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Invited review

Non-peptide G-protein activators as promising tools in cell biology and potential drug leads

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Abstract – Heterotrimeric G proteins are molecular switches connecting cell surface receptors with effectors but also eliciting modulatory functions on various endomembranes. A hallmark of G proteins is their guanine nucleotide sensitivity. While GDP is bound to Gα in its inactive form, ligand bound receptors stimulate Gα to release GDP and to bind GTP which triggers activation of the G protein. In addition to heptahelical receptors, other cellular proteins, some bacterial and insect toxins, and an array of pharmaceutical compounds were reported to activate or inhibit G proteins. Based on these observations low molecular weight alkyl-substituted amino acid amides and analogues were designed as potent G protein activators. This review details the development of non-peptide receptormimetics from the lead compound 2-(3-chlorophenyl)histamine **3** to compounds like N-(2,5-diaminopentyl)dodecylamine **42** indicating first selectivity among G-protein isoforms. These compounds act similar to heptahelical receptors by catalyzing the release of GDP from Gα. Therefore the successful development of G-protein activators supports the concept of employing specifically designed pharmaceutical tools for direct G protein modulation. © Elsevier, Paris

direct G-protein activator / G-protein-dependent diseases / G-protein modulation / receptor mimetics / signal transduction

1. Introduction

A great number of ligands act through binding to G-protein-coupled receptors (GPCR) [1–5]. These signaling molecules comprise endogenous ligands of remarkable chemical heterogeneity, e.g., aminergic or amino acid neurotransmitters, peptide- or lipid-based hormones, chemokines, or nucleotides. Moreover, sensory chemical or physical stimuli like odorants, flavors, or light are recognized by this class of receptors. Even bacterial cell wall constituents trigger heptahelical receptors of the infested organism, and viruses like the human immunodeficiency virus (HIV) bind to this class of cell surface receptors augmenting their reception into mammalian cells [6, 7]. In some cases viruses themselves express GPCR to control the host cell [8]. These pivotal and universal roles of heptahelical receptors easily explain why two out of three drugs prescribed in Germany are assumed to exert their actions through interfering with GPCR signaling [9].

Transmembrane signaling elicited by ligand bound GPCR requires the sequential and hierarchical intermolecular interaction of at least three signaling modules, (i) the heptahelical cell surface receptor as the sensor, (ii) the heterotrimeric G protein as the signal transducer, and (iii) various cellular effectors such as enzymes, transporters,

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Abbreviations: amino acids: ala, alanine; asn, asparagine; asp, aspartate; cys, cysteine; glu, glutamate; leu, leucine; lys, lysine; trp, tryptophane; CT, cholera toxin, an exotoxin from vibrio cholerae; GABA, γ-amino butyric acid; GDI, GDP-dissociation inhibitor; GPCR, G-protein-coupled receptor; G protein, regulatory heterotrimeric guanine-nucleotide-binding protein; Gαβγ, G-protein alpha-, beta-, gamma-subunit complexed with GDP; GppNHp, guanosine 5'-*O*-[β,γ-imino]triphosphate; GRK, G-protein receptor kinase; GTPγS, guanosine 5'-*O*-[γ-thio]triphosphate; NEM, *N*-ethylmaleimide; M6P/IGF II receptor, mannose-6-phosphate / insulin-like growth factor II receptor; PKA, protein kinase A; PKC, protein kinase C; PT, pertussis toxin, an exotoxin from bordetella pertussis.

and ion channels [10–13]. With hundreds of receptors as well as dozens of G-protein isoforms and effector molecules known and more being discovered all the time the molecular diversity on each level of this signaling machinery is tremendous. However, the vast majority of therapeutically relevant pharmacological interventions are restricted to the receptor level (i.e., agonists, antagonists, and inverse agonists) and in a limited number of cases to the effector level (e.g., ion channel blockers, phosphodiesterase inhibitors). But the assumed number of heterotrimeric G-protein combinations based on currently known 23 Gα, 7 Gβ, and 11 Gγ isoforms may be more than a hundred assigned to distinct cellular pathways [14]. This proposed heterogeneity argues for G protein modulation as a useful principle for pharmacological intervention. Furthermore, a cell- or tissue-specific expression of various G proteins augments selective targeting by modulators. There are additional reasons to appreciate G proteins as the site of action of specific cellular tools to dissect their roles. On the one hand, a large body of evidence implies that G proteins represent not only switches for transmembrane signal transmission, but are also important intracellular regulatory modules located on endomembranes [15, 16]. While various functions have been assigned to intracellular G proteins the nature of the physiological G-protein stimuli remains to be determined [17, 18]. On the other hand, cell surface G proteins function as intersections of diverging and converging intracellular signaling pathways. The coupling of a given GPCR to multiple G proteins produces branch points enabling different signal transduction routes at the same time [19–21]. Accordingly a considerable number of receptors initiate more than one signaling pathway. For instance the histamine H_2 receptor was shown to couple simultaneously to two different G proteins thereby activating phospholipase C and adenylyl cyclase via G_q and G_s proteins [22, 23]. Even more impressive was the observation that the thyrotropin (TSH)-receptor coupled to G proteins of all four subfamilies [24]. Vice versa different receptors expressed in the same cell are functionally linked to the identical type of G protein. Various chemoattractants including C5a, formyl peptides, interleukin-8, LTB_4, or platelet-activating factors bind to distinct cell surface receptors of leukocytes which all stimulate G_i proteins initiating gradient sensing movement of neutrophils [25]. In this example G proteins can be illustrated as bottle necks of intracellular signal transmission. The indicated complexity of G-protein signaling suggests that it is not the receptor but the downstream G protein that determines the effector pathway eliciting different types of cellular responses. This is underscored by the fact that not only GPCR but also G proteins have been implicated as key elements in pathogenic processes including Alzheimer's disease or as oncogenes associated with numerous tumors [26–31] *(table I)*. Additionally, bacterial and insect toxins have been demonstrated to exert their biological actions by targeting G proteins [32–34].

This review therefore intends to encourage the concept of employing specifically designed pharmaceutical tools for direct G-protein modulation in the field of cell biology and pharmacology. The first section will give a concise overview on G-protein-dependent cellular signaling and physiological and pharmacological interventions. In the second section we will discuss recent developments of synthetic non-peptide G-protein modulators.

2. G-protein-coupled receptors

The functional hallmark of GPCRs or serpentine receptors is represented by its GTP-sensitivity of agonist binding, whereas characteristic structural features are the assumed seven-transmembrane-spanning helices connected by three extracellular and three intracellular loops [5, 35]. The N-terminus with potential N-glycosylation sites is located outside the cell. In some receptors parts of the C-terminus form a fourth intracellular loop through insertion of one or two cysteine-linked palmitates 12 to 15 residues downstream the seventh transmembrane helix into the cytoplasm membrane. The vast majority of receptor ligands bind to the extracellular and certain transmembrane portions of the receptor, initiating a conformational change which in turn forces the associated G protein to exchange GDP for GTP *(figure 1)* [9]. It is the nucleotide exchange which eventually triggers dissociation of the heterotrimeric G protein and its subsequent coupling to cellular effectors. The receptor structures interacting with the G protein include regions of the second and third intracellular loop as well as proximal parts of the C-terminus [36]. Currently only hypotheses speculate about the molecular mechanisms of ligand-induced receptor activation. For instance, it is proposed that ligand binding leads to charge neutralization within the transmembrane helices thereby enabling their lateral rearrangement, whereas other data suggest that receptor activation involves rigid body motion of transmembrane helices [37–39]. Nevertheless, it is generally believed that conformational changes of the intracellular loops lead to activation of the G protein. However, the molecular basis of the specificity of interaction of GPCR to one or more G proteins is not fully understood yet. Experimental evidence suggests that a receptor exists in multiple active conformational states; thereby each one may be responsible for a different step in

Table I. Human diseases linked to the G-protein pathway (data taken from [26–28]).

Disease	Defective G-protein coupled receptor [a]	Functional consequence [a]
Familial hypoparathyreoidism	Parathormone calcitonin (PTHR) receptor	↑
Neonatal severe hyperparathyreoidism	Parathormone calcitonin (PTHR) receptor (homozygous)	↓
Sporadic hyperfunctional thyreoid nodules	Thyrotropin (TSH) receptor	↑
Hyperthyreoidism (thyreoid adenomas)	Thyrotropin (TSH) receptor	↓
Familial hypothyreoidism	Thyrotropin (TSH) receptor	↓
Familial male precocious puberty	Luteinizing hormone (LH) receptor	↑
Male-pseudohermaphroditism	Luteinizing hormone (LH) receptor	↓
X-linked nephrogenic diabetes insipidus	V_2 Vasopressin receptor	↓
Retinitis pigmentosa	Rhodopsin receptor	↓↑
Congenital night blindness	Rhodopsin receptor	↓
Retinal degeneration	Rhodopsin receptor (point mutation lysine 296)	
Colour blindness, spectral sensitivity variations	Cone opsin receptor	↓
Familial glucocorticoid deficiency and isolated glucocorticoid deficiency	Adrenocorticotropic hormone (ACTH) receptor	↓
Hirschsprung disease	Endothelin B receptor	↓
Jansen metaphyseal chondrodysplasia	PTH/PTHrP receptor	↑
Congenital bleeding	Thromboxan A_2 (TXA_2) receptor	↓
Disease	**Defective G protein**	**Functional consequence [a]**
Albright's hereditary osteodystrophy and pseudohypoparathyreoidisms	$G_{s\alpha}$	↓
McCune–Albright syndrome	$G_{s\alpha}$	↑
Pituitary, thyroid tumors (*gsp* oncogene)	$G_{s\alpha}$	↑
Combined precocious puberty and pseudohypoparathyreoidism Ia		↓
Adrenal cortex adenomas	$G_{s\alpha} + G_{i\alpha}$	↑
Adrenocortical, ovarian tumors (*gip* oncogene)	$G_{i\alpha}$	↑

Abbreviations: gsp, gip oncogene: G_s or G_i-protein oncogenes
[a] ↑: gain of function; ↓: loss of function

G-protein activation like GDP release, GTP binding, Gα–Gβγ dissociation, and Gα-receptor release. Additionally, different receptor loop regions and their specific conformation exhibit preferences for different G proteins [3].

Intermolecular coupling of GPCR to G proteins allows a tremendous amplification of the hormonal signal since one ligand-bound receptor activates many G proteins within a short time despite the fact that cellular responses are usually rapidly attenuated. On the receptor level signaling is switched off by regulatory mechanisms such as desensitization, endocytosis, and down regulation of the receptor [40]. Involved regulators of G-protein-mediated signaling include serine/threonine kinases which can be subdivided on the basis of the underlying mechanism. Second messenger kinases like protein kinases A (PKA) and C (PKC) contribute to heterologous desensitization by receptor phosphorylation. Another mechanism of phosphorylation is mediated by G-protein receptor kinases (GRK) leading to agonist-dependent homologous desensitization in concert with cofactors termed arrestins. The latter are supposed to disrupt receptor-G-protein interaction and to be involved in agonist-induced receptor endocytosis. Interestingly, G proteins also contribute to these mechanisms since some members of the GRK family were shown to interact directly with G-protein βγ complexes [41].

