Alkyl-Substituted Amino Acid Amides and Analogous Di- and Triamines: New Non-Peptide G Protein Activators†

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Synthesis and pharmacological properties of new potent direct activators of heterotrimeric G proteins are described. Compounds were synthesized from protected amino acids with alkylamines using coupling reagents (CDI, DCC, and EDC). Alkyl-substituted amino acid amides and their corresponding di- and triamines were subjected to structure−activity analysis. All compounds activated membrane-bound HL-60 GTPases in a pertussis toxin-sensitive fashion. This suggests a specific effect of compounds on the carboxy terminus of a defined subclass of heterotrimeric G proteins, i.e., members of the $G\alpha_i$ subfamily. Elongation of the alkyl chain and increasing the number of amino groups enhanced the potency of compounds on HL-60 membrane-bound GTPase. *N*-(2,5-Diaminopentyl)dodecylamine (**21**) was selected to study its mode of action employing purified pertussis toxin-sensitive G proteins. It stimulated Gα subunits by inducing the release of bound GDP. In contrast to receptors $G\beta\gamma$ complexes were not required for **21**-mediated activation of Gα. Moderate isoform selectivity of its action was observed within a group of highly homologous members of the G_i subfamily with $G\alpha_{o1}$ being activated at lowest concentrations, whereas higher concentrations were necessary for the stimulation of $G\alpha_{i1}$ or transducin. We conclude that these compounds represent important tools for studying G protein-dependent cellular functions.

Introduction

Mammalian heterotrimeric G proteins (G proteins) consist of three subunits termed α, *β*, and *γ*. They transmit extracellularly recognized signals into the cell interior. Ligand binding to the cell surface receptor enables conformational changes of the receptor thereby activating G proteins. As versatile switches the latter regulate various cellular effectors.[1−3] Mechanisms like desensitization or down regulation of receptors as well as additional G protein modulators, such as effectors with GAP (GTPase-activating protein) activity or the recently discovered RGS proteins (regulators of G protein signaling), allow fine-tuning of the signaling events.[4,5]

Signal transmission from receptor to G protein includes interaction with several contact sites, i.e., domains located on the second and third intracellular loop of the receptor resulting in the release of bound GDP from the α subunit of the G protein.[6] Most likely this first event is the rate-limiting step of the G protein activation reaction followed by high-affinity binding of cytosolic GTP. Mg^{2+} is very tightly associated with GTP and Gα in this complex and is required for activation of the Gα subunit and for subsequent hydrolytic activity.[7] Upon G protein activation, the Gα subunit dissociates from the $G\beta\gamma$ complex. The latter does not dissociate under physiological conditions. Gα and $G\beta\gamma$ modulate effectors until the intrinsic GTPase activity of Gα cleaves the *γ*-phosphate of GTP. Following GTP hydrolysis, the inactive GDP-bound Gα subunit dissociates from the effector and reassociates with the $G\beta\gamma$ complex to form a $G\alpha\beta\gamma$ heterotrimer. This inactive heterotrimer becomes available for subsequent activation cycles. Interestingly the receptor-stimulated GDP/GTP exchange reaction varies with different G proteins: G_i proteins are fast nucleotide exchangers, G_s, G_z, and G proteins of the G_q subfamily show smaller rate constants, and for G_{12} and G_{13} very small guanine nucleotide exchange rates have been published.[8,9]

Additionally, receptor-independent modulators of G protein activity have been reported. The wasp venom mastoparan represents the best known member of G protein activators.[10,11] Others are naturally occurring peptides or synthetic agents, such as compound 48/80.[12−15] Mastoparan shows an amphiphilic α-helical tetradecapeptide structure which is thought to be similar to the third intracellular loop of G protein-coupled receptors. It is supposed to bind in a receptor analogous fashion to the amino (N) and carboxy (C) termini of Gα.[16,17] However, previous work has revealed its limited specificity affecting many cellular proteins.[18−20] Unfortunately, its amphiphilic structure disturbs biological membranes, hence producing unspecific effects.[21,22] G proteins are also located on intracellular membranes where they are assumed to regulate diverse cellular processes, such as protein trafficking or vesicular function.[22] However, activators of intracellular G proteins are unknown so far. Hence, for studying G protein-dependent functions, potent and specific non-peptide activators are desired.

Previously, we identified the potent histamine H_1 receptor agonist 2-(3-chlorophenyl)histamine and other 2-substituted histamine derivatives as receptor-independent direct activators of G proteins.[23−25] Systematic

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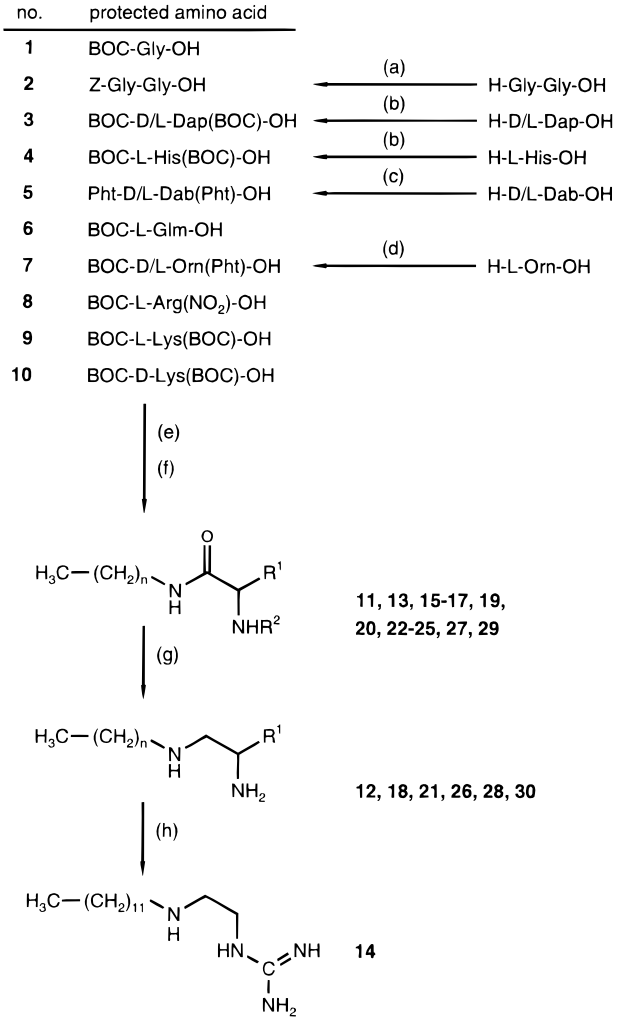
studies of the structure−activity relationships of these compounds suggest that the lipophilic domain is very important for the G protein-activating properties of histamine derivatives.[26−28] Interestingly, not only 2-substituted histamines but also other cationic amphiphilic compounds possess the ability to activate G proteins in a receptor-independent manner, i.e., substituted alkylamines.[29] Starting from these results it is generally assumed that both the lipophilic and the basic domain are required for receptor-independent G protein activation. These findings led us to study structure−activity relationships of some new alkyl-substituted amino acid amides and amines for direct G protein activation. Another aim of our study was to obtain more potent and selective G protein activators as important pharmacological tools for studying signal transduction *via* G proteins.

