 20 μM $AlCl_3$, 100 mM NaCl, and 10 mM Tris/HCl, pH 8.0.

Preparation of Lipid Vesicles. One hundred milligrams of *l*-α-phosphatidylcholine (*l*-α-Lecithin, type IV-S, from soybean, 20% or *l*-α-Lecithin, type IV-S, from soybean, purified 40%; Sigma, Deissenhofen, Germany) and 1 g of sodium cholate were solubilized in 10 ml of water. At 4°C, 60 μl of this solution was mixed with 60 to 80 pmol of heterotrimeric $G_{i/o}$ proteins to a final volume of 600 μl in a buffer consisting of 100 mM NaCl, 1 mM DTT, 1 mM EDTA, and 20 mM HEPES, pH 8.0, and kept on ice for 1 h. Vesicles carrying $G_{i/o}$ proteins were generated by passing the solution through a Sephadex G50 gel filtration column. Subsequently pooled fractions were used. To reconstitute heterotrimeric G_i or G_o proteins (Fig. 4), 60 to 80 pmol of Gβγ and 30 to 40 pmol of Gα were mixed with *l*-α-phosphatidylcholine and sodium cholate. In contrast, monomeric Gα subunits were added to preformed lipid vesicles prepared as described above.

Radioligand Binding Experiments. Equilibrium binding with the agonist [^{125}I]HPIA and the antagonist [^{3}H]DPCPX to A_1-adenosine receptor heterologously expressed in HEK 293 cell membranes were carried out as described previously (Waldhoer et al., 1998). In brief, for ^{125}I-HPIA binding, the reaction was carried out in a final volume of 40 μl containing 50 mM HEPES, pH 7.4, 1 mM EDTA, 2 mM $MgCl_2$, 8 μg/ml of adenosine deamidase, 8 to 10 μg of membrane protein, 1 nM radioligand, and the indicated concentrations of FUB86 and FUB132. After 90 min at 25°C, the reaction was terminated by filtration over glass fiber filters using a cell harvester (Skatron, Lier, Norway). The binding reaction with the antagonist [^{3}H]DPCPX was carried out analogously in a final volume of 80 μl containing 16 to 20 μg of membrane protein and 2 nM radioligand. Nonspecific binding was determined in the presence of 1 μM N^6-cyclopentyladenosine and amounted to less than 10% for both, specific binding of [^{125}I]HPIA and [^{3}H]DPCPX. This amounted to 2.7 to 3.2 fmol of [^{125}I]HPIA bound and to 15 to 18 fmol of [^{3}H]DPCPX bound. Data are means from three to four independent experiments carried out on different membrane preparations.

Measurement of Intracellular Calcium AND O_2^- Formation in HL-60 Cells. Determination of cytoplasmic Ca^{2+} concentrations ($[Ca^{2+}]_i$) in dbt-cAMP-differentiated HL-60 cells was performed as described previously using fura-2 as the dye (Leopoldt et al., 1997). O_2^- formation was basically analyzed as described by Seifert et al., 1994. In brief, dbt-cAMP–differentiated HL-60 cells (5×10^6 cells) were incubated for 3 min at 37°C in the presence of cytochrome *c* and cytochalasin B before the addition of fMLP at indicated concentrations.

Data Presentation. Averaged data are given as mean values ± standard deviation (S.D.) if not stated otherwise. Statistical significance was tested with Student's *t* test for paired or unpaired data.

Results

Stimulation of Purified $G_{i/o}$ Proteins by Synthetic Low Molecular Compounds. Almost 200 amphiphilic compounds were screened for their ability to stimulate the GTPase activity of $G_{i/o}$ proteins in heterotrimeric form. From this collection of compounds, we selected the lipoamine FUB132 (Fig. 1) for further analysis because it was more efficacious than its predecessor FUB 86 (Leschke et al., 1997). A direct comparison of these two compounds is shown in Fig. 2; FUB132 caused a pronounced stimulation of GTPγS binding to $rG\alpha_{i1}$ (Fig. 2A) and $rG\alpha_o$ (Fig. 2B). In contrast, both FUB86 and FUB132 stimulated binding of GTPγS to $rG\alpha_{ss}$ only modestly (by about 2-fold at most) at the highest concentration employed (Fig. 2C). Because it is not trivial to obtain $G\alpha_s$ in highly purified form from mammalian tissues, our comparison in Fig. 2 was based on employing recombinant G protein α-subunits that had been purified from bacterial lysates. FUB86 and FUB132 also stimulated G protein α-subunits purified from native sources (see below). Transducin, another member of G_i subfamily specifically expressed in the retina, was activated only weakly by FUB86 (association rate of GTPγS binding $k_{on,\ GTP\gamma S} = 0.007\ min^{-1}$ in the presence of 1 mM FUB86; EC_{50}, 155 μM; see also Nürnberg et al., 1999) and mastoparan (Ross and Higashijima, 1994). FUB132 had no effect on stimulation of GTPγS binding to transducin in concentrations below 1 mM (data not shown).