3. Structure and function of G proteins

3.1. General considerations

Guanine nucleotide binding proteins are members of a superfamily of GTPase enzymes (EC 3.6.1) which function as versatile cyclic molecular switches [42, 43, 44]. They are subdivided into monomeric GTPases and heterotrimeric G proteins (or G proteins). Monomeric GTPases fall into five subfamilies, namely Ras, Rab, ARF, Ran, Rho, and are found in the cytoplasm as well as membrane-associated [45]. They are involved in diverse

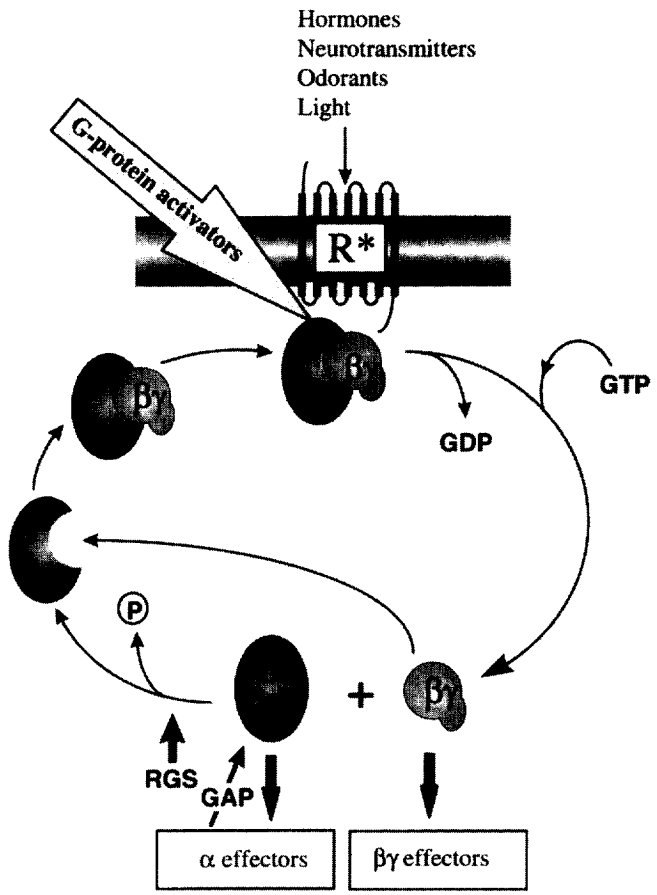


Figure 1. Activation and inactivation cycle of heterotrimeric G proteins. Abbreviations: GAP = GTPase activating proteins; RGS = regulators of G-protein signaling; α, β, γ: subunits of heterotrimeric G proteins; R*: receptor; →: interaction.

cellular events such as transmission of hormonal signals, modulation of cell growth and development, protein transport, cytoskeleton architecture, vesicular functions, and exocytosis [10, 11]. Essentially the same functions have been assigned for heterotrimeric G proteins which are best known as cytoplasmic membrane-tethered transducers though they are also key players on endomembranes [46].

G proteins are composed of three different subunits termed α, β, and γ, with molecular masses of approximately 39 to 52 kDa, 35 to 39 kDa, and 6 to 8 kDa, respectively [12]. On the basis of their Gα amino acid similarity they are classified into four subfamilies, i.e., G_s, G_i, G_q, and G_{12} *(table II)* [47]. Nucleotide sequence analysis shows that the genes of two subfamilies, i.e., G_i and G_q, are closely related, suggesting that the members of these subfamilies segregated as pairs of closely linked genes, whereas members of the G_s and G_{12} subfamilies segregated as unlinked genes during evolution [48]. Whereas G_i proteins, i.e., G_{i1}, G_{i2}, and G_{i3}, are highly homologous proteins (> 85% similarity of amino acid residues) the G_{12} proteins share only 67% similar residues. Meanwhile the number of cloned and sequenced Gβ and Gγ subunits have considerably increased comprising 7 Gβ and 11 Gγ isoforms (see *table II*) [49, 50]. Among Gβ subunits, $G\beta_5$ exhibits the largest difference in amino acid sequence with 53% identity to other Gβ subunits, whereas the deduced amino acid sequences of $G\beta_1$ to $G\beta_4$ display a high degree of homology with at least 79% identity [51]. Interestingly, $G\beta_5$ was proposed to selectively couple only to members of the $G\alpha_q$ subfamily [52]. Gβ subunits also contain essential structural attributes necessary for G-protein-subunit and G protein–protein interactions. They belong to the family of WD-repeat proteins [53]. Their common feature is a conserved core of the repeating unit (n = 4–8) of 36 to 46 amino acids usually ending with a Trp-Asp (WD). WD-repeat proteins regulate different cellular functions, such as cell division, cell-fate determination, gene transcription, transmembrane signaling, mRNA modification, and vesicle fusion. Furthermore, Gβ subunits contain coiled-coil forming structures interacting with other subunits at their N-terminal domain (Met^1–Ser^{31} of $G\beta_1$) [54–56]. Coiled coils are stabilized protein structures resulting from the interaction of two or more right-handed α-helices that wind around each other in a left-handed supercoil *(figure 2)* [57–61]. In contrast to Gβ isoforms Gγ subunits exhibit a higher sequence diversity, e.g., the bovine retinal $G\gamma_1$ subunit and the $G\gamma_2$ subunit are only 36% identical [62]. The differences in primary structures argue for an important role of Gγ (beside Gα subunits) in signal transduction [49, 63, 64].

3.2. G-protein structure

Crystal structure analysis has gained three dimensional pictures of G proteins with an enormous impact on the understanding of how G proteins function on the molecular basis (see *figure 2*) [44, 65]. Recently, the first crystals were generated showing the interaction of G proteins with regulators such as phosducin and regulators of G-protein signaling (RGS) or effectors like adenylyl cyclase [58, 66]. Interestingly, only the Gα-subunit, but not Gβγ, undergoes major structural changes upon activation. Hence the conformation of the GDP-liganded Gα in a Gα,β,γ heterotrimer is different from GTPγS- or GDP-liganded Gα alone. The Gα-subunit consists of two major parts, i.e., a GTPase or Ras-like domain and an

Table II. (a) Classifying and functional properties of Gα-proteins.

Family	Subtype	Incidence	Effectors	PTX-/CTX-sensitivity
G_s	$G_{s(s)}$, $G_{s(l)}$ [a]	ubiquitous	AC ↑ Ca^{2+}-channels ↑	CTX
	$G_{s(xl)}$ [a]	?	AC ↑	CTX
	G_{olf}	olfactory epithelium	AC ↑	CTX
G_i	Transducin ($G_{tr,c}$)	retina	cGMP-PDE ↑	CTX, PTX
	Gustducin	taste cells	cGMP-PDE ↑ ?	CTX, PTX
	G_{i1}	predominantly neuronal	AC ↓ K^+-channels ↑	PTX, (CTX)
	G_{i2}, $G_{i2(l)}$ [a,b]	ubiquitous	AC ↓ PI3Kγ ↑ K^+-channels ↑	PTX, (CTX)
	G_{i3}	predominantly non-neuronal	AC ↓ PI3Kγ ↑ K^+-channels ↑	PTX, (CTX)
	$G_{o1,2,3}$	neuronal neuro-endocrine, heart	Ca^{2+}-channels ↓	PTX, (CTX)
	G_z	platelets neuronal	AC ↓?	
G_q	G_q	ubiquitous	PLC-β ↑	
	G_{11}	non-haematopoietic cells	PLC-β ↑	
	G_{14}	testis, spleen, kidney	PLC-β ↑	
	$G_{15/16}$ [c]	haematopoietic cells	PLC-β ↑	
G_{12}	G_{12}	ubiquitous	Rho-proteins	
	G_{13}	ubiquitous	Rho-proteins	

Abbreviations and symbols: AC, Adenylyl cyclase; cGMP-PDE, cGMP-dividing phosphodiesterase; PLC-β, phospholipase C-β; PTX, pertussis toxin; CTX, cholera toxin; ↑ = stimulation; ↓ = inhibition; PI3Kγ, phosphoinositide-3-kinase γ

[a] (s) short, (l) long, and (xl) extra long splice variants of Gα subunits

[b] PTX-insensitive

[c] G_{15} and G_{16} species variants in mouse and human, respectively

α-helical domain connected by two flexible linker loops. Between these two domains lies a deep cleft in which the guanine nucleotide is tightly bound. Despite a low level of amino acid identity the structure of the GTPase domain

Table II. (b) Gβ and Gγ subunits of heterotrimetric G-proteins.

Gβ subunits		Gγ subunits		
Subtype	Incidence	Subtype		Incidence
$β_1$	ubiquitous	Family I:	$γ_1$	retina (rods)
$β_2$	ubiquitous		$γ_9$	retina (cones)
$β_3$	ubiquitous		$γ_{11}$	non-neuronal
$β_{3(s)}$	? platelets			
$β_4$	neuronal, lung			
$β_5$	neuronal	Family II:	$γ_5$	placenta, liver
$β_{5(l)}$	retina		$γ_{10}$	ubiquitous
		Family III:	$γ_2$	ubiquitous
			$γ_3$	neuronal, testis
			$γ_4$	widespread
			$γ_7$	widespread
			$γ_8$	neuronal, olfactory cilium
			$γ_{12}$	ubiquitous