Chemistry

Due to activation of the carboxylic moieties of amino acids, it is necessary to introduce the latter with protected amino and side-chain function. The following protecting groups for the amino function were used: phthalyl (Pht), *tert*-butyloxycarbonyl (BOC), and benzyloxycarbonyl (Z), while the guanidino function was protected with a nitro group (NO_2). The introduction of protective groups succeeded in the case of diglycine (Gly-Gly-OH) with benzyloxycarbonyl chloride according to Bodanszky et al.,[30] in the case of 2,3-diaminopropionic acid (Dap) and histidine (His) with di-*tert*-butyl dicarbonate according to Bodanszky et al.,[31] and in the case of 2,4-diaminobutyric acid (Dab) with *N*-(ethoxycarbonyl)phthalimide.[32,33] In the case of ornithine (Orn) the following sequence was used:[34] Masking the carboxylic and primary amino groups with $CuSO_4$,[31] protecting the side-chain amino group with *N*-(ethoxycarbonyl)phthalimide,[32] and unmasking and selectively protecting the primary amino function with di-*tert*-butyldicarbonate (Scheme 1).[35]

Starting from these protected amino acids, we prepared some new *N*-dodecyl, *N*-tetradecyl, or *N*-hexadecyl amino acid amides. Protected alkyl-substituted amino acid amides were synthesized by coupling amino acids with alkylamines using *N*,*N*′-carbonyldiimidazole (CDI),[36,37] *N*,*N*′-dicyclohexylcarbodiimide (DCC),[38] or *N*′-[3-(dimethylamino)propyl]-*N*-ethylcarbodiimide hydrochloride (EDC)[39] in the presence of 1-hydroxybenzotriazole (HOBT) as coupling reagents. Removal of the protecting groups and purification by rotatory chromatography or recrystallization yielded the amino acid amides in the form of free base, hydrochloride, hydrogen maleate, or trifluoroacetate salt. Reduction of selected amino acid amides (**11**, **17**, **20**, **24**, **27**, **29**) with $LiAlH_4$ in THF gave the appropriate alkyl-substituted diamine (**12**) or triamines (**18**, **21**, **26**, **28**, **30**). *N*-(2-Guanylethyl)dodecylamine (**14**) was prepared by guanylation of *N*-(2-aminoethyl)dodecylamine (**12**) with 3,5-dimethylpyrazole-1-carboxamidine nitrate.[40] The sequence of synthesis and the physicochemical properties are shown in Scheme 1 and Table 1.

Scheme 1. Synthesis of Alkyl-Substituted Amino Acid Amides and Alkyl-Substituted Di- and Triamines[a]

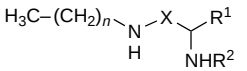


[a] n = 11, 13, or 15; for R^1 and R^2, see Table 2. Reagents: (a) benzyloxycarbonyl chloride, NaOH, 5−20 °C; (b) di-*tert*-butyl dicarbonate; (c) *N*-(ethoxycarbonyl)phthalimide; (d) 1. $CuSO_4$, 2. *N*-(ethoxycarbonyl)phthalimide, 3. di-*tert*-butyl dicarbonate; (e) method A (**11**, **15**−**17**, **20**, **22**, **23**, **27**, **29**)—CDI, 1-hydroxybenzotriazole hydrate (except **20**), THF, argon, 30−60 min, then dodecyl-, tetradecyl-, or hexadecylamine, 12 h at room temperature; method B (**13**, **19**)—DCC, 1-hydroxybenzotriazole hydrate, THF (for **13**) or THF/CH_2Cl_2 (for **19**); method C (**24**, **25**)—EDC, 1-hydroxybenzotriazole hydrate, CH_2Cl_2/H_2O, 24 h at ambient temperature; (f) removal of protecting groups—BOC, 1 N HCl (for **19** trifluoroacetic acid, for **24** and **25** 5−6 N HCl); Pht, hydrazine hydrate, 1 N HCl; NO_2, H_2, Pd/C; Z, NH_4HCOO, Pd/C; (g) $LiAlH_4$, THF, argon, 5−10 h reflux; (h) 3,5-dimethylpyrazole-1-carboxamidine nitrate.

Results and Discussion

Screening of GTPase-Activating Compounds. All compounds were screened for their ability to affect high-affinity GTPase activity in dibutyryl-cAMP differentiated HL-60 membranes (Table 2). The effects were compared with the known G protein activator mastoparan. Mastoparan stimulated GTPase activity half-maximally with a pEC_{50} value of 5.5 and 2-fold activation at 10 μM (Table 2 and Figure 1). These data confirmed previous results.[27] All compounds increased GTPase activity showing half-maximal activation with pEC_{50} values between 5.1 (**19**) and 6.6 (**28**, **30**). Maximal effects ranged between 1.8-fold (**30**) and 2.5-fold (**22**) stimulation. The compounds displayed typical structures of known GTPase-stimulating agents, i.e., a hydrophilic cationic moiety and a lipophilic tail. The GTPase-activating properties of the alkyl-substituted amino acid amides were dependent on the number of basic groups in the amino acid partial structure. Com-

Table 1. Physicochemical Properties of Alkyl-Substituted Amino Acid Amides and Alkyl-Substituted Di- and Triamines



no.	*n*	X	R^1	R^2	yield[a] (%)	mp (°C)	$[\alpha]^{20}_D$ (deg)[b]	formula[c]	M_r
11	11	C=O	H	H	61	107−108		$C_{14}H_{30}N_2O\cdot HCl\cdot 0.25H_2O$	283.4
12	11	CH_2	H	H	68	165−167		$C_{14}H_{32}N_2\cdot 2C_4H_4O_4$	460.6
13	11	C=O	H	C(=O)-CH_2-NH_2	25	176−180		$C_{16}H_{33}N_3O_2\cdot HCl$	335.9
14	11	CH_2	H	C(=NH)-NH_2	44[d]	119−121		$C_{15}H_{34}N_4\cdot 2HNO_3$	396.5
15	11	C=O	CH_2-NH_2	H	26	129−130		$C_{15}H_{33}N_3O\cdot 2C_4H_4O_4$	503.6
16	11	C=O	CH_2-4-imidazolyl	H	58	125−126	+13.0	$C_{18}H_{34}N_4O\cdot 2C_4H_4O_4\cdot 0.5H_2O$	563.6
17	11	C=O	$(CH_2)_2$-NH_2	H	20	128−130	*e*	$C_{16}H_{35}N_3O\cdot 2C_4H_4O_4\cdot 0.5H_2O$	526.7
18	11	CH_2	$(CH_2)_2$-NH_2	H	21	140−141	*e*	$C_{16}H_{37}N_3\cdot 3C_4H_4O_4\cdot 1.5H_2O$	646.7
19	11	C=O	$(CH_2)_2$-C(=O)-NH_2	H	48	97−98		$C_{17}H_{35}N_3O_2\cdot 0.9CF_3COOH$	416.1
20	11	C=O	$(CH_2)_3$-NH_2	H	58	54−55	*e*	$C_{17}H_{37}N_3O\cdot 0.5H_2O$	308.5
21	11	CH_2	$(CH_2)_3$-NH_2	H	56	100−102	*e*	$C_{17}H_{39}N_3O\cdot 0.8H_2O$	299.9
22	11	C=O	$(CH_2)_3$-NH-C(=NH)-NH-NO_2	H	34	95−96	+10.5	$C_{18}H_{37}N_6O_3\cdot H_2O$	403.5
23	11	C=O	$(CH_2)_3$-NH-C(=NH)-NH_2	H	63[f]	146−147	+7.9	$C_{18}H_{39}N_5O\cdot 2C_4H_4O_4\cdot 0.5H_2O$	582.7
24	11	C=O	$(CH_2)_4$-NH_2	H	44	105−106	+16.6	$C_{18}H_{39}N_3O\cdot 2.3HCl$	397.4
25	11	C=O	$(CH_2)_4$-NH_2	H	37	106−107	−17.2	$C_{18}H_{39}N_3O\cdot 2.2HCl$	393.7
26	11	CH_2	$(CH_2)_4$-NH_2	H	33	136−139	*e*	$C_{18}H_{41}N_3O\cdot 3C_4H_4O_4\cdot 0.75H_2O$	661.2
27	13	C=O	$(CH_2)_4$-NH_2	H	68	80	+12.0	$C_{20}H_{43}N_3O\cdot 2C_4H_4O_4\cdot H_2O$	591.8
28	13	CH_2	$(CH_2)_4$-NH_2	H	54	149−150	*e*	$C_{20}H_{45}N_3\cdot 3C_4H_4O_4$	675.9
29	15	C=O	$(CH_2)_4$-NH_2	H	27	152	+12.7	$C_{22}H_{47}N_3O\cdot 2C_4H_4O_4\cdot 1.5H_2O$	628.8
30	15	CH_2	$(CH_2)_4$-NH_2	H	26	158−160	*e*	$C_{22}H_{49}N_3\cdot 3C_4H_4O_4$	703.9

