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Review

G proteins as drug targets

C. Höller, M. Freissmuth* and C. Nanoff

Institute of Pharmacology, University of Vienna, Währinger Strasse 13a, A-1090 Vienna (Austria),
Fax + 43 142 77 9641, e-mail: michael.freissmuth@univie.ac.at

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Abstract. The structure and function of heterotrimeric G protein subunits is known in considerable detail. Upon stimulation of a heptahelical receptor by the appropriate agonists, the cognate G proteins undergo a cycle of activation and deactivation; the α-subunits and the $\beta\gamma$-dimers interact sequentially with several reaction partners (receptor, guanine nucleotides and effectors as well as regulatory proteins) by exposing appropriate binding sites. For most of these domains, low molecular weight ligands have been identified that either activate or inhibit signal transduction. These ligands include short peptides derived from receptors, G protein subunits and effectors, mastoparan and related insect venoms, modified guanine nucleotides, suramin analogues and amphiphilic cations. Because compounds that act on G proteins may be endowed with new forms of selectivity, we propose that G protein subunits may therefore be considered as potential drug targets.

Key words. G protein subunits; modified guanine nucleotides; receptor-derived peptides; mastoparan and related venoms; suramin analogues; amphiphilic cations.

Intercellular communication as well as the input from the environment is achieved by signaling via receptors. Work that has been carried out in the past 3 decades has led to delineation of the major classes of receptors, that is ion channels, oligomerizing receptors with intrinsic or associated kinase activity, DNA-binding receptors and G protein-coupled receptors. The latter share the general structural feature that they have a hydrophobic core composed of seven transmembrane-spanning α-helices, and they are therefore also referred to as heptahelical or serpentine receptors. The family of G protein-coupled receptors is probably the largest (e.g. the human genome encodes far more than 1000 different types); intracellular signaling pathways that are under the control of G proteins regulate the function of virtually every organ and tissue. Hence, they are of preeminent importance in clinical pharmacotherapy, and a large proportion of the currently employed drugs affect G protein-dependent signal transduction; this occurs predominantly at the level of individual receptors (by appropriate agonists and antagonists) and to some extent at the level of enzymes that remove second messengers (e.g. phosphodiesterases). Here, we will outline the arguments supporting the hypothesis that G proteins per se are also potential drug targets and summarize the structural prerequisites and mechanistic information on G protein ligands.

* Corresponding author

G protein diversity

Heterotrimeric G proteins consist of an α-, β- and γ-subunit and function as signal transducers that couple membrane-bound (cell surface) receptors for neurotransmitters, hormones, autacoids as well as photons and olfactants to their intracellular effector systems such as enzymes regulating second messenger levels or

ion channels. The G protein subunits display a large degree of molecular diversity. Currently, more than 20 individual G protein α-subunits are known which can be assigned to the following structurally and functionally related groups [1]:

α_s group:	stimulate the isoforms of adenylyl cyclase
$\alpha_{i/o/t}$ group:	
$-\alpha_{i/o/z}$:	inhibit some isoforms of adenylyl cyclase; inhibit and stimulate neuronal calcium channels and potassium channels, respectively (an effect which is due to the release of free $\beta\gamma$-dimers)
$-\alpha_{t/g}$-:	transducins and gustducin, which stimulate the retinal cyclic guanosine-monophosphate (cGMP)-phosphodiesterases and presumably a related gustatory effector
α_q group:	activate the β-isoenzymes of phospholipase C and non-receptor tyrosine kinases of the btk family
$\alpha_{12/13}$ group:	regulate low molecular weight G proteins of the rho family (which affect the cytoskeleton) and the Na/H exchanger (a direct interaction has not been proven in a cell-free assay)

In addition there are 5 G protein β-subunits and at least 12 γ-subunits. Thus, the number of distinct G protein heterotrimers that can be produced by combinatorial association of α-, β- and γ-subunits is large, although clearly smaller than theoretically possible, because some β and γ combinations fail to form functional dimers.

While it was originally thought that effector regulation was exclusively accomplished via the activated α-subunit, work carried out in the early 1990s firmly established that the $\beta\gamma$-dimer per se can also regulate effectors [2]. G protein $\beta\gamma$-dimers participate in the regulation of the following effectors: conditional stimulation (i.e. requiring the concomitant presence of activated $G_{s\alpha}$) of type II-like adenylyl cyclase isoforms [3, 4] and inhibition of type I-like adenylyl cyclase; stimulation of phospholipase Cβ (predominantly the β2-isoform; see refs. [5–7]), stimulation of potassium channels (G-protein-regulated inward rectifying potassium channel, GIRK [8]); inhibition of neuronal Ca^{2+} channels [9–11]. In addition, they play an important role in linking G protein-coupled receptors to the activation of the MAP kinase (mitogen-activated protein kinase) via a cascade of protein interactions which has not been completely defined [12–14]. Other potential G$\beta\gamma$ effectors include dynamin I and the nonreceptor protein tyrosine kinases Btk and Tsk [15, 16]. Finally, G protein $\beta\gamma$-dimers provide docking sites for proteins that mediate (mostly) negative feedback regulation, including phosducin and phosducin-like proteins [17–19] and GRK (G protein-coupled receptor kinases; see [20]).