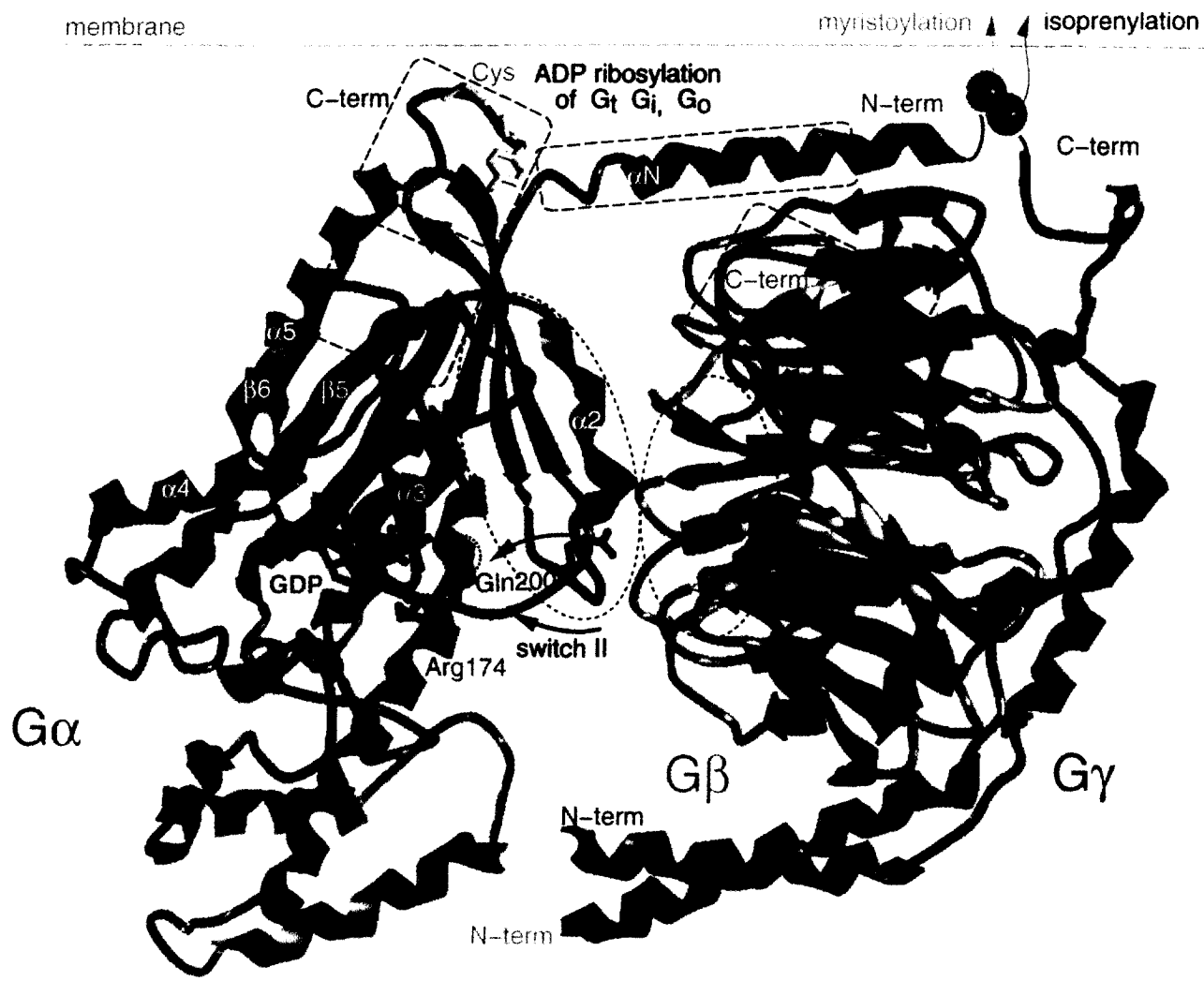


Figure 2. Structure of heterotrimeric G protein based on $G\alpha_t\beta\gamma$ [57] in the inactive state, with bound GDP (magenta) in Gα-subunit (blue), complexed with Gβ-subunit (orange) and Gγ-subunit (red). Incorporation of GTP with a γ phosphate moiety (dotted magenta circle) causes side chain movement of Arg174 and Gln 200 in Gα subunit. Reorganisation of helix α2 towards the γ phosphate takes place (switch II), followed by the dissociation of Gα and Gβγ. Binding regions for intracellular loops of heptahelical receptors are indicated by boxed long-dashed lines in Gα (N-terminal α-helix-αN; C-terminal α5-helix prolongation, α4–β6 loop) and Gβ (C-terminal region). Binding regions for effectors, indicated by oval short-dashed lines and occupied in the complexed state, are only properly accessible in Gα and Gβ subunits in the dissociated state. Helix-like conformation of the last ten C-terminal residues (green) of Gα subunit is homologously modeled based on structures Gα–RGS complex [58], $G\alpha_{i1}$–GDP complex [59], $G\alpha_s$–GTPγS complex [60]. ADP ribosylation at the cystein (yellow) occurring four residues before the C-terminus of $G\alpha_t$, $G\alpha_i$, and $G\alpha_o$ hinders the binding of receptors in the boxed regions of the Gα subunit. Conclusions that these areas must be orientated towards the membrane are supported by the close proximity of myristoylation (N-terminal of Gα, pink sphere) and isoprenylation sites (C-terminal of Gγ, violet sphere) anchoring Gαβγ onto the membrane. This common membrane anchor region is acting as a pivot to keep the interacting points in the right orientation towards the membrane.

is almost the same within other members of the GTP-binding class of proteins [67–70]. The helical domain has no known function, but there is evidence that it might act as an internal GAP (see below). Whereas most amino acids of Gα remain in the same conformation regardless whether GDP or GTP is bound, some of the residues show flexibility depending on the nucleotide binding state. Three segments, i.e., switch I, II, and III regions,

and the N- and C-terminus change their conformation upon guanine nucleotide exchange. While effector specificity is primarily determined by switch II helix α2 and the α3–β5 loop (see *figure 2*, oval short-dashed lines), receptor-contact sites include the C-terminal prolongation of helix α5, the α4–β6 loop, and the N-terminal helix αN of Gα (see *figure 2*, boxed long-dashed lines). Of the amino acids interacting with the guanine nucleotide glutamine^{200} and arginine^{174} (Gln^{204} and Arg^{178} in $G\alpha_{i1}$) are mobile residues which do not bind nucleotides in the ground state but interact tightly with substrates (GTP and bound water) during hydrolysis of GTP. Arg^{174} stabilizes developing charge in the transition state while Gln^{200} orients and polarizes the attacking water in GTP hydrolysis. Both residues are present on comparable loci found in all heterotrimeric Gα subunits. They were previously predicted as a prerequisite for GTPase activity of various G proteins based on site-directed mutagenesis experiments [71]. This arginine residue is sensitive to cholera toxin (see below). Interestingly, the monomeric GTPase p21ras has no arginine in a position analogous to Arg^{174} (in $G\alpha_t$) thus explaining the enzyme's weak hydrolytic activity and its inability to bind the G-protein-activating AlF_4^- complex [72].

Gβγ behaves as a functional monomer dissociating only under denaturing conditions. The Gβγ complex binds and stabilizes only the GDP-bound form of Gα (see *figure 2*). The Gβ subunit folds into a highly symmetric β propeller formed by amino acid sequences belonging to the WD-repeat motif (see above) [73]. Each of the seven propeller blades consists of a small four-stranded twisted β sheet, where the innermost β strand is positioned nearly parallel to the axis of a central tunnel. The conserved GH-X-WD cores of the WD-repeat motif predominantly contribute to three inner strands of each blade, which can be viewed as connectors linking the conserved cores and form the loop connecting the two outer strands and the most outer strand. Hence, each WD repeat does not form one blade structure, but rather overlaps two β blades. WD repeats 2 and 6 are the most divergent among the seven. The N-terminal domain including some 47 amino acids forms an extended polypeptide chain that girds the top of the propeller. The interface between Gβ and the serpentine Gγ which makes very few contacts with itself is extensive. The parallel α-helical coiled coil formed by the N-termini of the two subunits is a notable feature that had been predicted from sequence analysis (see above). Most of the Gγ subunit is stretched along the side and bottom of the Gβ subunit containing blades 5, 6, 7, and 1 (see *figure 2*). The loops and turns on this surface must retain the ability to bind Gγ and to discriminate among different Gγs. The docking of Gα to Gβγ involves extensive contacts, i.e., binding of the Gα N-terminal α helix to the side of the Gβ propeller parallel to its central tunnel and binding of the catalytic domain of Gα to the top surface of the β propeller.

Recently a splice variant of $G\beta_3$ was discoverd coding for a short $G\beta_3$ ($G\beta_{3s}$) lacking 41 amino acids from the middle of the peptide chain [74]. Surprisingly, the aberrant splice removes one of seven propeller blades which nevertheless folds a functional Gβ subunit in cells which may significantly contribute to the risk of hypertension. The existence of a functional $G\beta_{3s}$ suggests that the apparently rigid propeller structure of Gβ is more plastic than previously anticipated [75].

The heptahelical receptor with its cationic cytoplasmatic loops has probably free access to a large surface of Gβ which is remarkably negative and includes C-terminal blades 6 and 7 [76]. The cytoplasmic loops may also contact much of Gγ and the mouth of the tunnel through the propeller. The receptor probably interacts with the N-terminus of Gα, displacing it from the surface of Gβγ and thus promoting subunit dissociation [36]. Also, interaction of the receptor with the C-terminus of Gα is clearly important. Experimental data obtained from Gα chimeras show that the last five C-terminal residues of Gα mainly determine the selective recognition of Gα subtypes (e.g., G_i, G_s, G_q) by receptors [77].

In its heterotrimeric conformation G-protein subunits are not able to interact with effectors. Since Gα blocks interaction of Gβγ with all its effectors known, without inducing a conformational change in Gβγ, it is likely that Gα sterically interferes with binding of Gβγ to effectors. While Gα- and effector-binding sites may overlap, they are surely not identical, as peptides from Gβ sequences 84–143 interferred in transmitting signals from Gβγ to adenylyl cyclase [78]. This proposes a common effector binding surface on Gβγ. Mutational analysis of Gβ further suggests that each signaling partner for Gβ such as effectors or other Gβγ binding proteins relies on a different subset of Gβ residues for its interaction and hence, creates a set of unique 'footprints' on Gβ [79]. The Gα-binding surface on Gβγ may not be the only region of effector interaction. Other Gβγ regions of effector interaction include the coiled-coil N-termini of Gβ and Gγ and the C-terminus of Gβ. Regions of $G\alpha_s$ identified to interact with the cytoplasmic loops of adenylyl cyclases are the switch II helix (residues 225 to 240) and the α3–β5 loop [58]. In addition, the α4–β6 loop may also interact with adenylyl cyclases [80].

Research employing the oligonucleotide antisense technology (see below) revealed a surprisingly high specific G-protein subunit composition transducing signals from receptors to effectors [14]. However, the ob-

Table III. Effectors and corresponding references.

	Gα	Gβγ	Reference
Adenylyl cyclases	↑↓	↑↓	[84]
Phospholipases C-β	↑	↑	[85]
Phospholipase A_2		↑	[86]
cGMP-phosphodiesterase	↑		[87]
Phosphatidylinositide-3-kinase γ	↑	↑	[88, 89]
ERKs (over src-like tyrosine kinases)		↑	[90]
β-adrenergic receptor kinases		↑	[91]
Ca^{2+}-channels	↑	↓	[92, 93]
K^+-channels (GIRK)	↓	↑	[94, 95]
Na^+-channels	↑↓		[96, 97]
trpl-cationic channel	↑		[98]
'Pheromone response' (yeast)		↑	[99]

ERK = Extracellular-signal-regulated kinase, GIRK = G-protein-coupled inwardly rectifying K^+ channel, trpl-cationic channel = transient receptor potential-like cationic channel; ↑ = stimulation, ↓ = inhibition

served specificity of heterotrimer subunit composition appears to be restricted to the integrity of the cell and is partially lost in isolated cell membranes [81].

3.3. Activation of G proteins

G proteins undergo a cycle of activated and inactivated states allowing reversible and specific transmission of hormonal signals (see *figure 1*) [13, 82]. Activation of the G protein is stimulated by its interaction with the GPCR, resulting in the release of bound GDP followed by high affinity binding of cytosolic GTP. In the absence of a receptor the first event is rate-limiting. 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 [83]. Upon G-protein activation, Gα dissociates from the Gβγ complex which does not necessarily result in a physical separation (see below). Both the activated Gα subunit and the Gβγ complex modulate effector proteins *(table III)* until hydrolysis of the GTP bound to the Gα subunit turns off signaling (intrinsic GTPase activity). For receptor-activated G proteins this is the rate-limiting reaction. Hence, the kinetic of hydrolysis is subjected to physiological modulation by cellular components that function as negative regulators of G protein signaling (see below). Following GTP hydrolysis, the inactivated GDP-bound Gα subunit dissociates from the effector and reassociates with Gβγ. This heterotrimer becomes available for subsequent activation cycles. Recently the firmly established hypothesis that GPCR govern their effectors indirectly via a shuttling mechanism involving the exchange of heterotrimeric G proteins or their subunits between ephemeral receptor–G protein and G protein–effector complexes is challenged by experimental evidence suggesting the existence of receptor–G protein–effector complexes [100, 101].