[a] Yield related to amount of free base. [b] 5 mg/mL in methanol. [c] All compounds were analyzed for C, H, and N and are within ±0.4% of the theoretical values. They were characterized through ^{1}H NMR and $^+$FAB-MS. [d] Yield related to amount of dinitrate. [e] Racemate. [f] Yield related to amount of dihydrogen maleate.

pounds with one amino group (**11** and **13**) show low GTPase activity (pEC_{50} = 5.4 and 5.3). The introduction of an amide group (**19**) or a weak basic imidazole ring (**16**) in the amino acid partial structure did not influence the GTPase activity on HL-60 membranes, but a second strong basic primary amino group increased GTPase-stimulatory properties (**17** *vs* **19**). Elongation of the alkyl chain between both amino groups (Dap, **15**, pEC_{50} = 5.6 *vs* Dab; **17**, pEC_{50} = 5.9 *vs* Orn; **20**, pEC_{50} = 5.6 *vs* Lys; **24** or **25**, pEC_{50} = 6.1) resulted in an increase in GTPase activity of the substances (0.5 log unit). Only from the diaminobutyric acid derivative to the ornithine derivative did we observe a slight decrease in potency. The activity of the dodecyl lysine amide was independent of the absolute configuration (**24** and **25**) of the lysine moiety. In the case of the arginine derivative **23** exchange of the ω-amino group of the ornithine derivative **20** by a strongly basic guanidino group resulted in increased GTPase activity, but exchange by a nitroguanidino group (**22**) dramatically decreased activity.

In order to study the importance of the chain length of these compounds, we synthesized substances with longer alkyl chains. From earlier work it is known that the alkyl chain should be longer than 10 C atoms.[28] When the dodecyl chain of the lysine derivative **24** was replaced by a tetradecyl or hexadecyl chain, the potency of the compounds (**27** and **29**, pEC_{50} = 6.3) was higher than that of the dodecyl derivative with pEC_{50} of 6.1. These tetradecyl and hexadecyl derivatives are the most potent compounds in the class of alkyl-substituted amino acid amides in the present study. Replacement of the amide moiety of amino acid amides by the corresponding secondary amine group again increases GTPase activity (**11** *vs* **12**, **17** *vs* **18**, **20** *vs* **21**, **24** and **25** *vs* **26**, **27** *vs* **28**, **29** *vs* **30**; 0.2−0.6 log unit). The triamines **28** and **30** are the most potent G protein activators known so far and 12 times more potent than mastoparan.

To get an insight into the compounds' specificity of action, we pretreated HL-60 cells with pertussis toxin (PTX). PTX is known to functionally uncouple $G_{i/o}$ proteins and transducin from receptors by modifying a cysteine at the C-terminus of the Gα subunit.[41] Figure 1 (top) shows immunoblots and autoradiograms after PTX-mediated [^{32}P]ADP ribosylation of PTX-pretreated and control HL-60 cells. Membrane proteins fractionated on SDS−PAGE exhibited one immunoreactive band at 40 kDa after incubation with an antiserum (AS 8, anti-α_{com}) detecting all major Gα isoforms. Proteins of similar gel electrophoretic mobility were also recognized by an antiserum (AS 266, anti-α_{icom}) exclusively interacting with all known $G\alpha_i$ isoforms confirming the expression of predominantly G_i proteins in HL-60 cells. In addition, immunoblot analysis revealed that PTX treatment of cells did not alter G_i protein concentration of membranes as roughly estimated from immunostain intensity (Figure 1, top). PTX-mediated [^{32}P]ADP ribosylation of control membranes, but not membranes of PTX-pretreated cells, showed strong autoradiographic signals of electrophoretic mobility similar to the immunoreactive signals (Figure 1, top). Evaluation by a phosphor-imaging system (Fuji BAS 1500, Straubenhardt, Germany) confirmed an almost quantitative ADP ribosylation of G_i proteins (>95%) in PTX-pretreated HL-60 cells. Next, we studied the effects of mastoparan on GTPase activity of membranes obtained from control and PTX-pretreated HL-60 cells (Figure 1, bottom). In accordance with previous reports[10] PTX greatly diminished the stimulatory activity of mastoparan by approximately 85% and shifted the pEC_{50} value from 5.5 to 4.9 (Figure 1, bottom). The PTX-insensitive activation of GTPase by mastoparan seen in this study confirmed previous reports on a broad array of effects of mastoparan on different cellular proteins including members of the GTPase superfamily.[22] In contrast, the newly synthesized compounds tested were unable to

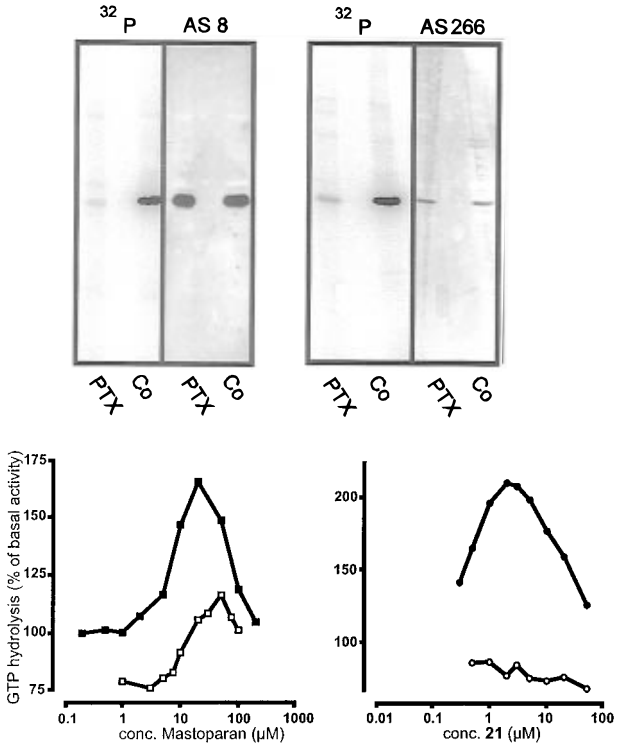


Figure 1. Activation of HL-60 membrane proteins by mastoparan and compound **21**. (Top) PTX-catalyzed [^{32}P]ADP ribosylation of HL-60 membrane proteins. Shown are typical autoradiograms and immunoblots of PTX-pretreated and control HL-60 cells (Co) after PTX-mediated [^{32}P]ADP ribosylation (^{32}P) or after incubation with antisera AS 8 (anti-α_{com}) and AS 266 (anti-α_{icom}). Proteins were fractionated by SDS−PAGE as described in the Experimental Section. (Bottom) Effect of G protein activators on steady state GTPase of HL-60 cell membranes. Membranes of HL-60 cells incubated in the absence (■,●) or presence (□,○) of pertussis toxin were treated with increasing concentrations of mastoparan (■,□) or **21** (●,○). GTP hydrolysis was quantified as detailed in the Experimental Section. Shown is one representative experiment each (n = 3−11). Data represent mean values from triplicate determinations.

