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Peptide G protein agonists from a phage display library

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Abstract

G proteins may serve as targets for pharmacological activation of signaling pathways bypassing the regulatory events that may counteract the effect of the external stimulus on the level of receptors. We sought to identify peptides as lead structures interacting with G proteins utilizing a commercially available phage-display library. The heptapeptide library was screened for binding to human $G\alpha_{i1}$ which was modified with a hexahistidine tag and immobilized on a Ni^{2+}-coated surface. After several rounds of phage selection a number of phage clones with consensus sequences were found. Peptides with such sequences were chemically synthesized and their effect on [^{35}S]GTPγS binding to $G\alpha_{i1}$ and other G protein α subunits was determined. Through this process two peptide 'families' with the capability to activate G proteins were identified. The peptides showed no structural similarity to known peptide or nonpeptide G protein activators with a cationic amphiphilic structure like mastoparan or alkylamines. The functional relevance of the peptide-G protein interaction was shown by an increased sensitivity for guanine nucleotides of high-affinity agonist binding to A_1 adenosine receptors. The peptide G protein activators may, therefore, serve as novel tools for further investigation of receptor-independent activation of G proteins.

Keywords: G protein; Signal transduction; Peptide; Phage-display library; Adenosine receptor

1. Introduction

The physiological activation of a G protein is the consequence of agonist binding to one of a vast number of G protein-coupled receptors (GPCR) which can be divided into families according to sequence homology and the molecular characteristics of their ligands [1–3]. GPCR receive an extracellular signal and modulate *via* G proteins the activity of various effectors like adenylyl cyclase, phospholipase C or ion channels, and are also implicated in signaling to the nucleus [4–6]. These signaling pathways are indispensable targets for modern drugs and it is estimated that 60% of therapeutically used substances act through GPCR [3]. In chronic pharmacotherapy, such drugs are typically receptor antagonists utilized to block specific signaling pathways. Although activation of a receptor might be a desirable strategy in many instances, drug therapy with agonists is usually successful in acute intervention only. During chronic treatment long-term activation often fails due to regulatory mechanisms that compensate for permanent signal activation by desensitization [7,8]. These mechanisms cause a blunted or even an abolished response after chronic exposure to an agonist resulting in tachyphylaxis to the respective drug. Mechanisms involved in desensitization mostly operate on the receptor level leaving open the theoretical possibility to avoid tachyphylaxis by turning on a specific signaling pathway downstream from a receptor on the level of a G protein, making this family of signaling proteins an interesting drug target. G proteins have been proposed as drug targets for a number of other reasons including distinct signaling specificity compared to GPCR [9,10].

Receptor-independent activation of G protein-mediated signaling cascades is a mechanism found in nature. The wasp venom mastoparan is capable of mimicking an agonist-occupied receptor and thereby inducing G protein activation [11]. This rather nonspecific activity appears to be related to the cationic nature of this lysine-rich peptide as other cationic compounds like various alkylamines, putrescine, spermidine, and amiodarone have also been

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Abbreviations: CCPA, 2-chloro-N^6-cyclopentyladenosine; GPCR, G protein-coupled receptor.

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found to activate G proteins [11–13]. The goal of our study was to identify novel peptides that activate G proteins independent of such a cationic nature. We screened a phage display library for phages bearing peptides that bind to a specific Gα subunit. In our study α_{i1} was immobilized on a nickel surface *via* a hexahistidine tag and was then utilized to select for such phages. With this method we were able to identify a number of G protein-activating peptides which do not share the cationic amphiphilic nature of previously known G protein activators.