3.4. Molecular mechanisms of G-protein activation

An assumed scenario is that distinct conformational states of the receptor loops seem to influence different steps during the mode of activation of Gα. Structures of such transition states of activation are not available yet. However, superimposition of the dissociated state [67] and the complexed inactive state [57] of Gα reveal clear conformational changes at the α4–β6 loop and the C-terminus (see *figure 3*). Both sites are covered from two synthetic peptides of the last eleven residues and residues 311–329 of $G\alpha_t$ (red coloured in *figure 3*), which compete with $G\alpha_t$ for binding on activated rhodopsin. In addition to the above-mentioned receptor-coupling ability of the N-terminal helix αN, the $G\alpha_{i1}$–GDP structure-complex suggests that the N-terminus might function as a GDP-activated switch [59]. The C-terminal residues are not cristallized in both structures mentioned. There are several hints taken from the RGS bound Gα structure [58], from $G\alpha_{i1}$–GDP structure-complex, and from homology modeling that these residues possess propensity for helix-like conformation. These assumptions were underlined very recently by structure-study of the C-terminal sequence of $G\alpha_t$ (340–350) by means of transfer-NOE spectroscopy [102]. The C-terminal α5-helix formed in the GDP-bound state is partially unwinded during activation and has been proposed to function as an internal GDP-dissociation inhibitor (GDI) [103, 104]. Taken together, the vicinity of C-terminus, α4–β6 loop and helix αN of Gα play a key role in triggering GDP release. A collision coupling of receptor

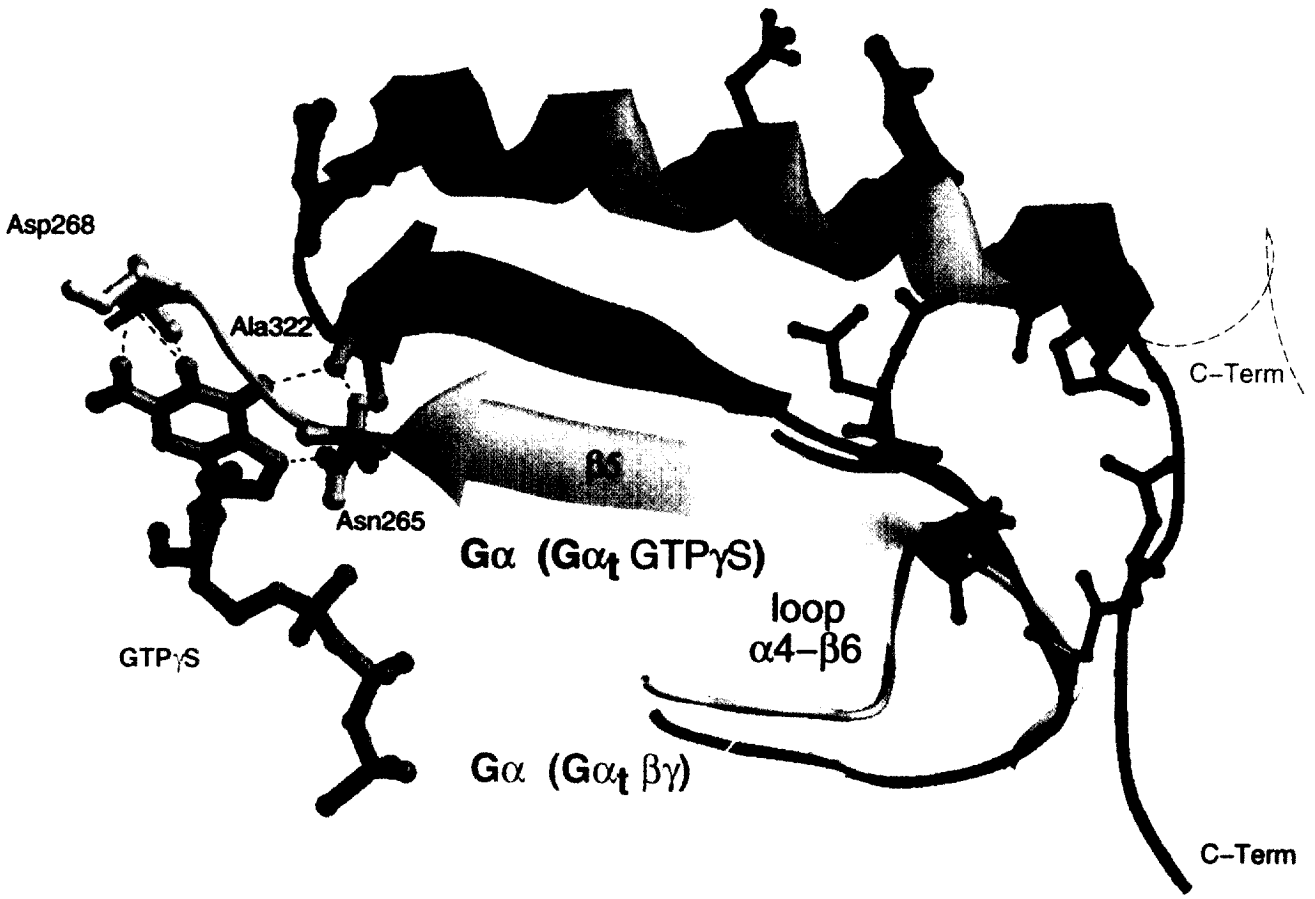


Figure 3. Structural detail of the C-terminal and α4–β6 loop region of the Gα subunit. Superimposition of the active, dissociated state in grey, magenta and red $G\alpha_t GTP\gamma S$ [67], and the inactive, complexed state in blue $G\alpha_t\beta\gamma$ [57]. The putative helix-like conformation of the last ten Gα C-terminal residues, missing in the $G\alpha_t\beta\gamma$ structure, is drawn as a blue dashed line. Red parts represent peptides in $G\alpha_t$ sequence, which bind to Rhodopsin and antagonize signal transduction. Clear conformational changes occur at the α4–β6 loop and the C-terminus region, indicating an additional involvement in receptor binding. Conformational changes upon receptor binding are transduced along the β-strands β5 and β6 towards the highly conserved residues (Ala322-backbone, Asn265, Asp268) at their C-terminal ends interacting with the nucleotide GDP or GTP. During the transition state (release step) restraints on GDP release are relieved, finally hydrogen bonds are broken, and GDP is released. After association of GTP (yellow, GTPγS) and reassembly of the hydrogen bonds, a dissociation step via conformational switches I, II and III (not shown, II: see *figure 2*) causes the detachment of the Gα subunit from the Gβγ complex. Non-peptide Gα-activators probably interact with the Gα subunit at the same site with a similar mode of action.

loop(s) at this site alters temporary conformations by relieve of restraints on GDP release. Structural details in *figure 3* support the hypothesis of nucleotide exchange by transducing conformational changes upon receptor coupling at the C-terminus vicinity along the β-strands β5 and β6 towards the highly conserved residues (Asn^{265}, Asp^{268}, and backbone of Ala^{322}) at their C-terminal ends interacting with the nucleotide GDP or GTP. As a consequence hydrogen bonds are obviously broken, and GDP is released. After association of GTP and reassembly of the hydrogen bonds, a dissociation step via conformational switches I, II, and III causes the detachment of Gα subunit from the Gβγ complex and finally the ability of coupling to the effectors. Non-peptide Gα-activators (see below) probably interact with the Gα subunit at the same site with a similar mode of action *(figure 4)*.

Interestingly, the GDP/GTP-exchange reaction varies among different G proteins: G_i proteins are fast nucleotide exchangers, G_s, G_z, and G proteins of the G_q-

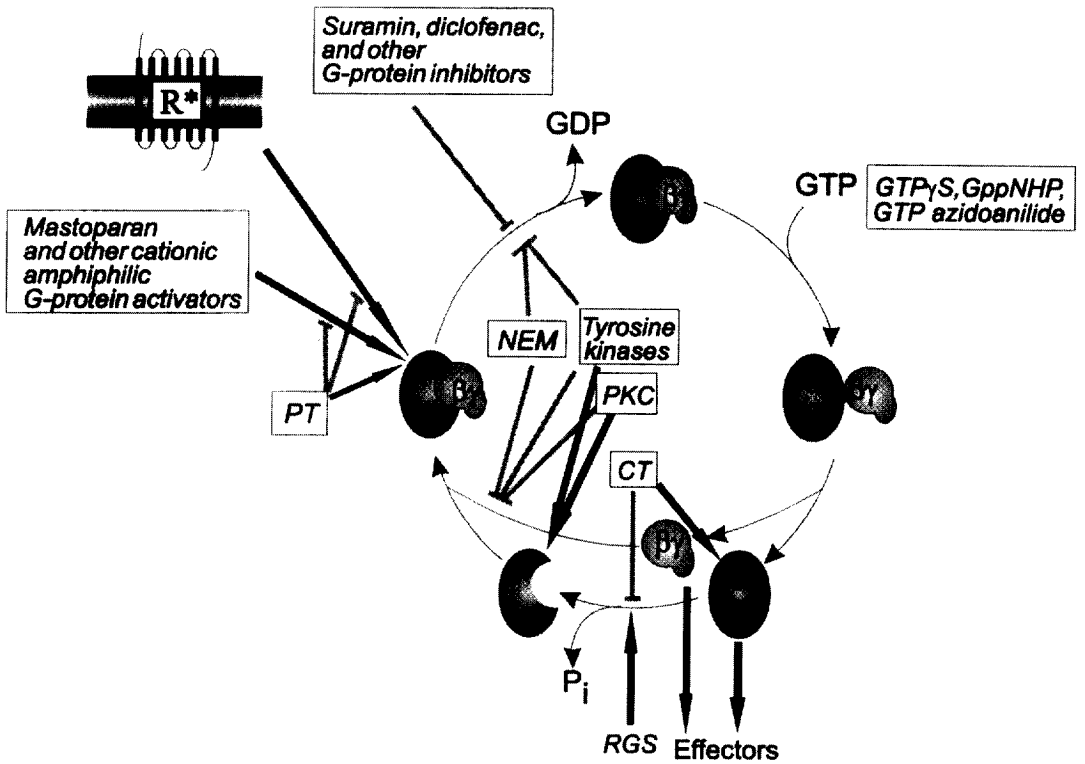


Figure 4. Modulation of G-protein activity by cross-talk and toxins. For details see text. Abbreviations: α and βγ: α and βγ subunits of heterotrimeric G proteins; PT: pertussis toxin; CT: cholera toxin; NEM: N-ethylmaleimide; PKC: protein kinase C; RGS: regulators of G-protein signaling; GTPγS, GppNHP and GTP azidoanilide, GTP analogues; →: interaction; —|: blockade.

subfamily show smaller rate constants, and for transducin, G_{12}, and G_{13} very small guanine nucleotide exchange rates have been reported [105–107]. Supplementary to this well established mechanism of G-protein activation, an alternative route was postulated [108]. In addition to the aforementioned reaction, it involves Gβ subunits as high energy phosphate acceptors. Following the GTPase reaction, the phosphate is transferred to a histidine residue of a Gβ subunit, resulting in a phosphoramidate which allows rapid phosphate transfer to a second GDP bound Gα-subunit [109].

As mentioned above the GTPase activity of the Gα subunit is modulated by some of its downstream targets, i.e., cGMP-dependent phosphodiesterase and phospholipase C-β which thereby function as GAPs (like the GTPase-activating proteins of the ras family). In addition, a large family of proteins with GAP activity termed RGS (regulators of G-protein signaling) have arisen recently from molecular cloning [110]. These proteins dramatically increase GTP hydrolysis hence interrupting signaling of activated G proteins. In addition, they may function as effector antagonists [13, 111]. However, so far only isoforms specific for G_i- and G_q-subfamily members have been described. The predicted G-protein regulator phosducin does not directly affect GTPase activity of Gα but instead is thought to sequester free Gβγ subunits hence interrupting the coupling of receptors to G proteins [112, 113]. While receptor kinases and arrestins are involved in the rapid, short-term down-regulation of the pathway, phosducin has been proposed to play an important role in long-term regulations of G-protein signaling [66].