The binding reaction in Fig. 2 was carried out under non-equilibrium conditions; the incubation times were 3 min for $rG\alpha_{i1}$ and $rG\alpha_{ss}$ and 1 min for $rG\alpha_o$. Under the conditions employed (2 mM $MgCl_2 \approx 1$ mM free Mg^{2+}, 20°C), the corresponding half-lives of the association binding reaction were ~40, 12, and 3 min for $rG\alpha_{i1}$, $rG\alpha_{ss}$, and $rG\alpha_{o1}$, respectively, in the absence of any compound. Thus, because the incubation period was substantially shorter than the half-life of the reaction, basal binding was quasi-linear with time and any increase in binding was assumed to reflect a stimulation of the exchange of prebound GDP for GTPγS. This was directly verified by employing $G\alpha_o$ (purified from bovine brain); a representative experiment is shown in Fig. 3. As expected, under basal conditions, the time course of GDP-release and of GTPγS-binding were essentially identical (Fig. 3A, squares) with kinetic parameters of the exchange rates ($k_{off,\ GDP}$ and $k_{on,\ GTP\gamma S}$) in the range of 0.26 min^{-1}. More importantly, FUB132 accelerated to a similar extent both the release of GDP and the association of GTPγS. For technical reasons, it is difficult to obtain a precise estimate of the reaction rate, because more than 50% of the GDP and of the GTPγS was released and bound, respectively, at the earliest time point (30 s). However, from the available data, we estimated exchange rates ($k_{off,\ GDP}$ and $k_{on,\ GTP\gamma S}$) in the range of 2.8 min^{-1}, indicating that the reaction rates were enhanced by at least 10-fold. Thus, the stimulation by FUB132 of GTPγS binding to $G\alpha_o$ was fully accounted for by the enhancement of GDP release. This was also true for FUB86 (Fig. 3A, triangles; exchange rate estimated at ~ 1.4 min^{-1}). Accordingly, when done in parallel, both compounds stimulated GDP-release from and promoted GTPγS-binding to $G\alpha_o$ with reasonably similar concentration-response curves (Fig. 3, B and C).

Comparison of the Stimulatory Effect of Lipoamines to the Action of Mastoparan. The wasp venom mastoparan is the prototypical direct G protein activator and serves as a useful reference (Freissmuth et al., 1999; Höller et al., 1999; Nürnberg et al., 1999). G protein activation by mastoparan (and related peptides) requires the presence of lipids (Higashijima et al., 1990). In contrast, a comparison of the

Mastoparan H_2N-INLKALAALAKKIL-$CONH_2$

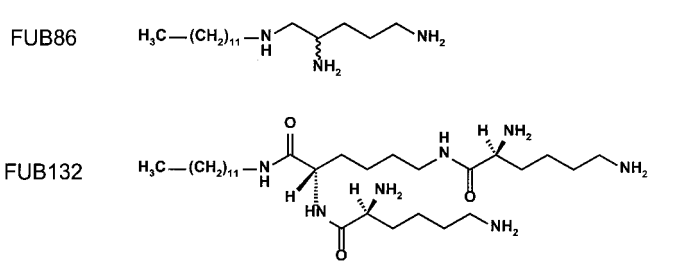

Fig. 1. Structure of mastoparan, FUB86, and FUB132. The synthesis of FUB132 is outlined under *Experimental Procedures*.

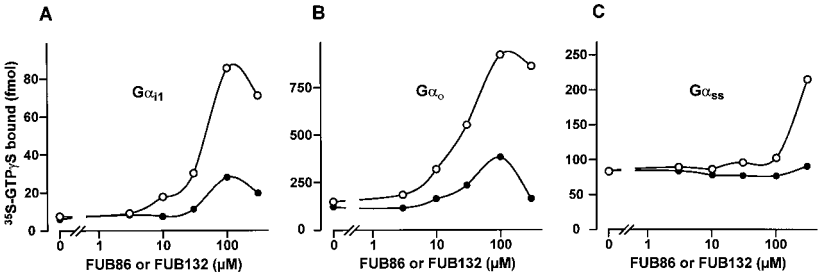

Fig. 2. Stimulation of [^{35}S]GTPγS binding to G protein α-subunits by FUB132 and FUB86. Purified *r*Gα$_{i1}$ (A; 30 ng/assay), *r*Gα$_o$ (B; 80 ng/assay), and *r*Gα$_{ss}$ (C; 90 ng/assay) were incubated for 1 (B) and 3 min (A and C) in buffer (final volume, 50 μl) containing 50 mM HEPES·NaOH, pH 7.4, 1 mM DTT, 1 mM EDTA, 2 mM $MgCl_2$, 0.01% Lubrol, 0.1 μM [^{35}S]GTPγS, and the indicated concentrations of FUB86 (●) and of FUB132 (○). Bound and free ligand were separated by filtration over nitrocellulose filters. Data are means from duplicate determinations in a representative experiment, which was repeated twice with similar results.

data summarized in Figs. 2 (absence of lipid) and 3 did not suggest that the stimulatory effect of FUB 132 depended—to a major extent—on the presence of lipids. However, the two preparations (i.e., *r*Gα$_o$ and bovine brain Gα$_o$) are not strictly comparable because purified bovine brain preparations contain a mixture of several isoforms of Gα$_o$ (Exner et al., 1999). We have therefore directly assessed the effect of lipids by carrying out the determination with purified bovine brain Gα$_o$ in parallel; it is evident from Fig. 4, A and B, that the addition of lipid vesicles had no appreciable effect on the concentration-response curve of FUB132 or of FUB86.

Mastoparan requires not only lipids for efficient G protein activation but also the presence of G protein βγ-dimers. To compare the mechanism of action of FUB lipoamines to that of mastoparan, we have therefore reconstituted purified bovine brain G$_{i/o}$ in oligomeric form into lipid vesicles. For technical reasons (i.e., because of the higher sample throughput), we analyzed the stimulation of the intrinsic GTPase rate; this rate of hydrolysis is limited by the rate of GDP-release, k_{off}, and by the intrinsic rate of cleavage, k_{cat}, under basal and maximally stimulated conditions, respectively. Both basal and FUB132- and mastoparan-stimulated GTPase rates were linear up to 30 min (Fig. 4C) and the same was true for FUB 86 (not shown); the extent of stimulation achieved by a saturating concentration of FUB132 approached that induced by mastoparan. This is also evident from the concentration-response curves shown for each individual compound in Fig. 5A; mastoparan and FUB132 stimulated the GTPase activity with EC_{50} values of 11 μM and 17 μM, respectively. When one compound was employed at a half-maximally effective concentration and increasing amounts of the other activator were added, the maximum effect was similar regardless of which of the combination partners was present at the fixed concentration (Fig. 5B). Furthermore, the maximum effects observed with the combinations did not exceed those observed after stimulation with addition of single compounds (Fig. 5, A and B). Finally, if a maximally effective concentration of mastoparan was present in the reaction mixture, no further increase in GTPase activity was induced upon addition of FUB132 (Fig. 5C) or of FUB86 (Fig. 5D) and vice versa.