stimulate GTPase of PTX-pretreated HL-60 membrane proteins (Table 2 and Figure 1, bottom). The PTX sensitivity of the compounds strongly argues for a narrow spectrum of targets among GTPases, i.e., PTX-sensitive G proteins. Furthermore, PTX sensitivity of the drugs' action points to the C-terminus of the Gα subunit as the putative domain affected by this new class of GTPase activators. For further analysis we employed recombinantly expressed or purified G proteins of the G_i subfamily.

Non-Peptide Alkylamines Activate PTX-Sensitive G Proteins. In order to prove stimulatory activity of the compounds on heterotrimeric PTX-sensitive G proteins, we isolated cell membranes from Sf 9 cells infected with baculoviruses encoding mammalian $G\alpha_{i1}\beta_2\gamma_2$. This expression system allows selective overexpression of recombinant proteins, while concentrations of endogenous insect proteins decrease during infection.[42] Membranes were incubated with increasing concentrations of mastoparan or compound **21**, and their effect on GTPγS binding to $G\alpha_{i1}\beta_2\gamma_2$ was measured. Both compounds significantly stimulated GTPγS binding to $G\alpha_{i1}\beta_2\gamma_2$ in a concentration-dependent fashion (Figure 2). Corresponding to their effect on HL-60 GTPase, **21** was more potent than mastoparan. Compound **21** exhibited maximum stimulatory activity at 300 μM, whereas mastoparan did not maximally stimulate $G\alpha_{i1}\beta_2\gamma_2$ at 1 mM. Compared to GTPase of HL-60 cell membranes, potencies of **21** and mastoparan on $G\alpha_{i1}\beta_2\gamma_2$ activation, however, were weaker. This difference is likely to depend on the different compositions of mammalian HL-60 and insect Sf 9 cell membranes. The influence of different membrane lipids on the potency of mastoparan activating G proteins is well documented.[13,43] However, at this point we did not seek optimized conditions thus tolerating differences in the potency of the compounds in different assays.

Mechanism of Action of G Protein Activators. According to a stimulatory effect of **21** on GTPγS binding to recombinantly expressed $G\alpha_{i1}\beta_2\gamma_2$, we also found **21** able to concentration-dependently increase the steady state GTPase of a mixture of G_i proteins purified from bovine brain membranes after reconstitution into phospholipid vesicles (Figure 3). Then we looked at the time course of GTPγS binding to purified bovine brain $G\alpha_{i1}$ subunits in the presence of lipid vesicles and to heterotrimeric G_{i1} reconstituted into phospholipid vesicles (Figure 4). Stimulation of GTPγS binding was also seen in the absence of G$\beta\gamma$ complexes (Figure 4, left panel) confirming that Gα is the target of the compound.

Release of bound GDP as the time-limiting step of G protein activation precedes GTP binding to Gα.[44] Thus we recorded [^{35}S]GTPγS binding to and [^{32}P]GDP release from purified bovine brain $G\alpha_{o1}$ which is the predominant PTX-sensitive G protein in the central nervous system (Figures 5 and 6). Both steps of G protein activation were stimulated by **21**. We therefore conclude that **21** activates PTX-sensitive G proteins by interaction with the C-terminus of the Gα subunits resulting in a stimulated exchange of GDP for GTP.

G Protein Selectivity of G Protein Activators. The newly synthesized compounds described in this paper are completely inactive on G proteins modified by PTX suggesting significant specificity (Table 2 and Figure 1, bottom). Among some two dozen different G proteins belonging to four G protein subfamilies, only members of the G_i subfamily are sensitive to PTX. They include ubiquitously expressed G_i proteins, G_o proteins found in neuronal and neuroendocrine tissues, and G proteins confined to sensory cells, such as transducin and gustducin. They share a high degree of amino acid identity of at least 80%. Nevertheless, data obtained from testing G_{i1}, G_{o1}, and transducin indicate selectivity of **21** within this highly homologous group of G protein isoforms (Figure 7). For activation of G_{o1} lower concentrations than for G_{i1} or transducin were necessary (EC_{50}: G_{o1} (20 μM) < G_{i1} (90 μM) = transducin). Interestingly, transducin, which lacks basal GTPγS binding or GTPase activity, was significantly stimulated by **21**. Since all G proteins tested exhibit subtle amino acid differences at their Gα C-terminus, future work should be able to identify the amino acids of Gα interacting with this compound.

Conclusions

The newly synthesized amino acid amides and analogous di- and triamines are potent and selective activators of heterotrimeric PTX-sensitive G proteins. They show significantly higher potency as compared to mastoparan, which is known to exhibit limited selectivity.

Table 2. GTPase Activity of Alkyl-Substituted Amino Acid Amides and Alkyl-Substituted Di- and Triamines

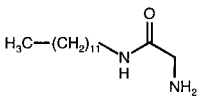
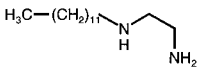
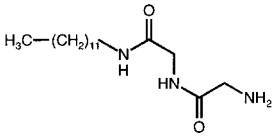
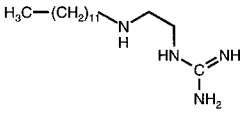
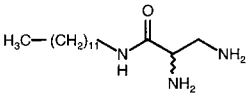
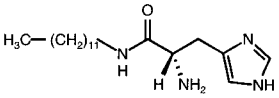
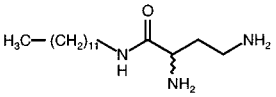
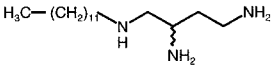
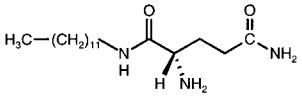
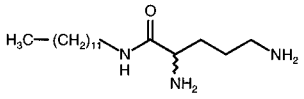
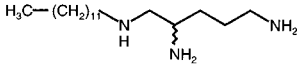
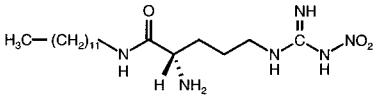
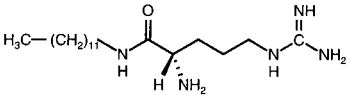
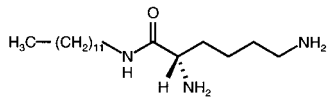
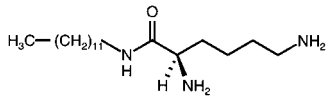
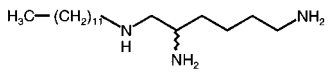
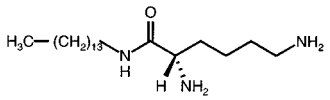
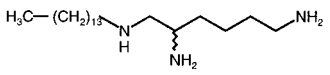
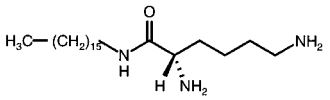
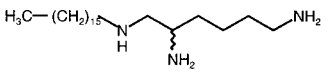
		GTP hydrolysis (HL-60 membranes)		
no.	compound	n^a	efficacy[b] [%] (conc.[c])	pEC_{50}[d]
11		4	100 (20 μM)	5.4 ± 0.11
12		4	92 (5 μM)	6.0 ±0.01
13		2	120 (30 μM)	5.3 ± 0.02
14		2	128 (7.5 μM)	6.0 ± 0.04
15		2	131 (10 μM)	5.6 ± 0.06
16		2	125 (20 μM)	5.5 ± 0.10
17		4	105 (10 μM)	5.9 ± 0.19
18		3	128 (3 μM)	6.2 ± 0.03
19		2	110 (30 μM)	5.1 ± 0.18
20		5	117 (10 μM)	5.6 ± 0.09[e]
21		8	126 (5 μM)	6.0 ± 0.02[f,g]
22		2	156 (300 μM)	-[h]
23		4	92 (7.5 μM)	6.1 ± 0.08