Non-hydrolyzable guanine nucleotides such as GTPγS, GppNHp, or the photoreactive GTP azidoanilide are experimental tools to activate G proteins [106, 114]. They shift the equilibrium of heterotrimeric G proteins towards the dissociated active forms of Gα and Gβγ. Careful interpretation of results obtained with GTPγS is required, since kinases do utilize nucleoside thiophosphates. Activation of heterotrimeric G proteins, but not monomeric GTPases, is accomplished by incubation with AlF_4^- [72, 115]. However, AlF_4^- also affects various other enzymes.

Table IV. Co- and posttranslational modifications of G-protein subunits.

Acylation of Gα	
Myristoylation [a] (cotranslational, irreversible)	
enhances protein–protein-interactions	$G\alpha_{i1\text{-}3}$, $G\alpha_{o1\text{-}2}$, $G\alpha_t$, $G\alpha_z$
sequence motive:...M*G*XXXS/T...(N-terminus)	
enzyme: N-myristoyl transferase	
Palmitoylation [b] (posttranslational, reversible)	
as membrane anchor	$G\alpha_{i1\text{-}3}$, $G\alpha_{o1\text{-}2}$, $G\alpha_t$, $G\alpha_z$
sequence motive:...MG*C*...? (N-terminus)	$G\alpha_s$, $G\alpha_{11\text{-}13}$
enzyme: palmitoyl transferase (?) but also autoacylation	
Isoprenylation of Gγ	
*Sequence motive:...**CAAX*** [c] (C-terminus)	
(a) *farnesyl-transferase* (C-15)	
CAAX: X = Ala [d], Cys, Glu, Met, Ser	$G\gamma_{1,9?,11}$, rab [e]
(b) *geranylgeranyl-transferase I* (C-20)	
CAAX: X = Leu	$G\gamma_{2,3,4,5?,7?,8?,10}$

[a] Additional transfer of lauryl acid and unsaturated amino acids (C-14:2, C-14:1) to $G\alpha_t$ results in functional heterogeneity of transducin [116].
[b] In platelets transfer of arachidonic acid to $G\alpha_i$, $G\alpha_q$, $G\alpha_z$, $G\alpha_{13}$ also.
[c] A = aliphatic amino acid, X ≠ Φ.
[d] Abbreviations of amino acids: Ala = alanine, Cys = cysteine, Glu = glutaminic acid, Leu = leucine, Met = methionine, Ser = serine.
[e] Member of small GTPases.

Additionally, high concentrations of fluoride may change the free concentrations of Ca^{2+} and Mg^{2+} thus generating unspecific effects.

3.5. Modulation of G proteins by co- and post-translational modifications

Most Gα subunits are modified by co- and posttranslational events *(table IV)* [116–119]. These modifications are essential for membrane association and increase the stability of the heterotrimer complex. During elongation of nascent $G\alpha_{i/o}$ proteins, the initial methionine is cleaved, followed by myristoyl amidation of the glycine residue. In addition to myristate (C-14-carbon acid), various unsaturated C-14-fatty acids and the C-12-lauryl acid are linked to the glycine residue of the retinal Gα of transducin. Myristoylation appears to be irreversible. This protein modification on the one hand enhances lipophilicity of the Gα subunit, and on the other hand it contributes to the specificity of protein–protein interactions. Simultaneously, myristoylation enhances the potency of the Gα subunit to activate effectors. Palmitoylation of all Gα subunits except transducin and arachidonate-acylated Gα subunits in platelets were reported as a posttranslational, reversible modification occurring at a cysteine-residue near the N-terminus via a labile thioester-bond and possibly at other sites [120–124]. The autoacylation of Gα was shown in the absence of a specific enzyme, whereas for deacylation a cytoplasmic acyl-protein thioesterase was identified very recently [125, 126]. After cell fractionation, non-myristoylated Gα subunits carrying a palmitate (C-16-carbon acid) accumulated in the particulate fraction, whereas the protein devoid of fatty acid modification was found in the cytosol [127]. However, it remains questionable whether the only function of palmitoyl acylation is anchoring the Gα subunit in the membrane. For instance, the degree of palmitoylation of Gα subunits is receptor-dependently regulated [128]. Furthermore, non-palmitoylated chimeras of $G\alpha_s/G\alpha_t$ and $G\alpha_q/G\alpha_t$ are unable to stimulate their respective effectors, i.e., adenylyl cyclases and phospholipases C [129]. Very recently, it has been demonstrated that palmitoylated $G\alpha_z$ and $G\alpha_{i1}$ were resistant to respond to GAPs which may result in prolongation or potentiation of G-protein signaling [130].

G-protein βγ dimers exhibit a higher lipophilicity than Gα subunits. This correlates with isoprenylation of Gγ subunits by isoform specific protein prenyltransferases [131, 132]. The signal sequence causing this modification is a CAAX-box-motif at the C-terminus common to all Gγ subunits and some monomeric GTPases. This motif triggers a number of posttranslational events initiated by prenylation of the cysteine residue through a thioether bond by specific enzymes followed by endoproteolytic cleavage of the three C-terminal amino

acids (AAX) and methylation at the new C-terminus, which is the prenylated cysteine residue. Efforts are currently made to elucidate the mechanism for Gβγ assembly and functional consequences of the various posttranslational modifications [133, 134]. First results suggest that prenylation is not required for Gβγ assembly which occurs prior to the proteolytic processing of Gγ [135]. Interestingly, isoprenylation and presumably carboxymethylation of $G\gamma_1$ appear to contribute to the efficiency of membrane association, subunit interaction between Gα and Gβγ, and the functional coupling of the G protein with receptors or effectors [136–138]. Some Gγs, e.g., the retina-specific $G\gamma_1$ subunit, are modified by a C-15 sesquiterpene (farnesyl moiety), whereas other Gγ isoforms are modified by a C-20 diterpene (geranylgeranyl group). This difference in isoprenylation of Gγ affects lipophilicity of the Gβγ dimer, i.e., retinal $G\beta_1\gamma_1$ dimers are soluble without detergents in contrast to C-20-isoprenylated Gβγ dimers. Furthermore, the type of isoprenylation has an impact on all functions of Gβγ dimers studied so far. In general, C-20-isoprenylated Gβγ complexes appear to exhibit a higher potency in modulating effectors than C-15-isoprenylated $G\beta_1\gamma_1$.

Analysis of the resolved three dimensional structures of Gαβγ suggests that putative locations of myristate in Gα and the isopren in Gγ are probably not only in close proximity to each other, but most likely are also quite close to the receptor contact sites (see above and *figure 2*). This sterical proximity is consistent with the idea that the complexed Gαβγ has a common anchor region onto the membrane acting as a pivot to keep suitable interaction points for the receptors in the right orientation towards the membrane.

Prevention of protein prenylation is therefore likely to affect the function of the protein significantly. Correspondingly, inhibition of prenylation of monomeric GTPases such as Ras is such an attempt to affect tumorigenesis. This promising approach pursued by academic research groups and pharmaceutical companies is covered by the literature in various aspects and therefore will not be discussed here [139–144].

Various G proteins are modulated by phosphorylation though reports are still scarce. For the PT-insensitive G protein G_z, selective phosphorylation of a serine residue near the N-terminus by PKC is well established [145]. The affinity of phosphorylated $G\alpha_z$ to Gβγ decreases, which probably abolishes a functional receptor G-protein interaction *(figure 4)* [146]. A similar mechanism is suggested for PKC-induced phosphorylations of G_{i2}, G_{12}, and G_{13} [147–149]. Recently cGMP protein kinases were speculated to suppress thrombin-induced increase in inositol 1,4,5-trisphosphate, and cytosolic calcium by phosphorylation of G_i proteins [150]. G_s is modified at its N- and C-terminus by various tyrosine kinases [151, 152]. Tyrosine phosphorylation of $G\alpha_s$ initiated by cross-talk between receptor tyrosine kinase signaling and G-protein-coupled-receptor pathways interrupts receptor-induced activation of adenylyl cyclases *(see figure 3)* [153].

3.6. Toxins affecting G proteins

Bacterial toxins such as pertussis toxin (PT) and cholera toxin (CT) were basic tools leading to detection, identification, and understanding of the structure and function of G proteins [154, 155]. Today, both toxins are still appreciated as cell-identifying G-protein coupled pathways [33, 34, 114, 156]. They are both ADP-ribosyltransferases though the target G proteins and the site of action are different (see *figure 4*).

CT is produced by vibrio cholera which represents the causative agent of cholera. CT is an oligomeric protein of 84 kDa composed of one A and five B subunits. It specifically catalyzes the transfer of the ADP-ribose portion of nicotinamide adenine dinucleotide (NAD) to arginine, either free or as part of a protein. In all cells the major protein substrate is $G\alpha_s$ modified at arginine[201] though all members of the G_s subfamily, transducin, and $G_{i/o}$ are sensitive to CT (see *table II*). $G\alpha_s$ and $G\alpha_{olf}$ are modified by CT in a receptor-ligand-independent manner, whereas $G\alpha_t$ and $G\alpha_{i/o}$ are modified in a receptor-ligand-dependent manner. The functional consequence of the CT-catalyzed ADP-ribosylation of arginine is the abolishment of the endogenous GTPase activity (see above) resulting in continuous activation of the Gα subunit. A major functional result seems to be persistently high levels of cAMP.

PT (or islet-activating protein), a 105 kDa hexameric enzyme, is produced by bordetella pertussis the agent causing whooping-cough. PT catalyzes ADP-ribosylation of most Gα isoforms belonging to the G_i subfamily, but not members of the G_s, G_q, and G_{12} subfamilies. PT catalyzes both the hydrolysis of NAD^+ and the transfer of the resulting ADP moiety to a cysteine residue four positions upstream the C-terminal aromatic amino acid of the α subunit of G_i, G_o, and $transducin_{r,c}$ in the presence of Gβγ. PT-resistent G_i proteins lacking a C-terminal cystein include G_z and a splice variant of G_{i2} ($G_{i2(L)}$). Since the modified cysteine is located within the C-terminus, PT-catalyzed ADP-ribosylation prevents functional interaction of the transmembrane receptor with the G protein (see above). Nevertheless, PT-modified G proteins are capable of exchanging guanine nucleotides and of hydrolyzing GTP which allows interaction with

effectors though the time required to observe a maximal effect following stimulation with non-hydrolyzable GTP-analogues (see above) can be markedly increased [17, 157].

Interestingly, low concentrations of *N*-ethylmaleimide (NEM), a sulfhydryl alkylating agent, have been found to mimic the actions of PT [158]. Correspondingly NEM alkylates the PT-sensitive cysteine of Gα. In addition, an N-terminal cysteine (Cys^{108} of $G\alpha_o$) is also modified by NEM resulting in a decreased affinity of $G\alpha_o$ to $G\beta\gamma$. However, in contrast to PT NEM represents a rather unspecific agent limiting its applications as a cell biological tool.

4. Antisense DNA/RNA as cell biological tools

While peptides derived from G protein sequences or antibodies against G proteins are appreciated as valuable tools antisense DNA/RNA technology recently has become a promising experimental approach for assignment of certain G-protein-dependent pathways [159]. In principle it allows selectively to inhibit the biosynthesis of one molecular species and to investigate which cell function is lost at the cellular level [14]. For studying the physiological consequences of a loss of function in whole animals the gene knock out technology in mice has emerged as a successful technique.