A C-Terminal Site of Action and Blockade of Receptor/G Protein Coupling. Taken together the data in Fig. 5 are consistent with the interpretation that mastoparan and the lipoamines FUB132 and FUB86 act at a common site. Mastoparan contacts the carboxy (C) terminus of G protein α-subunits for G-protein activation; however, C- and amino (N) terminus are in close vicinity; accordingly, appropriate mastoparan analogs are readily cross-linked to the N-terminal end of G protein α-subunits (Higashijima and Ross, 1991; Tanaka et al., 1998). Similar to the action of mastoparan, activation of G proteins by FUB lipoamines was sensitive to pertussis toxin (PT). We have therefore tested if the C terminus is important for binding of FUB132 to Gα$_o$ by employing a peptide that comprised the last 14 amino acids of Gα$_{o3}$. Addition of this peptide (100 μM) to the reaction mixture

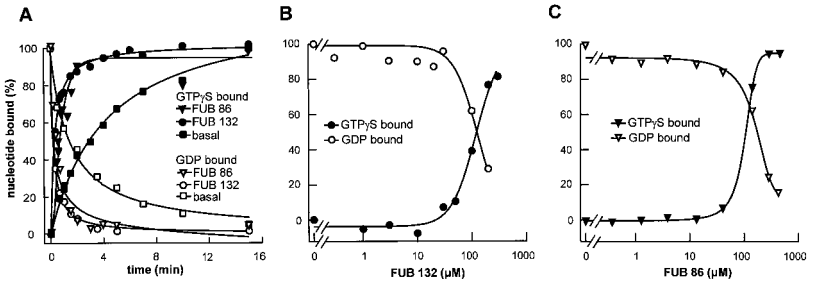

Fig. 3. Time- and concentration-dependent stimulation. FUB lipoamines of GDP-release from and GTPγS binding to Gα$_o$. Gα$_o$ subunits (20 nM) purified from bovine brain membranes were incubated in a final volume of 50 μl of buffer A containing phospholipid vesicles, 0.45 mM $MgSO_4$, 100 mM NaCl at 20°C. A. [α-^{32}P]GDP release from and [^{35}S]GTPγS binding (25 cpm/fmol; 0.1 μM) to Gα$_o$ in the absence (squares) or presence (circles) of 200 μM FUB132 or 200 μM FUB86 (triangles). The reaction was stopped at time points indicated. Maximal binding of [α-^{32}P]GTP and of [^{35}S]GTPγS to Gα$_o$ was 1.13 and 1.17 pmol, respectively. The k_{off} and k_{on} rates were calculated to be 0.26 and 2.8 min^{-1} for basal and FUB132-induced nucleotide exchange, respectively. B and C, determination of [α-^{32}P]GDP release from and [^{35}S]GTPγS (27 cpm/fmol) binding to Gα$_o$ induced by increasing concentrations of FUB132 (B, circles) and of FUB 86 (C, triangles) at 20°C for 1 min. To load Gα$_o$ subunits with [α-^{32}P]GDP, 1 pmol per sample was prelabeled in the presence of 1 μM [α-^{32}P]GTP (23 cpm/fmol) in a buffer A containing 10 mM $MgSO_4$ for 15 min at 20°C [α-^{32}P]GDP release was initiated (time = 0) by the addition of 100 μM unlabeled GTP. Maximal binding of [α-^{32}P]GTP and of [^{35}S]GTPγS to Gα$_o$ was 0.70 pmol and 0.62 pmol. The experiments shown are representative of two (GDP release) or three (GTPγS binding) independent experiments with duplicate determinations.

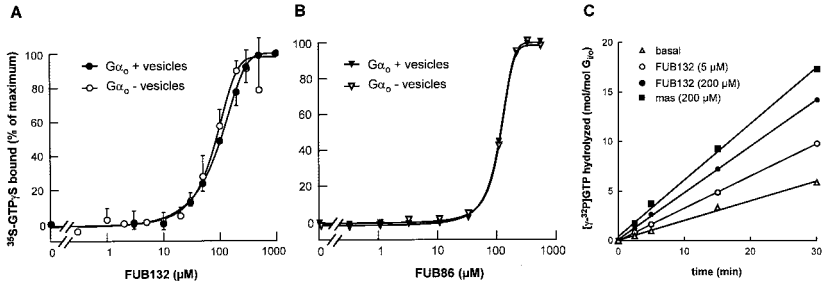

Fig. 4. A, FUB lipoamine-induced GTPγS binding to purified bovine brain $G\alpha_o$ in the presence or absence of phospholipid vesicles. $G\alpha_o$ subunits (1–1.5 pmol/tube) were incubated with increasing concentrations of FUB132 (A) or FUB86 (B) in the presence (■) or absence (□) of phospholipid vesicles in a final volume of 2 μl of buffer A containing 0.45 mM $MgSO_4$ and 30 mM NaCl at 21°C. The reaction was started with [^{35}S]GTPγS (300 nM, 23–30 cpm/fmol) and stopped after 20 s with 1 ml of ice-cold wash buffer. Data are mean values ± S.D. of four independent experiments (A) or one representative experiment (B), each carried out in duplicate. To normalize the different amounts of proteins used in individual experiments, binding in the presence of 1 mM FUB132 (540–800 fmol of [^{35}S]GTPγS/pmol $G\alpha_o$) was set 100%, basal binding was 112 to 200 fmol of [^{35}S]GTPγS/pmol $G\alpha_o$. C, linear rates of GTP hydrolysis of $G_{i/o}$ proteins induced by FUB132 and mastoparan for different incubation times. Purified $G_{i/o}$ proteins (0.2–0.3 pmol/sample) were reconstituted in phospholipid vesicles and incubated with FUB132 (5 μM and 200 μM) or mastoparan (200 μM) compared with basal GTP hydrolysis of $G_{i/o}$ proteins for the times indicated. Shown are means of triplicate determinations each.