2. Material and methods

2.1. Materials

A hepta-peptide phage-display library kit was purchased from New England Biolabs; [^{3}H]CCPA and [^{35}S]GTPγS were from NEN Life Science Products. All other unlabeled receptor agonists and antagonists were from Biotrend. All peptides were synthesized as C-amides by D. Palm, Biozentrum, University of Würzburg. Recombinant ras and ras-GAP were kindly provided by A. Wittinghofer, MPI of Molecular Physiology. The 96-well microplate filtration system (MultiScreen MAFC) and HATF-nitrocellulose-filters (0.45 μm) were obtained from Millipore, and Pierce nickel-coated microtiterplates were obtained from KMF. Ni-NTA-agarose from Quiagen was utilized. The Big Dye Terminator Cycle Sequencing Kit was obtained from PE Applied Biosystems. Cell culture media and fetal calf serum were purchased from PanSystems. Penicillin (100 U/mL), streptomycin (100 μg/mL), L-glutamine, and G-418 were from Gibco-Life Technologies.

2.2. G protein purification

The $G\alpha_{i1}$ protein bearing a hexahistidine tag was expressed in *Escherichia coli* BL21 utilizing the vector pQE60-α_i which was kindly provided by Christiane Kleuss, Institut für Pharmakologie, Freie Universität Berlin. After the bacteria were lysed by resuspension in 50 mM Tris, 10 mM β-mercaptoethanol pH 8.0 (buffer B) and sonification the homogenate was centrifuged at 130,000 *g* for 30 min. The supernatant was loaded onto a Ni-NTA-agarose column and the retained $G\alpha_{i1}$-His_6 was eluted with 100 mM imidazole in buffer B. The G protein fraction was further purified on a Q-Sepharose column by elution with a 0–300 mM NaCl gradient in buffer B. The peak fractions were pooled and dialysed against 20 mM Tris–HCl pH 8 containing 20 mM NaCl, 10 mM β-mercaptoethanol and 20% glycerol. The activity of the purified protein was determined by [^{35}S]GTPγS-binding.

G_o and its resolved α_o and βγ subunits were purified from bovine brain according to the method described by Sternweis and Robishaw [14] using a heptylamine Sepharose column. The amounts of functional G_o or α_o were determined by [^{35}S]GTPγS-binding. G_s from rabbit liver was purified as described by Feder *et al.* [15].

2.3. Immobilization of $G\alpha_{i1}$

For the screening of phage-display libraries, histidine-tagged $G\alpha_{i1}$ was immobilized on the surface of a Ni^{2+}-coated microtiter plate. The coating was carried out by overnight incubation of 100 μL of $G\alpha_{i1}$-His_6 (0.1 nmol) in 50 mM Na-phosphate buffer pH 8, containing 150 mM NaCl, 0.02% Lubrol WT (buffer A) and 1 mM β-mercaptoethanol. The coating solution was aspirated and the well was washed 6× with 300 μL of TBS (50 mM Tris–HCl pH 7.5, 150 mM NaCl) containing 0.1% Tween-20 or the respective concentration utilized in the following step.

2.4. Phage selection by biopanning

The peptide-bearing phages (2×10^{11}) were pipetted onto the $G\alpha_{i1}$-coated plate in a final volume of 100 μL TBS/0.1% Tween-20 and incubated for 2–3 hr at 4° with gentle rocking. Unbound phages were removed by washing 10× with TBS containing Tween-20 with increasing concentrations in each round of panning (0.1, 0.3, 0.5% in the first, second, and third round, respectively). Bound phages were eluted with 0.2 M glycine–HCl pH 2.2 for 10 min at room temperature and the eluate was neutralized with 1 M Tris pH 9.1 (15/100 μL eluate). *E. coli* ER2337 in 20 mL LB medium were infected with the eluted phages (50 μL with 10^5–10^7 pfu) and amplified for 4.5 hr at 37° on an orbital shaker at 200 rpm. The bacteria were pelleted by centrifugation for 10 min at 13,000 *g* and 16 mL of phage-containing supernatant were removed. The phages were then precipitated by overnight incubation with 2.6 mL of 20% (w/v) PEG-8000/2.5 M NaCl at 4°. The precipitated phages were centrifuged and resuspended in 1 μL TBS. The precipitation step was repeated with 166 μL of PEG/NaCl, and phages were finally resuspended in 200 μL TBS/0.02% NaN_3. The phage titer was determined by counting plaques of bacteria infected with phage on agar plates. The phage preparation was then used for two additional rounds of biopanning which followed the same procedure. A fourth round of panning did not appear to improve selection of G protein-binding phages and was, therefore, omitted in our standard protocol. After the last round of panning, individual plaques were picked and the ssDNA was prepared by NaI/ethanol-precipitation and sequenced by automated cycle sequencing (Big Dye Terminator Cycle Sequencing Kit) utilizing a −96 primer.