5. Receptor-derived peptides

Amino acid stretches derived from several receptor families have been reported to directly activate G proteins (see *figure 4*). Concerning GPCR regions belonging to the second and third intracellular loop and the proximal C-terminal tail interact with G proteins (see above) [160]. Among these structures juxtamembrane segments of the third intracellular loop are most important for activation of G-proteins [36]. Depending on ligand binding to the receptor this region is subjected to conformational changes thereby governing G-protein activity. In contrast, when it is expressed as an isolated region, i.e., free of the conformational constraints imposed by the receptor's membrane spans, it assumes an activated conformation activating G proteins. Nevertheless, the exact structural features for selectivity and activation of G proteins by GPCRs are not defined. However, cationic charges and amphiphilicity are indispensable for stimulation of G proteins though almost any cationic compound, even ammonium ions, can somewhat accelerate nucleotide exchange [32].

It is conceivable that both structural elements are found in a broad spectrum of proteins. Consequently, numerous receptors belonging to other families than to the superfamily of GPCR were hypothesized functionally to couple to G proteins. Among them are receptors for insulin, IGF II, ANP, as well as proteins involved in the development of Alzheimer's disease such as the amyloid precursor protein or in neurite outgrowth like GAP 43 [161–165]. However, the physiological roles of these mainly in vitro findings remain to be clarified [166]. Even ligands for receptors including substance P, sphingoid bases, dynorphin, or bradykinin, which all meet the aforementioned structural features, were found to activate G proteins [167–171]. Interestingly, substance P analogues function as G-protein antagonists [172]. It should also be mentioned that G-protein activation is not only elicited by receptors or receptor mimetics but is also influenced by the composition of the lipid environment [173–175].

6. Non-receptor-derived peptides

Peptides bearing cationic amphiphilic regions like mastoparan, a tetradecapeptide (Ile-Asn-Leu-Lys-Ala^5-Leu-Ala-Ala-Leu-Ala^{10}-Lys-Lys-Ile-Leu-NH_2; M_r 1480) isolated from the venom from wasps (vespula lewisii) or hornets, function as receptor surrogates to initiate signaling [176–180]. Mastoparan was initially found to stimulate exocytosis from diverse mammalian cells. It causes not only secretion of histamine from mast cells, serotonin from platelets, catecholamines from chromaffin cells, prolactin from the anterior pituitary, but also stimulates proliferation of Swiss 3T3 cells [181]. Mastoparan is hypothesized to bind to the plasma membrane in a neuraminidase-sensitive fashion and to penetrate the lipid bilayer by electrophoretic transfer [180, 182]. The late Tsutomu Higashijima correlated the secretagogous effects of mastoparan with a direct stimulation of $G_{i/o}$ proteins and studied its molecular mechanism in detail. In fact, in the presence of phospholipids mastoparan can be modeled as a cationic amphipathic α-helix presenting three positive charges to the aqueous face. In this configuration it resembles G-protein-interacting cytosolic domains of GPCR capable of directly stimulating G proteins (see above and *figure 5*) [183]. With striking similarity to receptor-induced G-protein activation the effects of mastoparan were blocked by PT *(figure 6)* [180, 184]. In addition mastoparan blocked the ability of G_o to increase the affinity of muscarinic agonists, suggesting that mastoparan and the receptor may compete for a common binding site on G_o [167]. Furthermore, mastoparan and analogues of sufficient length activate G proteins by stimulating the release of GDP from Gα without altering the rate of hydrolysis of bound GTP. Binding of masto-

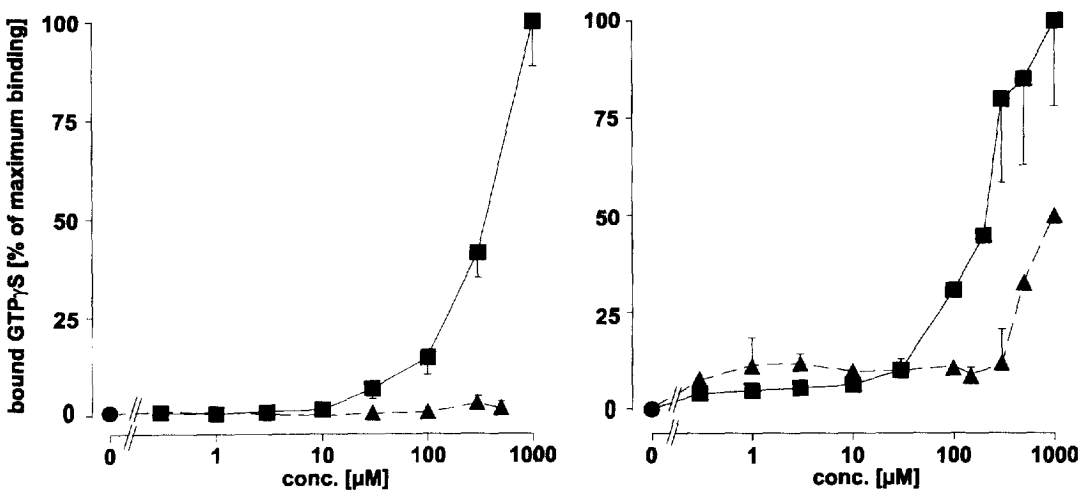


Figure 5. Influence of lipid vesicles on activation by direct G-protein activators. Binding of [^{35}S]-GTPγS (30 nM) to the α-subunit of transducin (1 pmol/tube) in aqueous solution (left) and in the presence of phospholipid vesicles (right) after stimulation with **42** (squares) and mastoparan (triangles) in 50 μL of solution containing 1 mM HEPES pH 7.6, 0.1 mM DDT, 100 μM $MgCl_2$ (left) and 10 mM HEPES pH 7.6, 1 mM DDT, 100 mM NaCl, 100 μM $MgCl_2$ (right). The reaction mixtures were incubated for 30 min at 30 °C. Shown are mean values ± SD.

paran to $G\alpha_o$ and activation of its GTPase activity is enhanced by the presence of Gβγ subunits. Correspondingly mastoparan interacts with N- and C-terminal regions of Gα [184, 185]. Recent evidence suggests that mastoparan-like compounds in part activate G proteins by unwinding some portions of the C-terminal α-helix [104]. Taken together these results revealed close mechanistic parallels between the activation of G proteins by mastoparans and ligand-bound receptors.

A lot of efforts were put in studying structure–activity relationships of mastoparans. Basically, mastoparan-like receptor mimetics have to be cationic amphiphilic with lysine residues known to be crucial for activity. Helix-breaking residues or charged residues on what should be the hydrophobic face of the helix both diminish activity significantly [32]. Mastoparan itself predominantly activates $G_{i/o}$ proteins and is less effective in stimulating transducin, G_s or G_q proteins. However, MP-S, a mastoparan derivative with a kinked helical conformation at residue 9, activates G_s more markedly than $G_{i/o}$ [186].

Mastoparan has become the gold standard for a group of diverse compounds bypassing the receptors and activating directly G proteins in eliciting its response on cells. Thus, mastoparans and other related regulatory peptides appear to mimik hormone-bound receptors and have been covered by patents claiming them as useful molecular tools to dissect G protein-dependent pathways and for the development of potential drugs [187, 188]. Though they are excellent experimental tools for studying purified proteins they exert limited specificity since mastoparan among other G-protein-activating peptides shows pleiotropic effects on various proteins including calmodulin, nucleoside diphosphate kinase, small GTPases such as Rho and Rac, and guanylyl cyclases [16]. An additional disadvantage of mastoparan-like peptides appears to be their amphiphilic character which disturbs the lipid bilayer of biomembranes at concentrations similar to those used to stimulate G proteins [189]. Furthermore, potential therapeutic use of this class of peptides is limited by unfavourable pharmacokinetic properties.

7. Non-peptide agents modulating G proteins

7.1. Empirically found compounds

As already outlined almost every cationic amphiphilic compound exhibits G-protein-activating properties despite otherwise considerable structural diversity [190]. For instance, natural polyamines or the synthetic polyamine compound 48/80, the preservative benzalkonium chloride as well as some taste substances such as the sweetener sodium saccharin or the bitter tasting quinine activate G proteins in vitro; the latter at concentrations used to elicit taste [167, 182, 191–194]. Among therapeutically relevant drugs arpromidine-derived guanidines, β-adrenoceptor, and muscarinic receptor an-

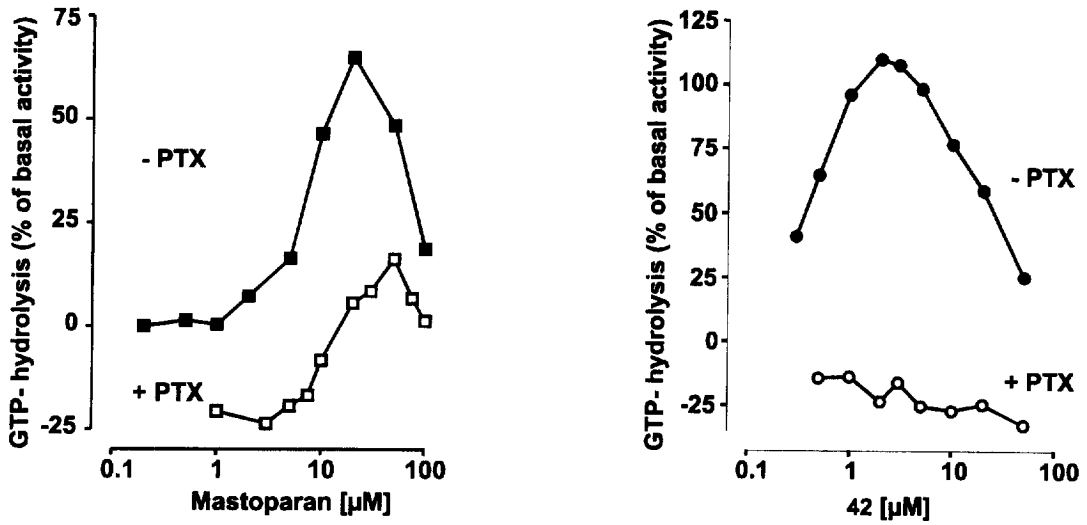


Figure 6. Activation of GTPases by **42** and mastoparan and their sensitivity to pertussis toxin (PT) in dibutyryl-cAMP (dbt-cAMP) differentiated HL-60 cell membranes. Concentration-dependent stimulation of GTP-hydrolysis in untreated (solid symbols) and in pertussis toxin pretreated (open symbols) dbt-cAMP-differentiated HL-60 and cell membranes by mastoparan (squares) and **42** (circles). Data taken from [213].

tagonists, histamine derivatives (see below), local anaesthetics as well as the antiprotozoic agent pentamidine [unpublished data] and the class III antiarrhythmic agent amiodaron activate $G_{i/o}$ proteins in vitro *(table V)* [190, 195–198]. They most likely share a common mechanism of G-protein activation (see above). In most cases the physiological relevance of these observations remains to be determined, while for some drugs direct activation of G proteins may explain unwanted drug actions.