Table 2 (Continued)

24		3	116 (5 μM)	6.1 ± 0.05[g]
25		2	108 (5 μM)	6.1 ± 0.04[g]
26		3	120 (2 μM)	6.3 ± 0.03[g]
27		3	100 (3 μM)	6.3 ± 0.02[g]
28		2	117 (0.75 μM)	6.6 ± 0.04[g]
29		4	90 (2 μM)	6.3 ± 0.15[g]
30		2	84 (0.75 μM)	6.6 ± 0.03[g]
	Mastoparan: H-Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2	4	100 (10 μM)	5.5 ± 0.05[i]

[a] Replicate number of determinations. [b] Efficacy is defined as maximal stimulation of GTP hydrolysis above basal activity. [c] Concentration of the compounds with maximal stimulation of GTP hydrolysis. [d] pEC_{50} is definied as −log of the half-maximal concentration, means ± SD. [e] Histamine H_1 receptor activity (guinea pig ileum):[24] −log K = 5.3. [f] Histamine activity:[24] H_1 receptor (guinea pig ileum), −log K = 5.4; H_2 receptor (guinea pig atrium), −log K = 4.1; H_3 receptor (guinea pig ileum), −log K < 6.0. Muscarine M_3 receptor:[24] −log K = 5.2. [g] The stimulation of GTP hydrolysis was PTX sensitive. [h] No saturation of the concentration−response curve could be obtained (300 μM). [i] The stimulation of GTP hydrolysis was partial PTX sensitive.

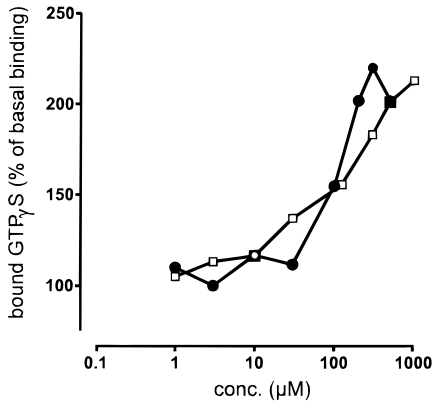


Figure 2. Effect of G protein activators on GTPγS binding of Sf 9 cell membranes. Shown is GTPγS binding to Sf 9 cell membranes containing mammalian $G\alpha_{i1}\beta_2\gamma_2$ in the presence of **21** (●) or mastoparan (□) as described in the Experimental Section. Data represent mean values from duplicate determinations.

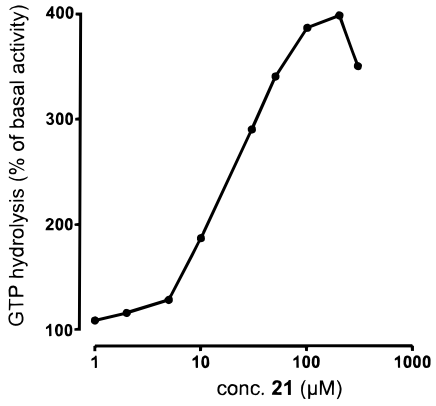


Figure 3. Effect of **21** on steady state GTPase of reconstituted PTX-sensitive G_i/G_o proteins. Effect of **21** on GTP hydrolysis by G_i/G_o proteins was determined in the presence of **21** at different concentrations as described in the Experimental Section. Shown is one representative experiment out of three with similar results. Data represent mean values from triplicate determinations.

Alkyl-substituted amino acid amides with a second basic (amino or guanidino) group show higher GTPase activity than amino acid derivatives with only one amino group, an imidazole ring or an amide structure in the amino acid moiety. Activity was increased (0.2−0.6 log unit) by replacement of the amide structure in the amino acid amide derivatives by a secondary amino group. *N*-(2,6-Diaminohexyl)tetradecyl- and -hexadecylamines (**28** and **30**) are 12 times more potent than mastoparan. The results of our present study show that basicity (two or three amino functions) and lipophilicity are of substantial importance for G protein activators.

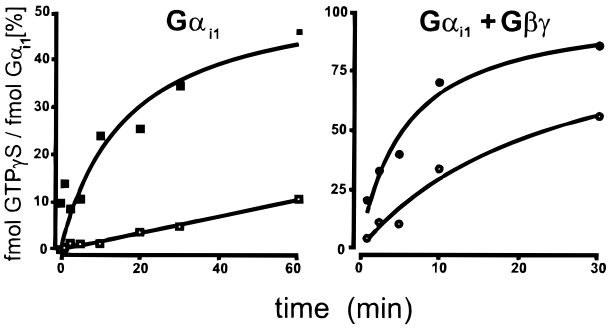


Figure 4. Effect of G$\beta\gamma$ complexes on the **21**-induced GTPγS binding to Gα_{i1}. Stimulation of GTPγS binding to Gα_{i1} or Gα_{i1} + G$\beta\gamma$ in phospholipid vesicles was determined in the presence (●,■) or absence (○,□) of **21** as described in the Experimental Section. Shown is one representative experiment each (n = 3−6). Data represent mean values from duplicate determinations.

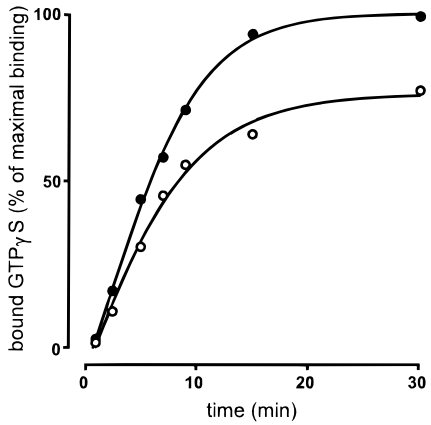


Figure 5. Binding of GTPγS to Gα_{o1} induced by **21**. Stimulation of GTPγS binding to Gα_{o1} in the presence (●) and absence (○) of **21** as described in the Experimental Section. Shown is one representative experiment out of three with similar results. Data represent mean values from duplicate determinations.

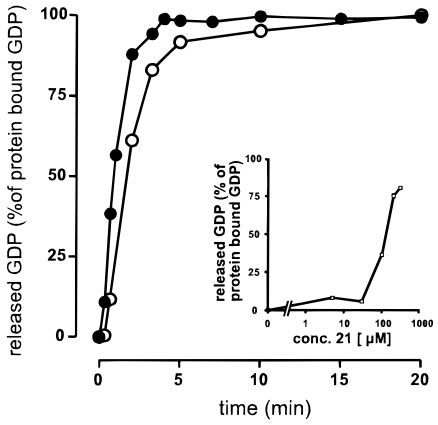


Figure 6. Release of bound GDP from Gα_{o1} induced by **21**. Release of GDP from Gα_{o1} mixed with phospholipid vesicles in the presence (●) or absence (○) of **21** as described in the Experimental Section. Shown is one out of three experiments with similar results. Data represent mean values from duplicate determinations. Inset: Release of bound GDP from Gα_{o1} induced by **21** in a concentration-dependent fashion. Release of bound GDP was measured in the presence of **21** after 1 min of incubation. Data were corrected for basal GDP release in the absence of **21**.