Structure of G protein subunits

The structure of two G proteins of the α_i-subfamily, namely $G_{t\alpha\text{-}r}$ (the rod isoform of transducin) and $G_{i\alpha\text{-}1}$ has been solved, and several different conformations of the proteins have been studied; these include the inactive guanosine diphosphate (GDP)-liganded form ($\pm$ bound Mg^{2+} [21–23]) the active GTPγS-liganded form [24, 22], the AlF_4-liganded form (which mimics the transition state; [25, 22]), a mutant form of $G_{i\alpha\text{-}1}$ which is trapped in the GDP.P_i-bound conformation [26], the $\alpha\beta\gamma$-oligomer and the free $\beta\gamma$-subunit [27–29] as well as the structure of a complex formed between $G_{i\alpha\text{-}1}$ and RGS4 (= regulator of G protein signaling, a protein capable of accelerating guanosine triphosphate (GTP) hydrolysis by constraining the residues involved in catalysis [30]): These studies have revealed that the β-subunit has a rigid propeller-like core composed of seven blades (arising from the seven WD40 repeats). The γ-subunit adopts an extended, mainly α-helical conformation where the amino terminal helices of the β- and γ-subunit form a coiled coil; the carboxyl terminus of the γ-subunit, which is modified by an isoprenoid lipid (farnesylated or geranylgeranoylated), is oriented towards the amino terminal α-helix of the G protein α-subunit [27, 28]. The N terminus of the α-subunit is also modified by lipids (N-linked myristate and a thioester-linked palmitate have been identified on most α-subunits). These lipid modifications are thought to participate in attaching the G protein subunits to the membrane; in addition, they may also serve more specific roles in protein–protein interaction (e.g. myristoylation of the subtypes of $G_{i\alpha}$ is absolutely required for inhibition of adenylyl cyclase; see [3]).

The G protein α-subunit is composed of a $p21^{ras}$-like domain and an α-helical domain that are separated by a cleft; the guanine nucleotide site is at the bottom of this cleft. The conformation of the G protein α-subunit is different in the GDP- and the GTP-bound form, these differences are accommodated by movements by three discontiguous loops termed switch I (connecting helix αF and strand β2), switch II (connecting α2 and β4), and switch III (connecting β4 and α3). Residues in switch II (and in the amino terminus of the α-subunit) interact with the G protein $\beta\gamma$-dimer (for biochemical

evidence see [31–33]). Switch II and the region adjacent to switch III also participate in the formation of effector binding site I and effector binding site II, respectively (effector binding site III is formed by helix $\alpha 4$ and the loop connecting $\alpha 4$ and $\beta 6$). This indicates that the binding of effector and $\beta\gamma$-dimer are mutually exclusive. In contrast to the pronounced changes that occur in the various conformations of the α-subunit, the $\beta\gamma$-dimer does not undergo any major structural rearrangements, irrespective of whether it is in its free form, complexed to the α-subunit [27, 28] or to the regulatory protein phosducin [34]. These two observations are consistent with the current concept that the rigid structure of the $\beta\gamma$-dimer is a scaffold for protein-protein interaction and that deactivation of the G $\beta\gamma$-dimer is achieved through reassociation with the α-subunit (see also below).

Mechanism of signaling

The basic mechanism of G protein mediated-signal transduction is understood in considerable detail (fig. 1) and the experimental observations can be summarized as follows [35]: In the basal state, the G protein exists as an $\alpha\beta\gamma$-oligomer; the α-subunit, which carries a high-affinity binding site for guanine nucleotides and possesses intrinsic GTPase-activity, contains tightly bound GDP; the β- and γ-subunits are tightly associated and cannot be separated under nondenaturing conditions and are thus considered as a single entity, the $\beta\gamma$-dimer. Under these conditions, that is in the absence of activation by a receptor, the rate of GDP dissociation limits the steady-state rate for GTP hydrolysis since the k_{off} for GDP release ($k \sim 0.01$/min) is 10–100 times slower than the k_{cat} for GTP cleavage. This unique kinetic feature, namely the very slow rate of GDP dissociation, functions as a switch that keeps the system shut off. Upon binding of an agonist to a receptor, the activated receptor interacts with the appropriate G protein and dramatically accelerates the rate of GDP release from the α-subunit. In the resulting ternary complex between agonist, receptor and G protein, the agonist is bound with considerably higher affinity than to the receptor alone. This complex, however, is ephemeral at the high intracellular GTP concentrations; GTP binds virtually instantaneously to the complex, and this reaction leads to formation of the activated α-subunit, α*.GTP.Mg, which dissociates from the $\beta\gamma$-dimer. The GTP-bound α-subunit and the free $\beta\gamma$-dimer interact with appropriate effector proteins and modulate their activity. The intrinsic GTPase activity of the α-subunit cleaves the terminal phosphate group, and the deactivated α-subunit reassociates with $\beta\gamma$, which is thereby deactivated. The system relaxes to the basal state. In this cycle of activation and deactivation of the α-subunit, the receptor operates as the switch which turns on the system and the intrinsic GTPase activity as the switch that turns it off again. This turn-off reaction of the G protein α-subunit can be accelerated either by the effector itself (documented for $G_{\alpha q/11}$ and phospholipase Cβ see [36]) or by a family of recently identified proteins termed RGS (regulator of G protein signaling; for review see [37, 38]); RGS proteins act as negative regulators of signaling by reducing the lifetime of the GTP-bound α-subunit [39–41]. This is achieved by stabilizing the transition state [42], which results in an increase of k_{cat} for GTP hydrolysis by about two orders of magnitude.