Interestingly some drugs were reported to inhibit G-protein activation. For example, isobutylmethylxanthine was suggested to directly inhibit G_i proteins [199]. In a series of papers Freissmuth and coworkers reported on suramin and analogues as potent and subtype-selective direct inhibitors of G proteins discriminating G_s- and $G_{i/o}$-dependent signaling pathways [200–202]. Essentially they directly act on Gα subunits blocking their activation by receptors. These observations demonstrate the feasibility of subtype-selective G-protein inhibition. However, this class of drugs was originally designed for the treatment of protozoal infections [203]. Corresponding to their broad spectrum of unwanted drug actions recognized during their clinical use they were found to act on a large variety of signaling molecules even at very low concentrations.

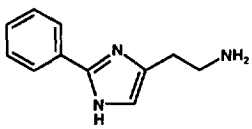
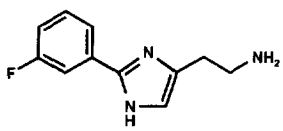
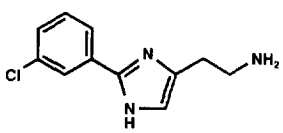
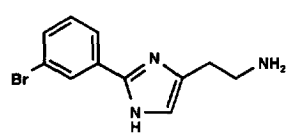
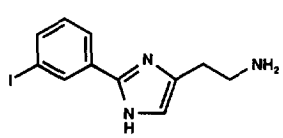
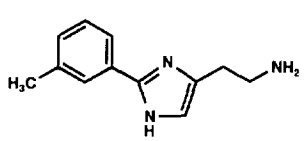
In recent years eicosanoid-independent actions of non steroidal anti-inflammatory drugs (NSAID) like salicylates were predicted to be elicited by direct interaction of these drugs with G proteins [204]. Salicylic acid, indomethacin, or piroxicam were found to interrupt transmembrane signal transduction of chemotactic receptors [205, 206]. Based on PT-sensitive effects the authors speculated that membrane-bound NSAID interfere with the C-terminus of Gα. Own work revealed that diclofenac also interrupts this signaling pathways in leukocytes by blocking activation of G_i proteins through inhibition of guanine nucleotide release.

7.2. Compounds designed as G-protein activators

7.2.1. 2-Phenyl-substituted histamines

Our research for receptor-independent G-protein activators started with the results derived from recent findings where we have shown that 2-(3-chlorophenyl-histamine) **3**, a potent and selective histamine H_1-receptor agonist, is able to activate pertussis toxin-sensitive heterotrimeric G proteins in a receptor-independent manner (see above) [207]. The 2-substituted histamines are cationic–amphiphilic, i.e., they have a basic domain (aminoethylimidazole) and a lipophilic moiety (phenyl residue) [190, 208–212]. As the structures of 2-substituted histamines can be modified in a logical manner they became a valuable starting point for the systematic analysis of structure–activity relationship of receptor-independent G-protein activators.

Table V. 2-Phenyl-substituted histamines as G-protein activators.

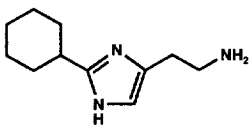
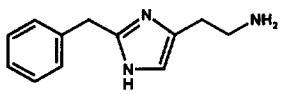
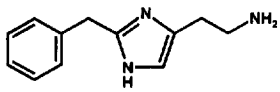
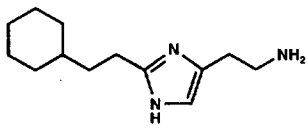
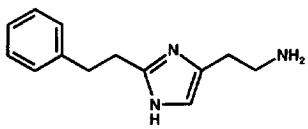
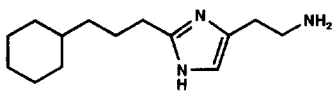
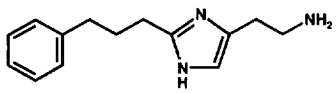
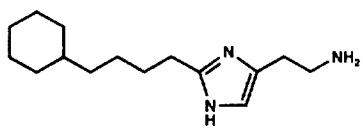
No.	Structure	pEC_{50}	Efficacy (%)	H_1-receptor agonist rel. act. (%)
1		3.5	36	31
2		–	38	85
3		3.5	75	96
4		3.7	80	112
5		3.8	80	96
6		3.8	50	30

All compounds were screened for their ability to affect high-affinity GTPase activity in dibutyryl-cAMP differentiated HL-60 membranes. The effects were compared with the standard G protein activator mastoparan. Mastoparan stimulated GTPase activity with a pEC_{50} value of 5.5 and 2-fold activation at 10 μM (see *figure 6*) [198, 213].

Table V shows the structural formulas of the substances and summarizes their effects at the H_1 receptors of the guinea-pig ileum and on GTP hydrolysis in HL-60 cell membranes. Compound **4** is the most potent H_1-receptor agonist in this series of compounds. Substitution on the phenyl ring with bulky and lipophilic halogens in the meta position, e.g., bromine (**4**) and iodine (**5**) or a bulky methyl group (**6**), increases GTPase activity. The effect of **2** on stimulation of GTP hydrolysis did not reach saturation, and pEC_{50} value could therefore not be calculated. Starting from these results we assumed that a bulky structure and lipophilicity are important determinants to obtain more potent compounds.

Exchange of the planar phenyl ring by a non-planar ring system (cyclohexane) and varying the spacer from one to six methylene groups between ring system and aminoethylimidazole enhanced GTPase-activating properties [198, 212]. Concerning the effects of **7–14** at the histamine H_1 receptors of the guinea-pig ileum *table VI* shows that **9** and **11** are poor H_1-receptor agonists while **7, 8, 10, 12–14** are weak H_1-receptor antagonists. Among

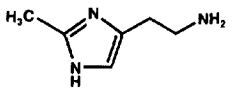
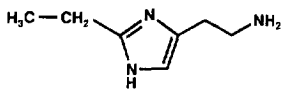
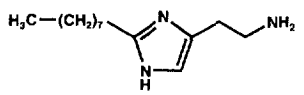
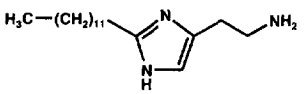
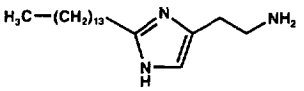
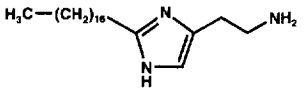
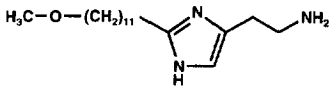
Table VI. 2-Substituted histamines as G-protein activators.

No.	Structure	pEC_{50}	Efficacy (%)	H_1-receptor agonist rel. act. (%)	H_1-receptor antagonist –log K_B
7		–	42		5.8
8		3.5	66		5.7
9		3.2	49	1	
10		4.0	100		5.3
11		3.4	74	3	
12		4.5	110		4.3
13		3.7	90		5.0
14		5.0	150		5.2

the phenyl derivatives (**9**, **11**, **13**) elongation of the spacer resulted in an increase in efficiency of substances in supporting G-protein activation. The same holds for cyclohexyl derivatives (**8**, **10**, **12**, **14**). When the type of ring system is considered, efficiency and potency of cyclohexyl derivatives were higher than those of the related phenyl derivatives. Considering the potency of the substances of *table VI*, compound **14** is the most active derivative.

More bulky ring systems like an adamantyl or norbornyl group were tested [212, 214]. These ring systems showed only a moderate increase in potency and efficacy

Table VII. 2-Alkyl-substituted histamines as G-protein activators.

No.	Structure	pEC_{50}	Efficacy (%)	H_1-receptor agonist rel. act. (%)	H_1-receptor antagonist $-\log K_B$
15		–	26	14	
16		–	24	7	
17		4.5	150		5.0
18		5.8	95		6.1
19		5.9	60		4.7
20		5.5	96		5.2
21		4.9	98		5.1

to stimulate GTPase activity. It appeared that lipophilicity was the more important determinant compared to the space demanding factor.

7.2.2. 2-Alkyl-substituted histamines

The novel 2-alkyl-substituted histamines showed good stimulatory effects on high-affinity GTP hydrolysis, whereas the histamine H_1 receptors were less affected [214].

Table VII illustrates that the tetradecyl chain (**19**) led to an optimum of the lipophilic moiety. Elongation to a heptadecyl chain (**20**) reduced potency. The introduction of an ether function (**21**) diminished potency about tenfold and had little effect on efficacy. 2- Octylhistamine (**17**) and 2-cyclohexylbutylhistamine (**14**) are clearly the most efficacious substances among the 2-substituted histamines presently available, but conversely 2-tetradecylhistamine (**19**) is the most potent substance. These data show that efficacy and potency of a receptor-independent G protein activator can be dissociated from one another and are independently determined drug parameters.

7.3. Chemistry of compounds (tables V–VII)

The condensation of α-hydroxyketones with activated derivatives of carboxylic acids is a common method of synthesizing imidazoles [215]. The substitution pattern required consisted of a lipophilic residue at the 2-position and a 2-aminoethyl or 2-hydroxyethyl group at the imidazole C-4. The synthesis of the compounds is outlined in *figure 7*. The α-hydroxyketone unit with the desired functionalized ethyl side chain is accessible from 1,4-butynediol by mercury-catalyzed addition of wa-

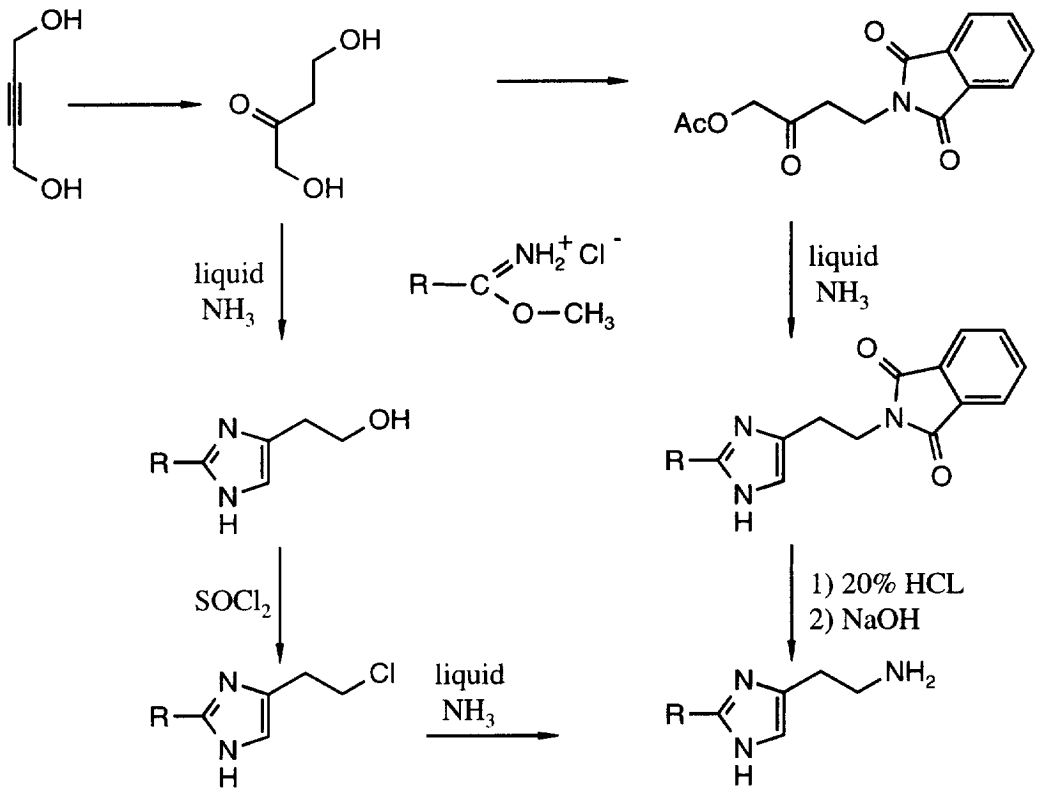


Figure 7. Synthesis of 2-substituted histamines.