The GTP hydrolyses of the amides and also of the amines were highly sensitive to pertussis toxin. Among them compound **21** was studied in more detail. The

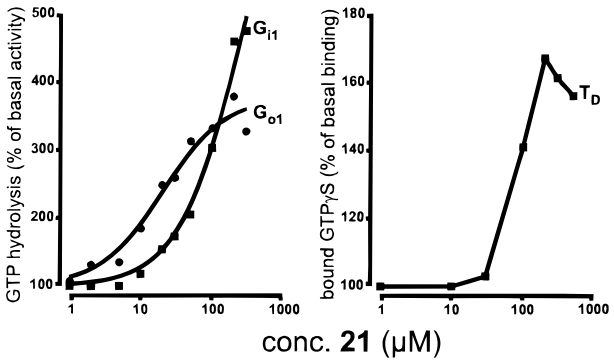


Figure 7. Activation of various PTX-sensitive G proteins by **21**. Concentration-dependent effect of **21** on GTP hydrolysis by G protein isoforms (G$_{i1}$, G$_{o1}$) and GTPγs binding (transducin) was measured as described in the Experimental Section. Shown is one out of 2−4 experiments with similar results. Data represent mean values from triplicate (left panel) or duplicate (right panel) determinations.

presented data suggest that **21** interacts with the extreme C-terminus of the Gα subunit, which is similar to receptor- or mastoparan-induced activation of G proteins. The release of bound GDP in exchange for GTP is induced by **21**. In contrast to receptors **21**-induced G protein activation does not require G$\beta\gamma$ dimers. Although PTX-sensitive G proteins exhibit a high degree of amino acid identity, **21** shows a 5-fold difference in potency among three members of the G proteins tested. These compounds are valuable tools to study G protein-dependent signal transduction pathways in cell biology and to elucidate the molecular mechanism of G protein activation.

Experimental Section

Chemistry. General Procedures. Melting points were determined on a Büchi 510 instrument or an Electrothermal IA 9000 digital apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker AC 300 (300 MHz) or a Bruker Avance-TM-DPX 400 (400 MHz) spectrometer. Chemical shifts are expressed in ppm downfield from internal Me_4Si as reference. ^{1}H NMR data are reported in the order: multiplicity (br, broad; s, singlet; d, doublet; t, triplet; m, multiplet; *, exchangeable by D_2O), approximate coupling constants in hertz, and number of protons. Mass spectra (FAB$^+$) were obtained on a Finnigan MAT CH5DF instrument (xenon/DMSO/glycerol). Elemental analyses (C, H, N) for all compounds were measured on a Vario EL instrument and are within ±0.4% of the theoretical values. Optical rotation was determined on a Perkin-Elmer 241 MC polarimeter. Yields are not optimized. TLC was performed on silica gel PF_{254} plates (Merck). Preparative, centrifugally accelerated, rotatory thin layer chromatography was performed using a Chromatotron 7924T instrument (Harrison Research) and glass rotors with 2 or 4 mm layers of silica gel 60 PF_{254} containing gypsum (Merck). Amino acids or protected amino acids were purchased from Sigma, Acros, or Neosystem Laboratoire. All compounds were characterized by ^{1}H NMR, FAB$^+$, and elemental analysis.

The dicyclohexylammonium salt of the N-protected amino acids (7.5 mmol) was dissolved in 10 mL of MeOH (0 °C) and 30 mL of 1 N HCl and stirred for 1 h. After evaporation of the MeOH in vacuo the solution was extracted three times with EtOAc. The combined organic layers were washed with water and dried over Na_2SO_4, and the solvent was evaporated to dryness under reduced pressure; yield 95%.

***N*-Alkyl Amino Acid Amides 11, 15−17, 20, 22, 23, 27, and 29.** Equimolar amounts of protected amino acids (**1, 3**−**5**, **7**−**9**) (3−11 mmol) in 20−50 mL of dry THF, 1-hydroxybenzotriazole hydrate (HOBT; for **16**, **27**, **29**), and *N*,*N*′-carbonyldiimidazole were stirred for 30−60 min under argon atmosphere. A solution of dodecyl-, tetradecyl-, or hexadecyl-amine

(equimolar amount) in 10−20 mL of dry THF was added, and the mixture was stirred overnight at ambient temperature. The solvent was removed in vacuo, and the residue was dissolved in CH_2Cl_2, extracted with saturated $NaHCO_3$ (3×), 10% acetic acid (3×), water (3×), dried over Na_2SO_4, and evaporated under reduced pressure. The resulting amino acid amides were deprotected by the following methods.

BOC Group: A solution of BOC-protected amino acid amides (3−11 mmol) in 15−25 mL of 1 N HCl was refluxed for 2 h and evaporated to dryness in vacuo. The residue was dissolved in 10−15 mL of water, alkalized with 2 N NaOH (pH 13), and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4 and evaporated to dryness under reduced pressure.

Pht Group: To a solution of phthalimido-protected amino acid amides (3−5.3 mmol) in 10−20 mL of EtOH was added hydrazine hydrate (1.1 equiv). After 1 h the mixture was acidified with 10 mL of 1 N HCl and refluxed for 2 h. The precipitate was separated, and the solution was alkalized with 2 N NaOH, extracted with CH_2Cl_2/2-propanol (3:1), dried over Na_2SO_4, and evaporated to dryness under reduced pressure.

NO_2 Group: A solution of nitro-protected arginine amide (0.60 g, 1.5 mmol) in 30 mL of MeOH, 10 mL of acetic acid, and 10% Pd/C (0.06 g) was stirred under hydrogen (1 bar) for 48 h. The mixture was filtered through Celite and washed with MeOH, and the solvent was evaporated under reduced pressure.

The deprotected alkyl amino acid amides or the nitroarginine derivative was purified by rotatory chromatography [eluent: CH_2Cl_2/MeOH (gradient from 19:1 to 15:1), ammonia atmosphere] and recrystallized from EtOH/Et_2O as a free base (**20, 22**), hydrochloride (**11**), or hydrogen maleate (**15**−**17**, **23**, **27**, **29**).

***N*-Hexadecyl L-lysine amide (29):** ^{1}H NMR (DMSO-d_6) δ 8.35 (br*, 2H, NH_2), 6.02 (s, 4H, 4 Mal-H), 3.66 (m, 1H, CH), 3.10 (m, 2H, N-CH_2), 2.74 (m, 2H, N-CH_2), 1.66−1.42 (m, 6H, 3 CH_2), 1.24 (br, 28H, 14 CH_2), 0.84 (t, J = 6.9 Hz, 3H, CH_3); MS m/z (FAB$^+$) 370 ([M + H]$^+$, 6), 84 (100), 82 (20), 55 (46), 43 (57), 41 (67), 30, (48). Anal. ($C_{22}H_{47}N_3O\cdot 2C_4H_4O_4\cdot 1.5H_2O$) C, H, N.