The scheme outlined above is clearly a simplification for several reasons: First, unliganded receptors have a

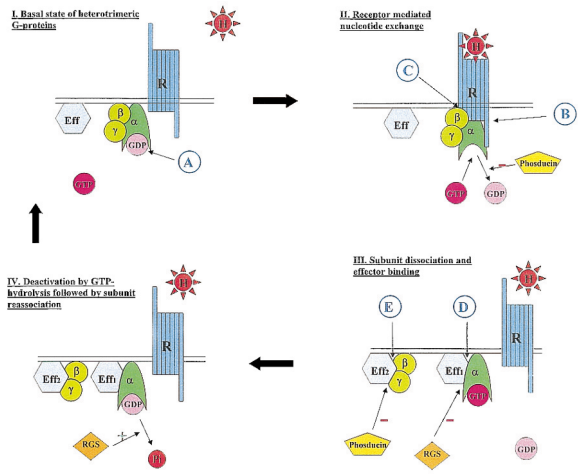

Figure 1. The mechanism of G protein signaling and potential drug target sites (A–E). Abbreviations used are: H, hormone (or the appropriate receptor agonist); R, receptor; α,β,γ, the subunits of heterotrimeric G proteins; Eff, effector proteins; RGS, regulators of G protein signaling (of which several types exist, see text for details); phosducin, the 33-kDa inhibitor of free $\beta\gamma$-dimers (see text), the role of phosducin, in inhibiting the receptor-catalyzed exchange reaction (as indicated in II) is not firmly established and may also be accounted for by scavenging free $\beta\gamma$-dimers; these are therefore unavailable for supporting the interaction of receptors with α-subunits. The sites of actions of direct G protein ligands are denoted by the letters as follows: A, guanine nucleotide binding pocket, GTP analogues: oGTP [70, 71], diaminobenzophenone-GTP [80]; B, receptor/G_α-interface: insect venoms: mastoparan and related peptides [53, 87, 89, 92, 95]; receptor-derived peptides [87–101], G_α-derived peptides [68, 102–105], substance P and analogues [95], amphiphilic cations and related synthetic compounds: benzalkonium [53], lipophilic β-adrenergic blockers, local anaesthetics and so on, [106–108], alkylamines [110], taste substances [111], suramin and analogues [113–118]; C, receptor/$\beta\gamma$ interface, L-AFC (*N*-acetyl-*S*-*trans*,*trans*-farnesyl-L-cysteine) [127, 128]; D, effector/G_α interface, suramin [115], G_α-effector binding site-derived peptides [143]; E, effector $\beta\gamma$ interface, effector-$G\beta\gamma$ binding site-derived peptide [136, 137].

spontaneous, 'basal' activity [43, 44]; it is evident that agonist-independent activation of a receptor is most readily detected upon overexpression of the receptor. However, it can also be unmasked by overexpression of the G proteins which are downstream of the receptor(s) [45]. Similarly, the basal activity of a given receptor can be greatly augmented by point mutations; these were originally generated by site-directed mutagenesis [44], but mutations that result in constitutive activation of a receptor have also been found to occur in human diseases [46]. Second, the model does not readily account for the observation that, in their native state in intact cells, receptors, G proteins and effectors may exist as large, highly organized complexes [47]; it is specifically still a matter of debate whether productive signaling requires the individual entities to dissociate completely from one another [47, 48]. Third, the model depicted in figure 1 assumes that the receptors can only exist in two states, that is an inactive and an active conformation; however, the kinetics of adenylyl cyclase activation by β-adrenergic receptor agonists [49] and experiments based on site-directed mutagenesis of the α_{1B}-adrenergic receptor [50] are incompatible with a simple two-state model; thus, the experimental evidence strongly favours a model in which receptors exist in more than one active state. These findings and earlier observations (summarized in [51]) have led to the proposal that there may be receptor agonists which selectively stabilize one of the active states; thereby, these agonists would favour the interaction of the receptor with one type of G protein (of several available), resulting in 'agonist-directed trafficking of receptor-stimulus' [51]. A recent analysis suggests that this type of agonism can indeed be observed [52]. Regardless of these limitations, the model shown in figure 1 highlights the fact that during the cycle of activation and deactivation, the G protein subunits interact sequentially with a series of reaction partners; thus, several binding sites for synthetic ligands can be postulated.

The concept of direct G protein ligands

In current pharmacotherapy, the intracellular signaling pathways that are controlled by G proteins are activated by the administration of appropriate receptor agonists; inhibition is achieved by the application of appropriate receptor antagonists. Here, we intend to argue that G proteins can per se be considered as drug targets. This concept is, of course, received with scepticism. The main two arguments that are raised against the idea of targeting G proteins are as follows:

1. Loss of selectivity. One of the driving forces that has led to the evolution of the very large number of heptahelical receptors (>1000) is their ability to bind ligands selectively, and this is exploited in the development of receptor agonists and antagonists with remarkable success. In contrast, the diversity of G proteins is much lower. Closely related members are highly homologous (e.g. $G_{i\alpha\text{-}3}$ and $G_{i\alpha\text{-}1}$ are 95% identical). In addition, many G proteins are expressed ubiquitously. Thus, a loss in selectivity is likely to be encountered if G proteins are targeted rather than receptors.
2. Membrane permeability. The site of action of G protein activators or inhibitors is intracellular because G proteins reside on the inner leaflet of the plasma membrane; hence, contrary to receptor ligands, G protein ligands have to overcome the plasma membrane, and membrane permeability clearly may be limiting for many compounds.

While these arguments are important, they do not represent insurmountable obstacles; in addition, some aspects of the arguments can be reversed and may be used in favour of the concept of G protein ligands: First, as outlined below, the wasp venom mastoparan is the prototypic direct G protein activator and selectively stimulates G_i and G_o [53]; the very fact that a class of proteins has been selected as the target of a venom during evolution suggests that it may also be targeted by synthetic drugs. Incidentally, the example of mastoparan (and of other compounds discussed below) highlights the fact that the problem of membrane permeability can be overcome.