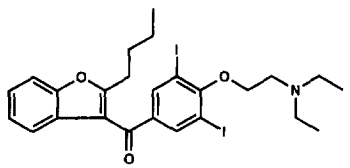
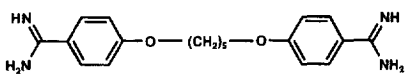
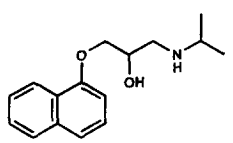
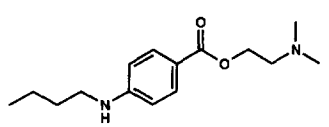
ter [216]. This was used as the hydroxy-terminated synthon. Subsequent reaction with acetic anhydride and phthalimide generates the protected N-terminated C-4 unit [212, 214, 217]. We used imidomethylesters for the C-1 unit of the imidazole carrying the lipophilic residue. These were obtained as hydrochlorides by the action of HCl in methanol or thionyl chloride in methanol on the corresponding aliphatic or araliphatic nitriles.

In liquid ammonia as solvent and reagent these units condensed to the hydroxyethyl derivatives and, after acid-catalyzed deprotection, the aminoethyl derivatives. Hydroxyethylimidazole could be converted to the histamine derivatives by reacting with thionyl chloride followed by ammonia *(figure 7)*.

7.4. Pharmacology of the G-protein activators

Since we thought that substances of the type of 2-substituted histamine derivatives act as receptor mimetics to the G protein and together with the finding of the importance of a long lipophilic chain domain, we tried to find out how amino acid derivatives, namely the basic ones, instead of histamine moiety behave [213]. Interestingly, nearly all compounds depicted in *table IX* showed an efficacy comparable to mastoparan (see *figure 6*). Since we found that dodecyl to hexadecyl is the optimum range for the alkyl chain only this will be considered here. Amides starting from amino acid and dodecyl amine were mainly used. In this class the amino acids with just one terminal amino group (**34–37**) showed potencies in the range of 5.1–5.5 with a slight decrease from glycine to amino octane. The group of compounds derived from α-amino acids with a terminal functional group (**26–29**, **31–33**) were between 5.1 and 6.1. Noteworthy is the result of **26** as the arginine compound versus the nitro arginine compound (**27**). It showed no saturation until 300 μM, whereas **26** had a pEC_{50} of 6.1. In this group compounds with no or just weak basic terminal groups tend to be less potent than substances with a basic terminal group. The most potent amino acid derivatives are the arginine and lysine substances (**26**, **28**, **29**) with a pEC_{50} of 6.1. Remarkably the enantiomers of the lysine

Table VIII. Various drugs as G-protein activators.

No.	Structure	pEC_{50}	Efficacy (%)
22	 Amiodarone		22
23	 Pentamidine	5.0	69
24	 Propranolol	3.7	90
25	 Tetracaine	> 3.5	> 90

compound (**28**, **29**) have the same value, whereas the little change in the amide structure – amide versus inverse amide (**28**, **29** versus **30**) – results in a significant decrease of potency and efficacy.

Another amide (**38**) of a similar structure showed that the inverse amide with a tertiary terminal amino group is less potent. Increasing the number of terminal amino groups results in higher potency (**39**).

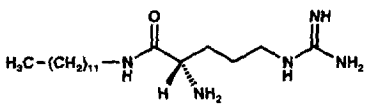
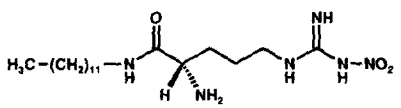
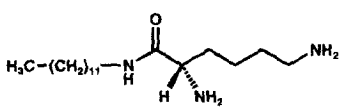
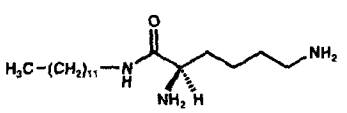
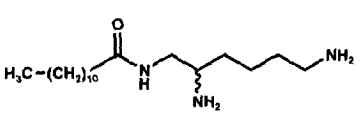
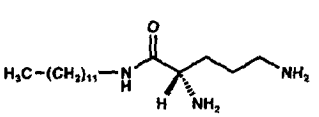
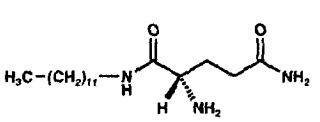
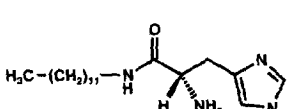
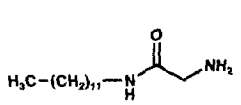
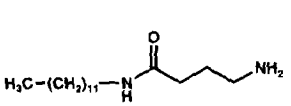
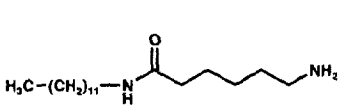
In the group of lipophilic amines depicted in *table X* the reduced glycine (**40**) and ornithine (**42**) derivatives showed a potency of 6.0, which is within the range of the best amino acid derivatives. Interestingly, the reduced diglycine compound (**41**) was 0.3 log units more potent and had a 30 percent higher efficacy, whereas glycine versus diglycine (unpublished result) were equipotent.

When comparing reduced derivatives of ornithine (**42**) and lysine (**43**), it is obvious that they have the same efficacies but different potencies (6.0 versus 6.3). Especially noteworthy is the comparison of **30** versus **43**, where an amide group causes a difference in potency of 0.8 log units; the amide is 6 times less potent. By modifying the length of the lipophilic chain of reduced lysine (**43–45**) we found a decrease in efficacy by the hexadecyl chain. The optimum chain length, both in potency and efficacy, was the tetradecyl chain. Thus, **44** is 13 times more potent compared to mastoparan, which was up to now the gold standard in G protein activation. In addition these compounds act as receptor mimetics stimulating release of GDP from Gα thereby allowing a rapid exchange for GTP (see *figure 2*). Interestingly in contrast to mastoparans these compounds do not rely on a lipophilic environment. They stimulate purified G protein α-subunits regardless whether phospholipids are present or not (see *figure 5*).

8. Outlook and therapeutical aspects

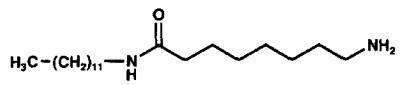
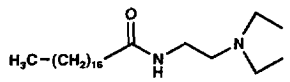
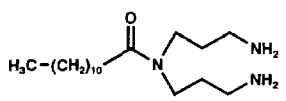
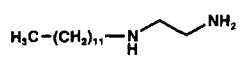
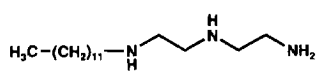
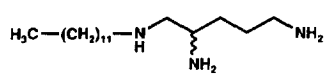
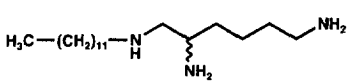
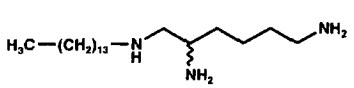
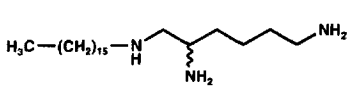
Potent non-peptide G-protein modulators with marked specificity for individual G proteins would be useful molecular tools in studies aimed at delineating the involvement of a particular G protein in specific biochemical pathways and, potentially, as leads for the development of G-protein-directed drugs. These compounds avoid labile bonds exhibiting chemical stability which results in superior pharmacokinetic features as compared

Table IX. Amino acid lipids as G-protein activators.

No.	Structure	pEC_{50}	Efficacy (%)
26		6.1	92
27		– [a]	156
28		6.1	110
29		6.1	108
30		5.5	78
31		5.6	117
32		5.1	110
33		5.5	125
34		5.4	100
35		5.5	101
36		5.3	110

[a] No saturation until 300 μM.

Table X. Lipoamines as G-protein activators.

No.	Structure	pEC_{50}	Efficacy (%)
37		5.1	133
38		5.2	64
39		6.0	115
40		6.0	92
41		6.3	125
42		6.0	126
43		6.3	120
44		6.6	117
45		6.6	84

to peptide precursors. First results indicate an improvement of the compounds, especially less membrane irritating effects and less unspecific effects compared to mastoparan. Further, compound **42** indicates first selectivity among G-protein isoforms. The currently published X-ray structures of heterotrimeric G proteins allow a more rational approach to design new compounds. Docking studies will lead to a better understanding of the mode of activation. Many G protein subunits are cell and tissue selectively expressed. Therefore direct G-protein activators may be capable of mimicking only distinct signaling pathways. Receptor inactivation like receptor desensitization or receptor internalization may be bypassed through direct G-protein stimulation *(figure 8)*.

In principle, direct G-protein modulators would represent important drugs. They should represent a new therapeutic strategy when the hormonal signaling pathway is disturbed at the receptor level. This pathological defect cannot be overcome by administration of receptor agonists. However, pharmacological interference at the G-protein level could bypass the inactive receptor. For instance distinct forms of diabetes insipidus are caused by the inability of the kidney functionally to process active GPCR for vasopressin although ligands and intracellular G proteins and effectors are present. Hence, it may be speculated that a receptor-independent stimulation of the vasopressin-receptor-uncoupled G protein should restore the impaired kidney function caused by the absence of cellular responses to vasopressin. Furthermore, in contrast to a genetic defect resulting in a loss of function other receptor defects, regardless whether they are inherent or acquired by mutation or viral infection, cause a

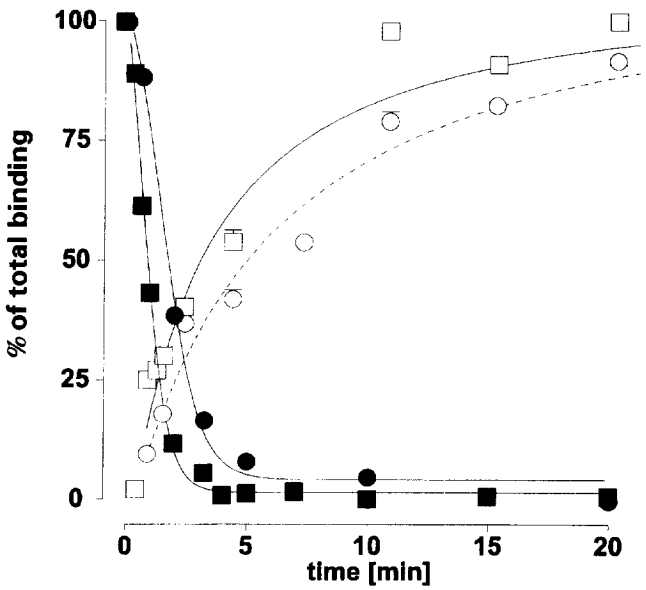


Figure 8. Stimulation of GDP/GTP-exchange of $G\alpha_{o1}$ by **42**. Time-dependent stimulation of [α-^{32}P]-GDP-release from (solid symbols) and ^{35}S-GTPγS-binding to (open symbols) purified $G\alpha_{o1}$ by 200 μM **42** (squares) versus control (circles). Data taken from [213].

gain of function by the appearance of constitutively active receptors. In the absence of agonists these receptors continuously stimulate the coupling G protein to a unphysiological extent, which in turn elicits pathological responses including the development of tumors. In these cases as well as for continuously active G-protein mutations administration of G-protein inhibitors could be a rational imagination.

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