***N*-Dodecyl Glycylglycine Amide (13).** A solution of dodecylamine (3.71 g, 20.0 mmol), Gly-Gly(Z)-OH (**2**) (5.33 g, 20.0 mmol), and HOBT (2.97 g, 22.0 mmol) in 150 mL of dry THF was cooled to 0 °C, and DCC (4.54 g, 22.0 mmol) in 10 mL of THF was added. The mixture was stirred at 0 °C for 1 h. Then the temperature was raised to ambient temperature, and the mixture was stirred overnight; 150 mL of EtOAc and 150 mL of MeCN were added. The mixture was heated to reflux and filtered hot by gentle suction. The solution was cooled to ambient temperature, and the precipitate was isolated (6.68 g of crude product). The crude product (1.00 g) was dissolved in 100 mL of MeOH, ammonium formate (0.60 g) and 10% Pd/C (0.20 g) were added, and the mixture was stirred for 12 h. The solution was filtered through Celite, the carbon was washed with MeOH, and the solvent was evaporated under reduced pressure. To the residue was added 50 mL of 1 N NaOH, and the solution was extracted with 50 mL of CH_2Cl_2 (3×). The organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was dissolved in the minimum amount of EtOH, acidified with 5−6 N HCl in 2-propanol; afterwards 50 mL of Et_2O was added, and the precipitate (0.40 g) was isolated. Recrystallization from EtOH/Et_2O gave **13** as the hydrochloride (yield 0.25 g, 25%): mp 176−180 °C; ^{1}H NMR (DMSO-d_6) δ 8.59 (br*, 1H, NH), 7.96 (br*, 3H, NH_3^+), 3.75 (d, J = 5.7 Hz, 2H, CH_2), 3.58 (s, 2H, CH_2), 3.10−3.00 (m, 2H, CH_2), 1.40−1.30 (m, 2H, CH_2), 1.30−0.90 (m, 18H, 9 CH_2), 0.86 (t, J = 6.7 Hz, 3H, CH_3); MS m/z (FAB$^+$) 300 ([M + H]$^+$, 11), 243 (22), 186 (22), 55 (46), 41 (75), 30 (100). Anal. ($C_{16}H_{33}N_3O_2\cdot$HCl) C, H, N.

***N*-Dodecyl L-Glutamine Amide (19).** To a solution of dodecylamine (1.90 g, 10.2 mmol) and BOC-L-Gln-OH (**6**) (2.5 g, 10.2 mmol) in 40 mL of dry THF was added HOBT (1.52 g, 11.2 mmol), and the solution was cooled to 0 °C. Then a solution of DCC (2.31 g, 11.2 mmol) in 10 mL of THF was added. Stirring at 0 °C was continued for 1 h, then the temperature was raised to ambient temperature, and the mixture was stirred overnight. The precipitated urea was removed, and the solvent was evaporated under reduced pressure. Then 100 mL of CH_2Cl_2 was added, and the solution was subsequently washed with 50 mL of 5% K_2CO_3, 50 mL of 2 N citric acid, and 50 mL of water and dried over Na_2SO_4. Evaporation of the solvent gave the BOC-protected product. A solution of 10 mL of trifluoroacetic acid in 10 mL of CH_2Cl_2 was added to the residue and stirred for 0.5 h. Evaporation of the solvent under reduced pressure, dissolving of the residue in 2 mL of EtOH, and dilution with 200 mL of Et_2O gave the crude product after cooling (−20 °C). Recrystallization in EtOH/Et_2O gave **19** as the trifluoroacetate (yield 2.10 g, 48%): mp 97−98 °C; ^{1}H NMR (DMSO-d_6) δ 8.42 (br*, 1H, NH), 8.19 (br*, 3H, NH_3^+), 7.44 (br*, 1H, NH_2), 6.92 (br*, 1H, NH_2), 3.74 (t, J = 6.2 Hz, 1H, CH), 3.20−3.00 (m, 2H, CH_2), 2.20−2.00 (m, 2H, CH_2), 2.00−1.80 (m, 2H, CH_2), 1.50−1.30 (m, 2H, CH_2), 1.30−1.10 (m, 18H, 9 CH_2), 0.86 (t, J = 6.3 Hz, 3H, CH_3); MS m/z (FAB$^+$) 314 ([M + H]$^+$, 33), 297 (33), 296 (24), 186 (20), 101 (31), 84 (100). Anal. ($C_{17}H_{35}N_3O_2\cdot 0.9CF_3COOH$) C, H, N.

***N*-Dodecyl L-Lysine Amide (24) and *N*-Dodecyl D-Lysine Amide (25).** To a solution of dodecylamine (1.63 g, 8.8 mmol, or 1.17 g, 6.3 mmol) in 80 mL of CH_2Cl_2 were added 90 mL of water and a solution of BOC-L-Lys(BOC)-OH (**9**) (3.07 g, 8.8 mmol) or BOC-D-Lys(BOC)-OH (**10**) (2.18 g, 6.3 mmol) in 10 mL of CH_2Cl_2. Finely powdered HOBT (1.19 g, 8.8 mmol, or 0.85 g, 6.3 mmol) was added, and the mixture was cooled down to 0 °C. Then EDC (1.87 g, 9.7 mmol, or 1.33 g, 6.9 mmol) was added, and the temperature was kept at 0 °C for 3 h. The ice bath was removed, and the mixture was stirred for 24 h at ambient temperature. To the reaction mixture was added 50 mL of 1 N HCl, and the organic phase was separated, subsequently washed with 50 mL of 0.5 N HCl, 40 mL of brine, 40 mL of 1 N $NaHCO_3$ (2×), and 30 mL of brine (2×), and dried over Na_2SO_4. Evaporation of the solvent under reduced pressure gave 4.24 g of oil. The oil was dissolved in 50 mL of CH_2Cl_2, and 50 mL of 5−6 N HCl in 2-propanol was added and stirred for 24 h. Evaporation of the solvent and recrystallization of the residue from EtOH/Et_2O gave **24** and **25** as dihydrochlorides (yield 1.50 g, 44%, or 0.91 g, 37%): mp 105−106 or 106−107 °C.

***N*-Dodecyl L-lysine amide (24):** ^{1}H NMR (DMSO-d_6) δ 8.58 (t*, J = 5.4 Hz, 1H, NH), 8.26 (br*, 3H, NH_3^+), 8.02 (br*, 3H, NH_3^+), 3.73 (t, J = 6.1 Hz, 1H, CH), 3.20−3.00 (m, 2H, CH_2), 2.80−2.70 (m, 2H, CH_2), 1.80−1.70 (m, 2H, CH_2), 1.50−1.10 (m, 22H, 11 CH_2), 0.86 (t, J = 6.7 Hz, 3H, CH_3); MS m/z (FAB$^+$) 627 ([2M + H]$^+$, 1), 314 ([M + H]$^+$, 28), 297 (3), 295 (3), 242 (2), 186 (15), 184 (4), 129 (4), 112 (5), 84 (100), 55 (49), 43 (72). Anal. ($C_{18}H_{39}N_3O\cdot 2.3$HCl) C, H, N.