Second, and more important, direct G protein ligands may be endowed with a new form of selectivity: In specialized cells, only a few different types of G proteins and receptors are expressed; this situation is epitomized in retinal rods (and cones), where a single type of receptor (rhodopsin or the cone opsins) interacts with a single molecular species of G protein (the rod and cone isoform of transducin). Most other cells express a large variety of both receptors and G protein subunits; the interaction between these receptors and G proteins is characterized by both exquisite specificity and extensive promiscuity. Many receptors couple to multiple G proteins; this is exemplified by the TSH (thyrotropin)-receptor [54], which can activate essentially all G protein α-subunits expressed in the thyroid (i.e. members of all subfamilies of G_α other than the retinal transducins, see above). In contrast, if receptor-dependent effector regulation is assessed, a stringent requirement of a given receptor for a given G protein oligomer ($\alpha_x\beta_y\gamma_z$) can be observed; this has primarily been documented in intact cells that were injected with antisense oligonucleotides to deplete defined G protein subunits (reviewed in [55, 56]). It is at present somewhat improbable that this highly selective interaction between the

receptor and the G protein subunits is specified by the receptor itself, because it cannot be recapitulated in biochemical reconstitution experiments. If purified (or defined) receptor and G proteins are allowed to interact, the selectivity that individual receptors display for various closely related G proteins is modest (e.g. for α-subunits [57–61]; for $\beta\gamma$-dimers [62, 63]); hence it is more likely that the stringent requirement of a receptor for a specific G protein heterotrimer arises from higher level organization of signaling components in membrane microdomains [64]. Regardless of the underlying mechanism, it is clear that, in many instances, a receptor not only regulates a single cellular effector, but activates multiple signaling pathways via several G proteins to produce the biological response. Receptor agonists (or antagonists) activate (or inhibit) all of these pathways. Thus, while compounds that act distal of the receptor may lack the selectivity that is inherent in the interaction between ligands (agonists and antagonists) and receptors, they may be endowed with new, potentially interesting types of selectivity:

1. G protein antagonists may impede the interaction of a given receptor with only one type of G protein oligomer and thus inhibit only one signaling pathway that is normally regulated by the receptor (biased inhibition of receptor/G protein tandem formation).
2. They may inhibit the interaction of G proteins with effectors (effector-selective inhibition).
3. G protein antagonists may inhibit the rate of spontaneous activation of the G proteins (inhibition of basal activity); this is presumably irrelevant under most physiological conditions, as the basal rate of GDP dissociation is low (see above); however, mutated, constitutively active forms of G proteins exist which play a role in human diseases (for review, see [46]). Direct G protein inhibitors may also block activation of G proteins by non-receptor-dependent mechanisms [65–67].
4. Finally, there are pathophysiological conditions in which multiple receptors impinge on a common signaling pathway to drive the long-term adaptive response of an organ. A well-known instance is the remodelling of the heart in response to a chronic increase in afterload. While this initially serves as a homeostatic mechanism to prevent a fall in peripheral perfusion, the hypertrophy of the failing heart ultimately accelerates disease progression. The chronic stimulation of several receptors that signal via G_q (e.g. for angiotensin II, endothelin, α_1-adrenergic, P_{2Y}-purinergic) is important in triggering cardiac remodelling. While it would be feasible to block each individual receptor with an antagonist, the alternative is to inhibit downstream signaling of all receptors by preventing their interaction with $G_{q\alpha}$. This has recently been achieved in a murine model where a (mini)transgene encoding the carboxy terminus of $G_{q\alpha}$ (amino acids 305–359) was placed under the control of the α-myosin heavy chain promoter (because this heart-specific gene is silent during embryonic and fetal development). The carboxy terminus of $G_{q\alpha}$ competes with $G_{q\alpha}$ (see also below) for binding to receptors and thereby prevents all receptors from signaling (i.e. activating phospholipase C-dependent inositol trisphosphate production). Expression of this transgene greatly reduced cardiac hypertrophy that resulted from pressure overload induced by aortic banding [68].

The guanine nucleotide binding pocket: modified guanine nucleotides

Guanine nucleotides bind extremly tightly to the G-protein α-subunit, and the binding site is buried in the cleft between the ras-like and the helical domain of $G_{\alpha;}$ nevertheless, substitutions are tolerated on the guanine ring [69]. Nonhydrolyzable GTP analogues (such as GppNHp or GTPγS) are widely used experimental tools to persistently activate G proteins. Theoretically, one can envision that the guanine nucleotide binding pocket may be targeted with appropriate compounds. In fact, GTP analogues that act as highly effective antagonists can be designed. The 2′,3′- dialdehyde analogue of GTP, oGTP, binds in a quasi-irreversible manner to G protein α-subunits (due to Schiff base formation between lysine side chains and the aldehyde groups of oGTP). When bound to the α-subunit, oGTP is hydrolyzed to oGDP; hence, oGTP can only support one round of G protein activation and subsequently traps the α-subunits in the inactive conformation [70, 71]. Because the guanine nucleotide binding pocket is highly conserved among various classes of GTP-binding proteins, these also bind oGTP and related periodate-oxidized guanine nucleotides [72–74]; hence, even if membrane permeable analogues of oGTP can be generated, they will presumably also block a host of other GTP-dependent processes such as *ras*-dependent cell growth, cytoskeletal dynamics, protein synthesis and translocation through the endoplasmic reticulum and the nuclear membrane as well as vesicle transport. Thus, although oxidized guanine nucleotides are useful experimental tools to dissect reaction pathways [75], the low selectivity that is inherent in their mechanism of action casts doubt on their usefulness in the search for therapeutically relevant G-protein antagonists.

Two amino acids are of critical importance in supporting the intrinisic GTPase of G_{α}-subunits by stabilizing the transition state, namely a conserved glutamine and

a conserved arginine residue (corresponding to Q^{227} and R^{201} in the long form of $G_{s\alpha}$). If each one of these is mutated, the intrinsic GTPase is greatly impaired and the protein becomes constitutively active because it is predominantly in the GTP-bound form [76, 77]. These mutations also occur in human diseases [78, 79]. Of interest, the defective GTPase activity of the $Q^{227}L$ mutation in $G_{s\alpha}$ can be restored by substrate-assisted catalysis, that is using the GTP analogue diaminobenzophenone-GTP, which bears the functionally relevant group of the Gln side chain [80]. These experiments point to a possible pharmacotherapeutic application of appropriately substituted GTP analogues. However, it is not clear how two major problems are to be surmounted: (i) cells are not permeable to GTP analogues and, more important, (ii) the stability of these compounds in biological fluids is limited. For example, GTP analogues that bind reversibly are readily destroyed by the 'rescued' GTPase activity and presumably all other (extra- and intracellular) nucleotidases.