resulted in a significant shift to higher FUB132 concentrations to stimulate GTPγS-binding to Gα (Fig. 6A, circles). In contrast, a peptide representing the C-terminal 12 amino acids derived from the PT-insensitive $G\alpha_q$ did not change the potency of FUB132 to stimulate GTPγS-binding to $G\alpha_o$ (Fig. 6A, triangles). It should be noted that both peptides reduced the binding of GTPγS to $G\alpha_o$ in the absence and in the presence of FUB 132. However, the fold-stimulation of GTPγS binding to Gα at maximum effective concentrations of FUB132 was similar in all cases (Fig. 6A, inset). These data suggest that the C terminus is involved in the interaction between FUB132 and Gα.

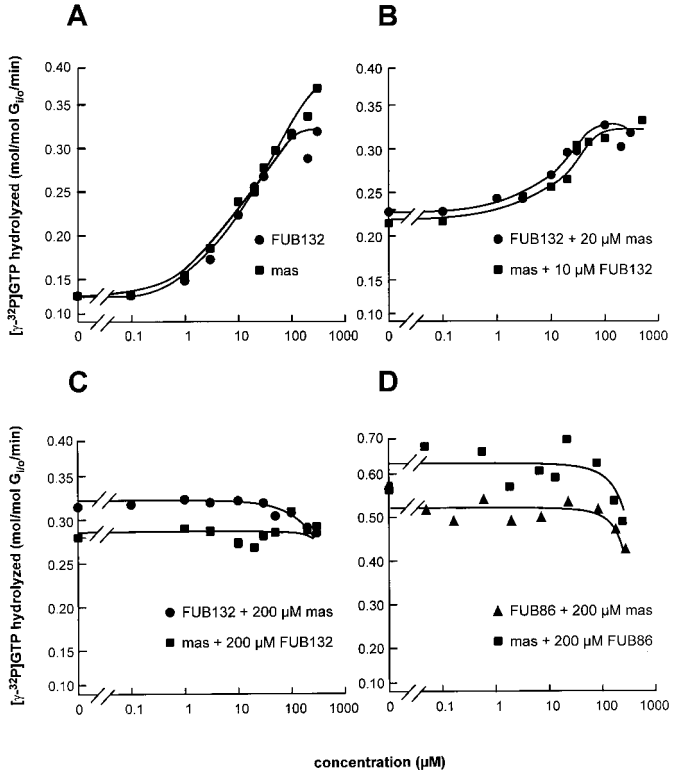

Fig. 5. Stimulation of GTP hydrolysis of $G_{i/o}$ proteins by coincubation of FUB lipoamines and mastoparan. Purified $G_{i/o}$ proteins (0.2–0.3 pmol/sample) were reconstituted in phospholipid vesicles and incubated with FUB 132 and mastoparan (A-C) or FUB86 and mastoparan (D). Increasing concentrations of FUB lipoamines were incubated in the absence (A) or presence of half-maximal (B, 20 μM), or maximal (C and D, 200 μM) stimulating concentrations of mastoparan. Vice versa, increasing concentrations of mastoparan were incubated in the absence (A) or presence of half-maximal (B, 10 μM FUB132), or maximal (C and D, 200 μM) stimulating concentrations of FUB lipoamines. In D, a different preparation of $G_{o/i}$ was used which had a higher basal GTPase (basal molar turnover = 0.2 min^{-1}). Data are representative of three independent experiments with triplicate determinations each.

If FUB132 and FUB86 targeted the very C terminus of Gα, the compounds should also compete with GPCRs for interaction with Gα (Freissmuth et al., 1999). To assess this possibility, we studied the impact of the synthetic G-protein activators on the binding of agonists to receptors (Beindl et al., 1996). High-affinity binding of agonists depends on the formation of the ternary complex HRG between agonist (H), receptor (R), and G protein (G). Accordingly, compounds that interfere with the coupling of a given receptor to its cognate G protein(s) are expected to suppress agonist binding (Waldhoer et al., 1998; 1999; Roka et al., 1999). The human A_1-adenosine receptor, a prototypical $G_{i/o}$-coupled receptor (Jockers et al., 1994) was stably expressed in HEK 293 cells, and membranes thereof were incubated with the agonist radioligand [^{125}I]HPIA in the absence or presence of increasing concentrations of FUB132 or FUB86 (Fig. 6B, closed symbols). Both compounds inhibited equilibrium binding of the agonist radioligand in a concentration-dependent manner (EC_{50}, 7–15 μM). In contrast, FUB132 and FUB86 did not block binding of the radiolabeled A_1-adenosine receptor antagonist [^{3}H]DPCPX (Fig. 6B, open symbols).