***N*-Dodecyl D-lysine amide (25):** ^{1}H NMR (DMSO-d_6) δ 8.60 (t*, J = 5.4 Hz, 1H, NH), 8.26 (br*, 3H, NH_3^+), 8.05 (br*, 3H, NH_3^+), 3.73 (t, J = 6.2 Hz, 1H, CH), 3.20−3.00 (m, 2H, CH_2), 2.80−2.70 (m, 2H, CH_2), 1.80−1.70 (m, 2H, CH_2), 1.50−1.10 (m, 22H, 11 CH_2), 0.86 (t, J = 6.6 Hz, 3H, CH_3); MS m/z (FAB$^+$) 627 ([2M + H]$^+$, 1), 314 ([M + H]$^+$, 36), 297 (3), 295 (4), 242 (3), 186 (41), 184 (7), 129 (3), 112 (2), 84 (90), 55 (60), 43 (100). Anal. ($C_{18}H_{39}N_3O\cdot 2.2$HCl) C, H, N.

Alkyl-Substituted Di- and Triamines 12, 18, 21, 26, 28, and 30. A solution of the amino acid amides (**11, 17, 20, 24, 27, 29**) (0.5−2.1 mmol) in 10 mL of dry THF was added to a suspension of $LiAlH_4$ (2 equiv; 4 equiv for **12** and **21**) in 20−50 mL of dry THF (activated *via* stirring for 30 min under reflux). The mixture was refluxed for 6−10 h under argon atmosphere. The $LiAlH_4$ was hydrolyzed with 1 N NaOH, and the organic layer was decanted. The inorganic pulp was washed three times with 40 mL of THF, and the THF phase was dried over Na_2SO_4 and evaporated under reduced pressure. The resulting oil was purified by rotatory chromatography [eluent: CH_2Cl_2/MeOH (2.5:1), ammonia atmosphere] and recrystallized as a free base (**21**) or the hydrogen maleate.

***N*-(2,6-Diaminohexyl)hexadecylamine (30):** ^{1}H NMR (DMSO-d_6) δ 6.05 (s, 6H, 6 Mal-H), 3.34 (m, 1H, CH), 3.08−2.70 (m, 6H, 3 N-CH_2), 1.54 (m, 6H, 3 N-CH_2-CH_2), 1.24 (m, 28H, 14 CH_2), 0.84 (t, J = 7.0 Hz, 3H, CH_3); MS m/z (FAB$^+$) 356 ([M + H]$^+$, 13), 98 (81), 96 (20), 84 (51), 82 (18), 81 (25),

79 (19), 70 (29), 69 (30), 67 (26), 55 (71), 43 (97), 41 (94), 30 (100). Anal. ($C_{22}H_{49}N_3 \cdot 3C_4H_4O_4$) C, H, N.

***N*-(2-Guanylethyl)dodecylamine (14).** To a solution of amine **12** (0.17 g, 0.74 mmol) in 7.5 mL of DMF was added a solution of 3,5-dimethylpyrazole-1-carboxamidine nitrate (0.30 g, 1.49 mmol) in 7.5 mL of DMF and 0.3 mL of Et_3N. The resulting solution was alkalized with Et_3N (pH 8−9) and stirred for 3 days. The solvent was evaporated under reduced pressure, and the residue was crystallized as the dinitrate from EtOH/Et_2O (yield 0.17 g, 44%): mp 119−121 °C; ^{1}H NMR (DMSO-d_6) δ 7.45 (br*, 1H, NH), 7.23 (br*, 3H, NH_3^+), 3.44 (t, J = 6.0 Hz, 2H, CH_2), 3.04 (t, J = 5.9 Hz, 2H, CH_2), 2.92 (t, J = 7.8 Hz, 2H, CH_2), 1.56 (br, 2H, CH_2), 1.25 (br, 18H, 9 CH_2), 0.86 (t, J = 6.9 Hz, 3H, CH_3); MS m/z (FAB$^+$) 271 ([M + H]$^+$, 57), 212 (13), 86 (51), 60 (69), 55 (52), 44 (92), 43 (100), 41 (98), 30 (51). Anal. ($C_{15}H_{34}N_4 \cdot 2HNO_3$) C, H, N.

Pharmacology. [^{35}S]GTPγS (1150−1400 Ci/mmol), [^{32}P]-NAD (800 Ci/mmol) and [^{32}P]orthophosphoric acid (8500−9120 Ci/mmol) were purchased from DuPont New England Nuclear (Bad Homburg, Germany). [γ-^{32}P]GTP was synthesized as described by Walseth and Johnson,[45] and each preparation was used for less than 3 weeks. The sources of all other reagents were of highest analytical grade quality or as described previously by Nürnberg et al.[46]

***In Vitro* Screening at Histamine H_1, H_2, and H_3 and Muscarine M_3 Receptors.** Selected compounds were screened for histamine H_1, H_2, and H_3 and muscarine M_3 receptor activity by standard methods described previously by Leschke et al.[24]

Preparation of HL-60 Membranes. HL-60 cells cultered in suspension were differentiated toward neutrophil-like cells with dibutyryl-cAMP (0.2 mM) for 48 h. For ADP ribosylation of G_i proteins HL-60 cells were incubated overnight with pertussis toxin (PTX; 200 ng/mL). HL-60 membranes were prepared as described previously by Wieland et al.[47]

GTPase Assay of HL-60 Membranes. GTP hydrolysis was determined as described by Wieland et al.[47] Activities were determined as triplicates, and data are calculated as mean values of at least three independent experiments. Standard deviation was less than 10%.

Sf 9 Cell Culture and Expression of Recombinant Proteins. Fall army worm ovary cells (Sf 9, from ATCC, Rockville, MD) were cultured in suspension with TNM-FH medium (Sigma) supplemented with 10% (v/v) fetal calf serum, lipid concentrate (1:200; Life Technologies, Karlsruhe), penicillin (1000 IU/mL), and streptomycin (1 mg/mL). Recombinant $G\alpha_{i1}\beta_2\gamma_2$ were expressed as detailed elsewhere using baculoviruses.[42]

Purification, Identification, and Reconstitution of G Proteins. For isolation of heterotrimeric G proteins previously published protocols were used.[48−50] Transducin α and $\beta\gamma$ subunits were from bovine retinae, whereas $G_{i/o}$, $G\beta\gamma$ complexes, $G\alpha_{i1}$, and $G\alpha_o$ were from bovine brain. Purities of G proteins were greater than 95% as determined by silver staining of 10% SDS−PAGE gels. The purified PTX-sensitive $G\alpha$ isoforms were identified by immunoreactivity to subtype-specific polyclonal antibodies[42,46,50−52] and proved to be devoid of contamination by other PTX-sensitive $G\alpha$ subunits as assessed by autoradiographic analysis after PTX-mediated [^{32}P]ADP ribosylation; standard protocols were applied for PTX-mediated ADP ribosylation.[41] [^{35}S]GTPγS binding to G proteins was performed as described elsewhere.[50] The mean value of all measurements was used for obtaining the concentration of a specific G protein preparation. Purified G protein preparations were stored at −70 °C. Heterotrimeric G proteins were reconstituted into phospholipid vesicles (Azolectin) as reported.[43] G proteins were added to the solubilized lipids. Lipids (1%, w/v) were solubilized together with a 10-fold excess of crystallized sodium cholate (w/v) in a buffer consisting of Hepes (20 mM, pH 8.0), NaCl (100 mM), DTT (1 mM), and EDTA (0.1 mM) at 4 °C. G proteins (40 pmol) were mixed with 60 μL of cholate-solubilized lipids to a final volume of 600 μL and kept on ice for 1 h. Vesicles containing G proteins were formed by passing the solution through an AcA 34 gel filtration column and used immediately after reconstitution.

GTPγS Binding, GDP Release, and GTPase Activity of Purified G Proteins. Determination of activities of purified G protein preparations was performed as described previously.[46,53,54]

Miscellaneous. Protein was determined according to the literature[55] with modifications.[56]

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