The receptor/G protein interface: receptor- and G protein-derived peptides and related compounds

A molecular description of how the agonist-liganded, activated receptor catalyzes guanine nucleotide exchange on the G protein α-subunit is not yet available; the following facts are well established [56]: the receptor binds both the α-subunit and the $\beta\gamma$-dimer; the interaction involves the C terminus of the α-subunit and the intracellular loops which connect the presumed transmembrane α-helix 1 (TM1) and TM2 (i_1), TM3 and TM4 (i_2), TM5 and TM6 (i_3). In addition, many receptors are believed to have a fourth intracellular loop (i_4) formed by a stretch of amino acids which connect the end of the last transmembrane helix (TM7) with one (or two) palmitoylated cysteine residues (the lipid side chain is thought to act as a membrane anchor). The receptor must somehow act at a distance since the intracellular loops in many receptors are too short to reach to the bottom of the cleft between the helical and ras-like domain of the α-subunit where GDP is bound. The activated receptor engages the C terminus and a less well defined additional region, but the stimulatory signal is presumably transferred to the residues involved in binding GDP via the G protein $\beta\gamma$-dimer [81]. This general model is most likely applicable to the whole class of G proteins and G protein-coupled receptors. It is, however, worth pointing out that only very few amino acids are conserved in the intracellular loops that are thought to contact the G protein (e.g. a triplet at the beginning of i_2, which is required for G protein activation, see [82]); hence, in spite of several attempts (e.g. see [83]) a clear-cut consensus sequence that would allow prediction of G protein specificity of a given receptor has not been deduced. More important, the contact sites that are formed by receptors and G proteins are apparently different in each complex of an individual receptor and a given G protein. This is supported by the following lines of experimental evidence. (i) Some but not all G_q-coupled receptors interact readily with a mutated $G_{s\alpha}$ in which the carboxy terminus has been modified to contain the last five amino acids of the $G_{q\alpha}$ [84]. (ii) The α_{2A}-adrenergic receptor can couple to both G_i and G_s but requires distinct segments of its second and third intracellular loop for interacting with G_s and G_i [85]. (iii) If the interaction of the α_{1B}-adrenergic receptor to the closely related G protein α-subunits $G_{q\alpha}$, $G_{\alpha\text{-}11}$ and $G_{\alpha\text{-}14}$ is being examined, distinct amino acids within the third intracellular loop of the receptor support G protein coupling [86]. It is evident that this type of specificity, which resides in the receptor-G protein interface, is of interest for the search of specific G protein inhibitors or activators.

Peptide venoms and receptor-derived peptides

An important initial finding was the observation that the wasp venom mastoparan, a peptide of 14 amino acids, was capable of directly activating G proteins [53, 87]. In aqueous solution mastoparan is a random coil, but in the presence of membrane lipids, mastoparan adopts an α-helical conformation [88]; the helix axis is oriented parallel to the lipid bilayer, and the three charged residues are found on the side of the α-helix which is opposite to the membrane. The same is true for mastoparan-X, a related peptide that is more potent in activating G_i and G_o [89]. A comparison of this phospholipid-bound structure with that observed when mastoparan X is liganded to a G protein α-subunit ($Gi_{\alpha\text{-}1}$) indicates that the conformation is very similar, that is mastoparan-X adopts a straight amphipathic α-helical conformation extending from residue Trp^3 to the C-terminal Leu [90, 91]. In contrast, mastoparan-S, an analogue that is capable of binding to and activating $G_{s\alpha}$, is kinked at residue Met^9 [92].

The activation of G_i and G_o by mastoparan resembles that induced by receptors (e.g. with respect to Mg^{2+}-requirement and pertussis toxin sensitivity), and mastoparan competes with receptors for binding to the G protein [53, 87]. Importantly, mastoparan and other peptides that are present in the venom of hymenopteroid insects do not indiscriminately activate all G protein α-subunits. For example, mastoparan is selective for the G_i/G_o subfamily and is inactive on $G_{s\alpha}$, but replacement of Ala^{10} by α-aminoisubutyric acid leads to mastoparan-S which selectively activates $G_{s\alpha}$ [92]; mellitin efficiently stimulates $G_{\alpha 11}$ and $G_{i\alpha\text{-}1}$ but

inhibits the spontaneous guanine nucleotide exchange of $G_{s\alpha}$ [93]. The juxtamembrane portions of the intracellular loops (in particular i_2 and i_3 and to some extent i_4) of G protein-coupled receptors are rich in basic residues; for various receptors, peptides have been derived from these regions and shown to activate purified G proteins; appropriate substitutions that disrupt the helical arrangement of positive charges lead to loss of activity or to peptides with antagonistic activities [94–98]. This has led to the hypothesis that the intracellular loops of heptahelical G protein-coupled receptors form amphipathic α-helices and that this conformation is required for efficient activation of G proteins. However, some observations are inconsistent with this assumption [99, 100]. More important, these experiments have verified that receptor-derived peptides interfere with receptor-G protein coupling in cell membranes as well.