Effect of Gβγ on the Stimulation of Gα by FUB 132. The data summarized in Figs. 2 and 3 indicates that FUB 132 accelerated the guanine nucleotide exchange rate Gα by up to >10-fold. However, the GTPase rate was stimulated to a substantially lesser extent (by about 3-fold; see Figs. 4B and 5). Two explanations can be invoked to reconcile this discrepancy: 1) FUB lipoamines accelerate nucleotide release but inhibit k_{cat} of the hydrolytic step. This seems unlikely because this would require FUB lipoamines to bind to a second site on the G protein α-subunit; the residues involved in catalysis are not near the C terminus. 2) Alternatively, this discrepancy is accounted for by the presence of Gβγ-dimers in the GTPase assays shown in Fig. 4B and 5. This was confirmed by determining the effect of Gβγ on the ability of FUB lipoamines to accelerate the rate of GTPγS-binding to $G\alpha_o$ in the absence of lipids (Fig. 7). Because Gβγ reduces the

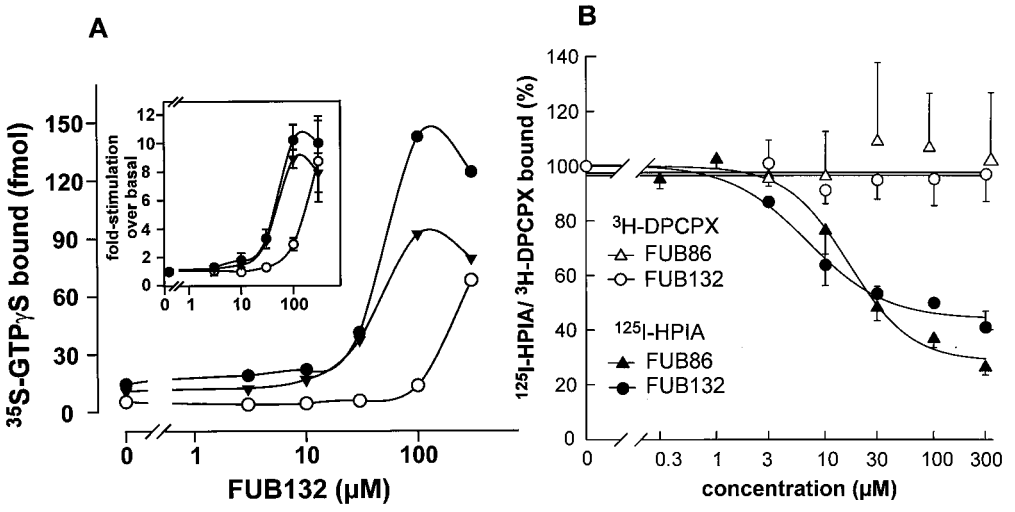

Fig. 6. A. Inhibition of FUB132-induced GTPγS binding to $r\text{G}\alpha_{i1}$ by peptides derived from C-terminal regions of Gα subunits. Purified $r\text{G}\alpha_{i1}$ (30 ng/assay) was incubated for 3 min in the absence (●) and presence of a peptide comprising the last 14 amino acids of $\text{G}\alpha_{o3}$ (100 μM; ○) or of a peptide comprising the carboxy terminus of $\text{G}\alpha_{q}$ (100 μM; ▲). Assay conditions were as outlined for Fig. 2A (i.e., the buffer contained 2 mM $MgCl_2$ and 0.1 μM [^{35}S]GTPγS). Data are means of duplicate determinations in a single experiment. In the inset, data have been normalized for fold-stimulation by setting the binding seen in the absence of FUB132 1.0; data are means from three independent experiments carried out in duplicate. Error bars indicate S.D. B, inhibition of agonist but not antagonist radioligand binding to the human A_1-adenosine receptor heterologously expressed in HEK 293 cells by FUB86 and FUB132. Inhibition of agonist radioligand binding, but not antagonist binding to HEK 293 cell membranes containing recombinant human A_1-adenosine receptor by FUB86 and FUB132. For [^{125}I]HPIA binding, the reaction was carried out in a final volume of 40 μl containing 50 mM HEPES, pH 7.4, 1 mM EDTA, 2 mM $MgCl_2$, 8 μg/ml of adenosine deaminase, 8 to 10 μg of membrane protein, 1 nM radioligand, and FUB86 or FUB132 at the indicated concentrations. After 90 min at 25°C, the reaction was terminated by filtration over glass fiber filters using a cell harvester (Skatron, Lier, Norway). The binding reaction with the antagonist [^{3}H]DPCPX was carried out in an analogous manner in a final volume of 80 μl containing 16 to 20 μg of membrane protein, 2 nM radioligand, and the indicated concentrations of FUB86 or FUB132. Nonspecific binding was determined in the presence of 1 μM N^6-cyclopentyladenosine and amounted to less than 10% for both binding of [^{125}I]HPIA and [^{3}H]DPCPX. This amounted to 2.7 to 3.2 fmol of [^{125}I]HPIA bound and to 15 to 18 fmol of [^{3}H]DPCPX bound. Data are means from three to four independent experiments carried out on different membrane preparations; error bars represent S.D.

rate of GDP-release from Gα at low Mg^{2+} concentrations (Higashijima et al., 1987), increasing the amount of Gβγ suppressed the basal rate of GTPγS binding (Fig. 7A). More importantly, with increasing concentrations of Gβγ, there was also a pronounced decrease in the stimulation by FUB132 and by FUB86 of the exchange reaction. In fact, the inhibitory effect of Gβγ on FUB132-stimulated GTPγS binding surpassed its inhibitory action on basal GTPγS binding to $\text{G}\alpha_{o}$. This is most readily evident in the replot shown in Fig. 7B, where GTPγS-binding at each concentration of Gβγ in the absence of FUB 132 was set 100%. Figure 7B also shows a representative curve for Gβγ-dependent inhibition of the action of a saturating concentration of FUB86 (200 μM; Fig. 7B, ■). As mentioned earlier, the efficacy of FUB86 in activating $\text{G}\alpha_{o/i}$ is lower than that of FUB132; however, if the concentration-response curves are compared at equivalent concentrations of the FUB lipoamines (i.e., at 200 μM each; Fig. 3B, squares), Gβγ was essentially equipotent in suppressing FUB86 and FUB132. This inhibitory effect was not caused by buffer components in the preparation of Gβγ. A control experiment was carried out in which denatured Gβγ subunits (100 nM) were combined with 20 nM Gα and the ability of FUB132 to accelerate GTPγS binding was assessed; the presence of heat-inactivated Gβγ did not shift the concentration-response curve of the FUB lipoamines. Similarly, the effect of Gβγ was also seen when Gβγ was purified from recombinant sources (i.e., appropriately infected Sf9 cells) and when $r\text{G}\alpha_{i-1}$ was used instead of $r\text{G}\alpha_{o}$ (data not shown).