Similarly, when introduced into a cell by expression from a minigene, a peptide comprising the third intracellular loop of the α_{1B}-adrenergic receptor blocked signaling via G_q-coupled receptors, but failed to do so for a G_s-coupled receptor. The amount of inhibition achieved differed even among the G_q-coupled receptors, namely α_1-adrenergic receptors and the M_1-muscarinic acetylcholine receptor [101]. These findings indicate that the receptor/G protein interface can be selectively targeted in an intact cell provided that the membrane barrier is overcome.

Peptides derived from G protein α-subunits

By analogy with receptor-derived peptides, one would predict that the receptor/G protein complex ought to be disrupted by G protein-derived peptides that comprise the amino acids contacted by the receptors. This is the case; peptides derived from the carboxy terminus of G protein α-subunits block effector regulation in membranes [102, 103]. For some—but not all—receptors (see [104]), this interaction between receptor and G protein-derived peptide results in the stabilization of the high-affinity state for agonist binding. This discrepancy highlights the difference in the mode by which individual receptors engage a given G α-subunit (see above). More important, the ability of peptides to trap the receptor in the high-affinity state can be exploited to screen for amino acid substitutions that enhance the inhibitory potency of the peptides [105]. Additionally, this approach has already been tested in vivo; as mentioned earlier, targeted expression of a peptide derived from the C terminus of $G_{q\alpha}$ led to 'class-specific' inhibition of G_q-mediated signaling in a murine model of cardiac pressure overload, thereby preventing subsequent myocardial hypertrophy [68].

Nonpeptide G protein activators

While the structural requirements for direct activation of G proteins by receptor-derived peptides are not fully understood, it is nevertheless clear that appropriately spaced positively charged residues are required. In addition, most peptides are amphipathic. The combination of positive charge and hydrophobicity is clearly important, because several positively charged organic compounds activate G proteins directly. The first example was benzalkonium [53], but other compounds which contain an aromatic ring system or related hydrophobic substituents (referred to as 'amphiphilic cations') have been reported as well. Because many biologically active compounds share these general structural characteristics, it is not surprising that many compounds have been observed to directly activate G proteins (at fairly high concentrations); this includes several drugs such as β-adrenergic receptor antagonists and local anaesthetics [106], the antiarrhythmic drug amiodarone [107] and H_1-receptor agonists [108]. Nevertheless, it is worth pointing out that both the peptides as well as the 'amphiphilic cations' display some selectivity, as they do not indiscriminately interact with all G proteins tested. The concentrations required to observe G protein activation by these lipophilic drugs are in the submillimolar to millimolar range and thus far above that expected to occur when these compounds are administered in pharmacotherapy. Hence, it is highly improbable that a direct effect on G proteins contributes to any of the actions of these compounds in an intact organism. Similarly, it is not clear whether substance P and other tachykinins are released in vivo in quantities sufficient to promote mast cell degranulation via a direct activation of G proteins [95]. The same argument holds true for polyamines (spermine and spermidine), which have been proposed as endogenous direct activators of G proteins, but which activate G proteins only at millimolar concentrations [109]. However, the potency of polyamines can be greatly enhanced upon increasing the lipophilicity by substitution with alkyl side chains; because these alkyl-diamines and -triamines activate G proteins with an EC_{50} comparable to the potency of mastoparan, they may be promising lead compounds [110].

In contrast, taste substances represent one example in which direct activation of G proteins may contribute to a physiological effect in vivo. Several compounds, in particular those with bitter taste, activate transducin at concentrations comparable to those required for taste sensation [111]. Gustducin is closely related to transducin, and transduscin is also present in taste buds [111, 112]; based on these arguments, it is conceivable that these compounds are indeed sensed due to a direct activation of G proteins.

Nonpeptide G protein inhibitors

Earlier work identified the polysulfonated naphthylurea-derivative suramin as an inhibitor of receptor/G protein coupling in membranes [113, 114]. A subsequent analysis revealed that suramin decreased the basal rate of GDP release from purified, recombinant G protein α-subunits with submicromolar to micromolar affinities [115]. Because the dissociation of prebound GDP is the rate-limiting step in G protein activation, any compound that inhibits the basal guanine nucleotide exchange reaction is—by definition—a direct G protein inhibitor. If suramin analogues that differed in their size but not in the number and in the positions of the sulfonic acids were examined, a modest selectivity for individual G protein α-subunits was observed [115]. Based on this observation, several distinct classes of suramin analogues, in which a variable number of sulfonic acids were attached to ring systems other than the naphthylamine rings (present in suramin), were screened for selective inhibition of $G_{s\alpha}$ versus $G_{i\alpha\text{-}1}$; this search led to the identification of two compounds that effectively blocked signaling via $G_{s\alpha}$; in membranes these compounds (NF449 and NF503) blocked adenylyl cyclase stimulation by $G_{s\alpha}$ and coupling of β-adrenergic receptors to G_s in the low micromolar concentration range; in contrast, ≥ 30-fold higher concentrations were required to block signaling via a G_i- and a G_q-coupled receptor [116]. These findings thus provided evidence for the feasibility of selective G protein inhibition.

In the initial report on the inhibitory action of suramin, a major discrepancy was observed [113], suramin blocked the activation of pertussis toxin-sensitive G proteins by the δ-opioid receptor in NG108–15 membranes but not by serum factors (not identified; but in hindsight presumably lysophosphatidic acid). This indicated that the inhibition by suramin depended on the nature of the receptor-G protein complex. This interpretation was substantiated by subsequent experiments which showed that the D_2-dopamine receptor was more readily uncoupled by suramin than the A_1-adenosine receptor, although both receptors interact with the same G protein subfamily, that is the pertussis toxin-sensitive G_i and G_o proteins [117]. This discrepancy is explained as follows: Receptors compete with suramin for binding to the G protein. The higher the concentration of active, agonist-liganded receptor in the membrane, the less likely the receptor is to be uncoupled by suramin. However, due to this competition, the affinity of the receptor for the G protein(s) determines the apparent inhibitory potency of suramin. This has been verified by appropriate experiments, in which the distinct susceptibility of the A_1-adenosine receptor and of the D_2-dopamine receptors was linked to their different affinities for the G proteins [118].