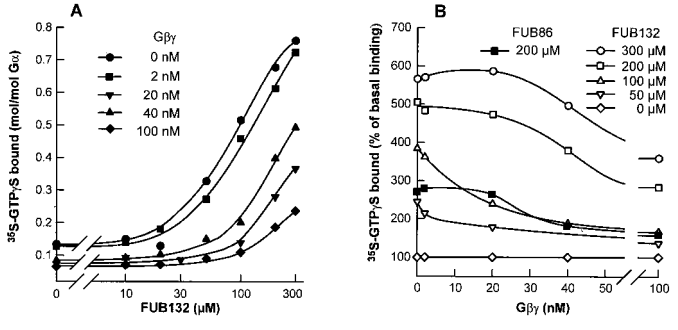

Fig. 7. FUB132-induced GTPγS binding to $\text{G}\alpha_{o}$ in the presence or absence of Gβγ complexes. $\text{G}\alpha_{o}$ subunits (20 nM) purified from bovine brain were incubated in a final volume of 50 μl of buffer A containing 0.45 mM $MgSO_4$ and 100 mM NaCl at 20°C. A, total binding of GTPγS in moles per mole of Gα after incubation with increasing concentrations of FUB132 with or without isolated bovine brain Gβγ complexes at different concentrations. B, relative stimulation of GTPγS binding to Gα after incubation with increasing amounts of Gβγ complexes in the presence of different concentrations of FUB132 or FUB86 (200 μM; ■). The reactions were started with [^{35}S]GTPγS (60 nM, 20 cpm/fmol) at 21°C for 20 s. The experiment shown is representative of three independent experiments with the same results and duplicate determinations.

FUB86 Inhibits G-Protein Signaling in Intact HL-60 Cells. The in vitro studies presented so far suggest FUB lipoamines as a potential new class of synthetic low molecular G protein modulators. Next, their ability to regulate PT-sensitive G protein signaling in vivo was examined. We used HL-60 cells, which contain a high concentration of G_i proteins that link chemoattractant receptors to stimulation of phospholipase C. Activation of this signaling cascade ought to result in a transient, PT-sensitive increase of the intracellular Ca^{2+}-concentration, an effect that can be readily measured in cells loaded with the Ca^{2+}-sensitive dye fura-2. As a control, we have used maximally effective concentrations of the chemoattractant receptor agonist *N*-formyl-methionyl-

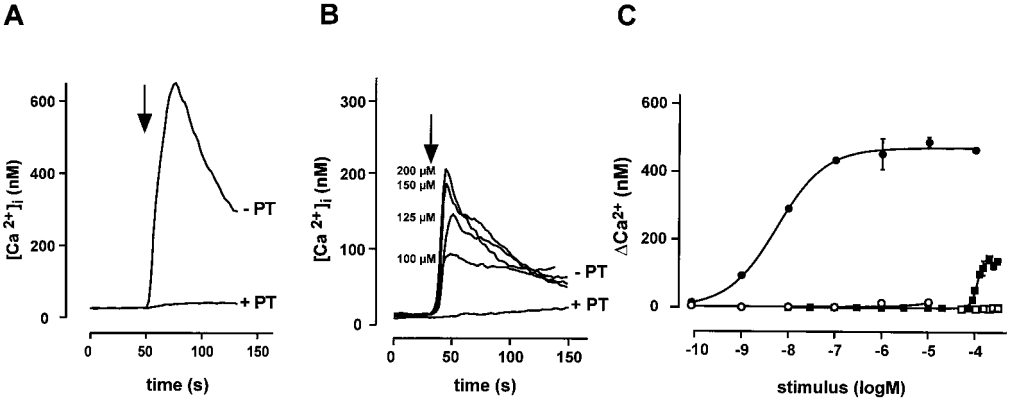

Fig. 8. Increase of intracellular calcium in HL-60 cells by fMLP and FUB86. A, typical time courses of intracellular $[Ca^{2+}]$ rise after stimulation with fMLP (1 μM) in cells without (−) or after (+) PT-pretreatment. B, similar experiments as described for A but with FUB86 as the stimulus at different concentrations indicated in the figure. FUB86 was applied at 100 μM when cells were pretreated with PT (+). Shown are typical experiments. C, concentration response curve of fMLP (circles) and FUB86 (squares) without (■) or after (□) PT-pretreatment. Shown is one of three similar experiments with triplicate determinations (mean ± S.D.).

leucyl-phenylalanine (fMLP), which transiently increased the intracellular Ca^{2+}-concentration of dbt-cAMP-differentiated HL-60 cells in a PT-sensitive fashion (Fig. 8A). Under our experimental conditions, only FUB86 was able to penetrate cell membranes; this is consistent with the observation that FUB86 is more lipophilic than FUB132 (see Figs. 1 and 9B; W. Schunack, K. Schimmelpfennig, and S. Rummel, unpublished observations). Starting at 100 μM, FUB86 increased $[Ca^{2+}]_i$ in a concentration-dependent fashion (Fig. 8, B and C). Notably, the rise in intracellular Ca^{2+} induced by FUB86 was transient (see Fig. 8B). This argues against nonspecific effects, in particular leakage of Ca^{2+}, due to permeabilization of cell membranes as observed for mastoparan at similar concentrations (data not shown). Furthermore, the stimulatory effect of FUB86 was completely blocked by pretreatment of cells with PT. This observation strongly suggests that FUB86 activates members of the G_i subfamily that are responsible for PT-sensitive Ca^{2+} transients in HL-60 cells.

The efficiency of FUB86 to stimulate the rise in $[Ca^{2+}]_i$ was much smaller than the response elicited by fMLP, indicating that FUB86 was a weak activator of G protein signaling in vivo (see Fig. 8C). Therefore, we surmised that FUB86 was able to reduce fMLP-induced cellular signaling by displacing the receptor from its G protein. Accordingly, equilibrated cells were first incubated with FUB86 and subsequently stimulated by a maximally effective concentration of fMLP (Fig. 9A, inset). This sequential addition allowed us to distinguish between the small stimulatory effect that FUB86 exerted per se and its inhibitory effect on the fMLP-induced rise in $[Ca^{2+}]_I$; at concentrations equal to or exceeding 100 μM, FUB86 elicited a transient increase in $[Ca^{2+}]_i$. This, however, had faded (Figure 8B) by the time the cells were challenged with fMLP (i.e., 170 s later). As shown in Fig. 9A, preincubation of cells with FUB86 inhibited the fMLP-induced rise in $[Ca^{2+}]_i$ in a concentration-dependent manner. At maximally effective concentrations of FUB86, the fMLP-induced rise in $[Ca^{2+}]_i$ was reduced to basal levels. Notably, the FUB86-induced inhibition was evident at concentrations (IC_{50} = 30 μM) much lower than those required to induce transient increases in $[Ca^{2+}]_i$ (Fig. 8C). However, the concentration range was in reasonable agreement with that observed for FUB86-dependent inhibition of agonist binding to the human A_1-adenosine receptor (see Fig. 6B). These data support the notion that in vivo FUB86 acts as a partial agonist/antagonist at the level of the G protein. If FUB86 competed with the fMLP receptor for binding to the G_i protein, the compound should also block other pathways regulated by fMLP-receptor-coupled G_i proteins with similar efficiency and potency. To verify this assumption, we examined the fMLP-induced production of O_2^- by HL-60 cells. This pathway is mediated by phosphoinositide-3-kinases and can be completely blocked by PT (Fig. 9C). Indeed, FUB 86 in-

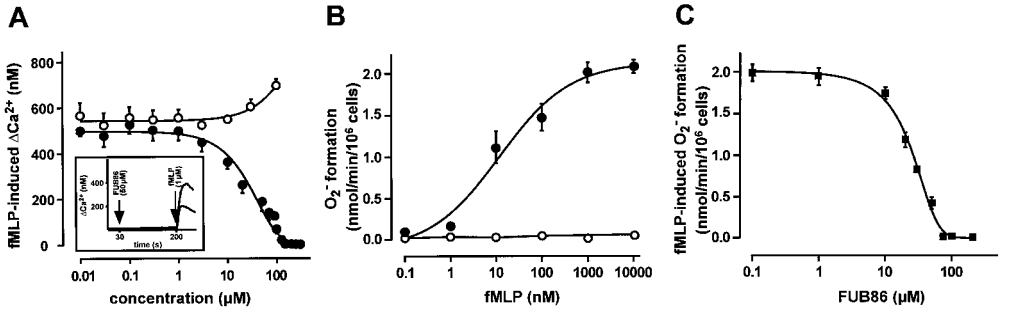

Fig. 9. Inhibition of fMLP-induced calcium release in HL-60 cells by FUB86. A, concentration response curve of FUB86 (●) and FUB132 (○)-mediated inhibition of fMLP (1 μM)-induced rise of $[Ca^{2+}]_i$. Shown is one of four similar experiments with triplicate determinations (mean ± S.D.). Inset, typical time courses of intracellular $[Ca^{2+}]$ rise after stimulation with fMLP (1 μM) in cells without or after pretreatment with 50 μM FUB86. B, concentration response curves of fMLP (left) and FUB86-mediated inhibition of fMLP (1 μM)-induced O_2^- formation in HL-60 cells. Shown are mean values (±S.D.) of three independent experiments.

hibited fMLP-induced O_2^--formation with an IC_{50} of 25 μM and completely blocked it at a FUB86 concentration of 100 μM.

Discussion

Low-molecular-weight G-protein activators are potentially useful tools to study G protein dependent signaling pathways in intact cells. Several compounds have been shown to directly activate G proteins (i.e., to accelerate the release of prebound GDP) (reviewed in Freissmuth et al., 1999; Höller et al., 1999; Nürnberg et al., 1999). The common structural feature of these compounds is a hydrophobic moment and a net positive charge. In the present work, we have therefore sought to identify more efficacious lipoamine analogs by increasing the number of amino groups. Of the series of compounds tested, FUB132 was the most efficacious; the maximum effect that FUB132 elicited with purified $r\text{G}\alpha_{i1}$ and $r\text{G}\alpha_o$ exceeded the effect of the reference compound FUB 86 (Leschke et al., 1997) by at least 2- to 4-fold. In contrast, there was not any appreciable difference in the extent to which FUB86 and FUB132 uncoupled the A_1-receptor form its G protein. This discrepancy can be rationalized if the mechanistic differences are taken into account; G protein activation relies on the ability of the lipoamines to stimulate the exchange reaction, whereas uncoupling of the receptor depends on the propensity of the compound to occupy the site on Gα that is contacted by the receptor. Compared directly, FUB86 and FUB132 differed little in their concentrations, eliciting half-maximum activation of $\text{G}\alpha_{i1}$ (see Fig. 2). We therefore conclude that the increased number of amino groups in FUB132 primarily increased the stimulatory efficacy, whereas the affinity was not enhanced. Finally, FUB132 and FUB86 were selective for G_i and G_o; activation of $\text{G}\alpha_s$ and $\text{G}\alpha_t$ required higher concentrations of the lipoamines and the extent of stimulation was much less pronounced. Thus, FUB lipoamines, in particular FUB132, fulfill essential criteria for a potentially useful G protein activator: efficient stimulation of guanine nucleotide exchange and subtype-selectivity.