In addition, this work also demonstrated that the didemethylated suramin analogue NF037 discriminated between the two receptors investigated; suramin and NF037 are equipotent in uncoupling the D_2-dopamine receptor, but 10-fold higher concentrations of NF037 than of suramin are required to prevent the interaction between A_1-adenosine receptors and G proteins [117, 118]. This difference persists, even if the receptors are forced to interact with the identical G protein α-subunit. Based on these observations, it is safe to conclude that the differences in the mode by which receptors engage a given G protein can be exploited to find suitable compounds that are selective for individual receptor-G protein complexes. The reverse finding, in which one compound suppresses the interaction of a given receptor with one G protein but not with another closely related G protein, has also been obtained (M. Waldhoer, C. Nanoff, and M. Freissmuth, unpublished observation).

The site to which suramin analogues bind is not known; however, it is clear that suramin binds in 1:1 stoichiometry [116]; in addition, the site contacted by suramin not only impedes the interaction of the receptor with the G protein α-subunit but also overlaps with the effector binding region on the G protein α-subunit. This interpretation is based on the finding that purified adenylyl cyclase relieves the suramin-dependent inhibition of the guanine nucleotide exchange reaction on $G_{s\alpha}$ [115]. The effect of suramin analogues on the basal guanine nucleotide-exchange rate and the uncoupling of receptors on a given G protein do not strictly correlate; this is in particular true for small analogues that are more effective in suppressing GDP release than in uncoupling receptor-G protein complexes [117]. This suggests that the area covered by suramin analogues is important in determining whether the compounds acts preferentially as a 'plug' that inhibits GDP release or is capable of inhibiting receptor and effector interaction.

Suramin, which was originally introduced into pharmacotherapy for its trypanocidal action (against African sleeping disease) and later also used as an anthelminthic (against *Onchocerca volvulus*, the causative agent of river blindness), is notorious for its many additional pharmacological effects, most of which have been observed in vitro (reviewed in [119]). Because of its polyanionic nature (six sulfonic acids), suramin does not permeate readily into cells. However, in some human tissues, most notably in the adrenal cortex, suramin does accumulate; the administration of suramin may result in adrenal insufficiency, and suramin has been successfully employed in the treatment of adrenocortical cancer [120, 121]. In cell culture, suramin not only depresses the growth of adenocortical cells [120, 122, 123] but also the release of steroid hormones in response to ACTH (adrenocorticotropic

hormone) [122–124]. It is attractive to speculate that these effects may be related to a suramin-induced block in the interaction between ACTH receptor and G_s and, thus, may represent an example of G protein inhibition in vivo.

Interfering with G protein $\beta\gamma$-dimers

As mentioned above, the receptor-catalyzed GDP/GTP-exchange reaction requires the presence of the G protein $\beta\gamma$-dimer, suggesting that the receptor also contacts the β- and/or the γ-subunit. This conclusion is also supported by the observation that a peptide derived from the third intracellular loop of the α_2-adrenergic receptor can be cross-linked to the C terminus of the β-subunit [125, 126]. The S-prenylated cysteine analogue *N*-acetyl-*S*-*trans*,*trans*-farnesyl-L-cysteine (L-AFC) inhibits the receptor-dependent activation of G_i (in HL60 membranes) and transducin (in membranes from rod outer segments); this is independent of the known action of L-AFC on carboxymethylation (required for the proper function of the G protein γ-subunit) and is not accompanied by a disruption of the interaction between α-subunit and $\beta\gamma$-dimers. The inhibition can be overcome by addition of $\beta\gamma$-dimers or by raising the concentration of active receptor in the membrane, suggesting that L-AFC targets the interface between $\beta\gamma$-dimers and receptor [127, 128].

Free G protein $\beta\gamma$-dimers are signaling molecules in their own right, and they are deactivated by GDP-liganded G protein α-subunits; deactivation is achieved because the α-subunit covers the surface of Gβ that binds various effectors; these include phospholipase Cβ2, the α-subunit of neuronal calcium channels, potassium channels and type II adenylyl cyclase. In addition, some isoforms of G protein-coupled receptor kinases (most notably the β-adrenergic receptor kinase β-ARK = GRK2) require $\beta\gamma$-dimers as a membrane anchor to phosphorylate the agonist-liganded, active conformation of the receptor (see [129] for review). Although the contact sites are clustered on one surface of the β-subunit, each reaction partner for Gβ nevertheless relies on a different subset of amino acid residues for its interaction [130]. This fact may also be exploited for the design of appropriate ligands which mimic or inhibit the action of $\beta\gamma$-dimers in regulating specific targets (see below).

Phosducin is the endogenous inhibitor of G protein $\beta\gamma$-dimers. This 33-kDa protein forms high affinity complexes with $\beta\gamma$-dimers [17, 131] and thereby makes them unavailable for other binding partners, including G protein α-subunits and βARK [19, 132]. The crystal structure of phosducin with transducin $\beta\gamma$ shows two domains of phosducin that wrap around $G_t\beta\gamma$ ($\beta\gamma$-dimers resolved from oligomeric transducin) to form an extensive interface [34]. Although discontiguous regions of phosducin contribute to deactivation of $\beta\gamma$-dimers [133–135], it has not yet been explored whether the interaction between phosducin and $\beta\gamma$-dimers can be exploited as a potential target site for drug action. In contrast, the carboxy terminus of β-ARK, which mediates the interaction with $\beta\gamma$-dimers via a pleckstrin-homology (PH) domain [136], is now widely used experimentally as an inhibitor of signaling via $\beta\gamma$-dimers. As the C terminus of β-ARK is about 220 amino acids in length, this domain can only be introduced into cells by transfection of an appropriate plasmid or by using the purified protein. However, a similar effect, that is inhibition of $\beta\gamma$-mediated signaling, can be achieved with a synthetic peptide encompassing amino acid residues 956 to 982 of type II adenylyl cyclase. Addition of this peptide inhibits not only $\beta\gamma$-mediated conditional activation of this enzyme but also $\beta\gamma$-mediated stimulation of PLC-β_3, of atrial K^+ channels, of β-ARK and $\beta\gamma$-mediated inhibition of type I adenylyl cyclase [137]. Thus, in spite of its lack of selectivity, this peptide of 22 amino acids may be used as a template for the search of more selective inhibitors. The fact that these effectors apparently contact distinct residues on the surface of Gβ [130] supports the conjecture that this should—in principle—be possible.

Effector-G_α interaction

The crystal structure of a complex between GTPγS-liganded (= activated) $G_{s\alpha}$ and an effector (= a nonphysiological dimer) of the catalytic (C) domains of adenylyl cyclase isoforms II (C2) and V (C1) has been solved [138]. The contact sites include the α2-helix (= switch II) and the α3–β5 loop of $G_{s\alpha}$. These contact sites had been predicted by mutational analysis of $G_{s\alpha}$ [139, 140], $G_{t\alpha}$ [141] and $G_{q\alpha}$ [142]. In addition, the α4–β6 loop of $G_{s\alpha}$ is also implicated as part of the domain required for effector activation [140]. This latter interaction has not been visualized in the crystal [138]; this may be due to the presence of additional contact sites on adenylyl cyclase, which are not within the catalytic domains. Activation of adenylyl cyclase by $G_{s\alpha}$ is thought to involve the insertion of the α2-helix of the α-subunit into a cleft of the catalytic domain (formed by the $\alpha1'$–$\alpha2'$ loop and the $\alpha3'$ helix of the catalytic C2–subunit). However, this interaction per se does not suffice to account for stimulation, because $G_{i\alpha}$-subunits do not activate adenylyl cyclase in spite of an essentially identical α2-helix. Hence, the other binding sites must support the activation. This is further highlighted by the earlier observation that a synthetic peptide corresponding to the amino acid residues 293–314 of $G_{t\alpha}$ (i.e.

comprising the helix $\alpha 4$ and the loop $\alpha 4/\beta 6$) directly activates the effector of $G_{t\alpha}$, the retinal cGMP-phosphodiesterase [143]. Since this region does not undergo any major conformational change upon activation of the G protein α-subunit [21, 22], effector activation by this short peptide predicts that inactive G protein should also activate effectors. This has been observed; GDP-liganded $G_{s\alpha}$ interacts efficiently with the catalytic domains of adenylyl cyclase and increases cyclic adenosine monophosphate (cAMP) synthesis, albeit with lower potency than the GTPγS-liganded form [144]. This finding stresses the importance of the interaction between α-subunit and $\beta\gamma$-subunit because it results in mutual inactivation. More important, based on this peptide approach, it is attractive to speculate that compounds may be identified which mimic or block the action of G protein α-subunits on effectors. It is evident that this strategy may result in selective compounds, because the various classes of G protein-regulated effectors share little, if any, sequence similarity and because the effector binding regions in $\alpha 3/\beta 5$ and $\alpha 4/\beta 6$ are also divergent (for overview see [145]). As mentioned above, suramin analogues do not only interfere with receptor/G protein coupling but they also block the activation of adenylyl cyclase by $G_{s\alpha}$ [115, 116]. It is at present unknown if this is also true for the regulation of other effectors.

As mentioned earlier, RGS proteins accelerate the GTPase reaction of the G protein α-subunit. In addition, RGS proteins inhibit effector regulation by the GTPγS-liganded G protein, that is under conditions where GTP hydrolysis cannot take place [146]. This finding is consistent with the observation that the surface covered by RGS on the three switch regions of the α-subunit overlaps with the effector binding site [30]. The switch regions are highly conserved in all α-subunits. Nevertheless, RGS4 and GAIP interact with $G_{i\alpha}$ and $G_{q\alpha}$ but not with $G_{s\alpha}$ [39–41, 146]. Similarly, the short form of RGS3 regulates G_i-, G_s- and G_q-dependent signaling, but the long form of RGS3 is only capable of negatively regulating G_i-mediated responses [147]. While the surface, which supports the interaction between α-subunits and RGS proteins, has not yet been probed with peptides or low molecular weight inhibitors, the observed specificities suggest that subtle differences within the binding site may be exploited to achieve a high degree of selectivity.

Perspectives

Significant progress has been made in delineating—mechanistically and at the structural level—the sites at which G protein subunits may be potentially targeted by drugs. Peptides (derived from signaling proteins, from insect venoms, and from substance P and related tachykinins) have been instrumental in this search; one can anticipate that they will play an important role in the future in providing leads that define specificity and selectivity of target sites. However, it is clear at present that nonpeptide compounds exist which interfere with well-defined reaction steps within the cycle of G protein activation and deactivation. Currently, the major obstacle is to find compounds that are both, sufficiently selective and cell permeable, to explore the short and long term consequences of direct G protein ligands in an intact organism. These experiments are obviously required to verify that the concept of targeting G proteins with drugs will ultimately be useful in clinical pharmacotherapy. Of equal importance from the perspective of experimental pharmacology and physiology, we expect that, during this quest, insights will be generated on the relative importance of G protein-dependent signaling in the control of organ and tissue function. It has, for instance, recently been appreciated that G protein-coupled receptors can bind signaling molecules other than G proteins [148, 149]. One can envision that inhibitors of receptor-G protein coupling augment signaling through these alternative routes.

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