However, the analysis of the mechanism of action of FUB lipoamines implies that the stimulatory action observed with purified Gα-subunits may not necessarily translate into G protein activation in intact cells. This conjecture is based on the following findings. Mastoparan and FUB lipoamines are likely to share a common site of action, because their action is nonadditive; in addition, FUB lipoamines block receptor/G-protein coupling because they inhibited high-affinity binding of the agonist I-HPIA to the $G_{i/o}$-coupled A_1-adenosine receptor. We can rule out the idea that FUB lipoamines blocked the ligand binding pocket of the receptor because the compounds did not affect binding of the antagonist; the most likely candidate mechanism is binding of FUB lipoamines to the C terminus of the G protein α-subunit, which impedes access to the receptor and thus prevents formation of the ternary complex of agonist, receptor, and G protein. Although FUB lipoamines, mastoparan, and receptors share a common site on Gα (i.e., the C terminus), mechanistically, their effects differ in several important respects. 1) mastoparan requires the presence of phospholipids to adopt its active conformation (Higashijima et al., 1983; Sukumar and Higashijima, 1992; Kusunoki et al., 1998). In contrast, the action of FUB lipoamines was not affected by the presence of lipids. Incidentally, this observation also rules out that the action of FUB lipoamines can be ascribed to a mere detergent-like action. 2) More importantly, mastoparan requires the presence of G$\beta\gamma$-dimers to efficiently activate Gα-subunits (Higashijima et al., 1990). This is also true for receptors; it has long been known that G$\beta\gamma$-dimers catalytically support the activation of transducin $\text{G}\alpha_t$ by the prototypical G protein-coupled receptor photoactivated rhodopsin (Fung, 1983). Gα-subunits alone do not suffice to stabilize high-affinity agonist binding to the A_1-adenosine receptor (Freissmuth et al., 1991). In fact, receptors contact simultaneously all three G protein subunits (α, β, and γ) in the heterotrimer (Kisselev et al., 1999). In contrast, FUB 132 efficiently activated the release of GDP from (and thereby promoted binding of GTPγS to) Gα in the absence of any G$\beta\gamma$-dimers. Moreover and surprisingly, G$\beta\gamma$ blunted the stimulation induced by FUB lipoamines. This observation suggests that the mechanism by which FUB lipoamines and a receptor (or a receptomimetic peptide) promote guanine nucleotide exchange differ in a fundamental way.

Receptors contact the very C terminus of Gα (i.e., the last 5–10 amino acids); in addition, the intracellular loops of the receptor also contact amino acids that are more amino-terminal (i.e., within the last 50 amino acids) up to the loop formed by helix α 4/strand β 6 (Hamm, 1998). Although the precise mechanism by which receptors induce the release of GDP from the α-subunit remains unclear, it is evident that the receptor cannot per se contact the residues that control access to the GDP-binding pocket because the intracellular loops of the receptor are too short; it has therefore been proposed that the receptor acts at a distance (Iiri et al., 1999). Two mechanisms can be envisaged (and these may actually work in concert): the receptor signal may be either transmitted through the C terminus by a network of interactions that results in a rearrangement of residues within the GDP-binding pocket such that an exit pathway is opened for the nucleotide. Alternatively (or in addition), the receptor may use the G$\beta\gamma$-dimer, which contacts the switch II-region of the G protein α-subunit, as a lever to pry apart the residues of Gα that cover the nucleotide (Iiri et al., 1998). This contrasts with the effect that G$\beta\gamma$ exerts on the release of GDP in the basal state, where it prevents GDP-release (by acting as lid that blocks the GDP exit pathway). There isn't any evidence that FUB lipoamines can bind Gα and G$\beta\gamma$ simultaneously. On the contrary, G$\beta\gamma$ quenches the effect of FUB lipoamines on Gα. This suggests that the stimulation exerted on Gα does not suffice to relieve the inhibition imposed by G$\beta\gamma$.

In solution, FUB lipoamines exert a marked stimulatory effect on the rate of guanine nucleotide exchange on Gα; this is much less pronounced with the heterotrimer. It is difficult to obtain precise quantitative estimates in cell membranes, because the measured rates of GTPγS binding and of GTP hydrolysis reflect the activity of many proteins. Nevertheless, in most studies the stimulatory effect of G-protein activators is very modest ($\leq$1.5-fold enhancement of the rate; for review, see Mousli et al., 1990, and Nürnberg et al., 1999); this was also true for FUB132 (not shown) and FUB86 (Leschke et al., 1997). We therefore conclude that in intact cells direct G-protein activators that are lipophilic enough to overcome the membrane barrier may only be weak activators of G proteins compared with the effect elicited by agonist-activated receptors. In fact, FUB86 was a weak stimulator of

calcium signaling in HL-60 cells compared with fMLP. In contrast, its predominant action may rather be blockage of signaling by receptors. This interpretation is substantiated by the observations that FUB86 efficiently blunted the action of fMLP-induced rise of $[Ca^{2+}]_i$ and O_2^--formation.

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