2.5. Cell culture and membrane preparation

Membranes for radioligand binding experiments were prepared from CHO cells stably transfected with human A_1 adenosine receptors. Cell culture and membrane

preparation followed the procedures exactly as described recently [16].

2.6. Binding studies

Binding of [^{35}S]GTPγS was measured as described by Freissmuth and Gilman [17]. In brief, 0.5 pmol of G_α or heterotrimeric G protein were incubated with 10 nM [^{35}S]GTPγS in buffer A containing the indicated concentrations of peptide for 30 min at 32°. In the case of recombinant ras 0.5 fmol ras-GAP were included. The reaction was stopped with 4 mL of ice cold stop buffer (20 mM Tris–HCl pH 7.4, 100 mM NaCl, 25 mM $MgCl_2$) and the reaction mixture was filtered over HATF-nitrocellulose-filters (0.45 μm). The reaction tubes and filters were washed 3× with 4 mL of stop solution. Filters were then dissolved in 2-methoxy-ethanol and bound radioactivity was determined by liquid scintillation counting.

Radioligand binding experiments at A_1 adenosine receptors were carried out in 96-well microplates utilizing the A_1 selective agonist 2-chloro-N^6-[^{3}H]cyclopentyladenosine ([^{3}H]CCPA, 1 nM) as described recently [16].

EC_{50}- and IC_{50}-values in the above binding experiments were calculated with the Hill equation. Hill coefficients in all experiments were near unity unless noted otherwise.

3. Results

A commercially available heptapeptide phage display library was screened for binding to $G\alpha_{i1}$ that was immobilized on a Ni^{2+}-coated surface *via* a hexahistidine tag. Table 1 summarizes a selection of sequences identified with this procedure. Based on identical and similar amino acids in a number of clones three consensus sequences (shown in bold in Table 1) were chosen for detailed analysis. In order to identify these peptide sequences in proteins potentially involved in regulation of G protein function, protein sequence databases were screened for these consensus sequences. Among the hits (typically 250–1000) only a few proteins implicated in signal transduction were found. Peptide *1a* (Table 1) was found in a yeast protein kinase A, peptide *2a* represents a sequence in the central portion of the third intracellular loop of the M1 muscarinic acetylcholine receptor, and peptide *3a* occurs in β-arrestin 1. Next, peptides with a phage-derived sequence were synthesized (peptides *1–3*) as well as peptides derived from the respective proteins mentioned above (peptides *1a–3a*) containing such a consensus sequence (Table 1). These peptides were then tested for their effects on [^{35}S]GTPγS binding as an indicator of G protein activation.

The effect of synthesized peptides is summarized in Fig. 1. At a concentration of 100 μM the phage-derived peptides *1* and *3* caused a stimulation of [^{35}S]GTPγS binding as opposed to peptide *2* which was inactive. The corresponding protein-derived peptides *1a* and *3a* were equally active whereas peptide *2a* had no effect. Peptide *3b* which is a hybrid between a phage peptide and a corresponding protein peptide (substitution of the "protein" F for the "phage" A in position 3) is as active as peptides *3* and *3a*.

The effect of active peptides was analyzed in [^{35}S]GTPγS saturation experiments at $G\alpha_{i1}$. Fig. 2 shows that peptide *1a* causes an increase of the apparent [^{35}S]GTPγS affinity with no change of the B_{max} value. Similar effects were found consistently for all active heptapeptides. The apparent K_D values for [^{35}S]GTPγS in the presence of peptides are summarized in Table 2. Active peptides caused a 4–5-fold shift to lower apparent K_D values in contrast to inactive peptides which did not affect [^{35}S]GTPγS affinity.

The concentration dependence for this effect on [^{35}S]GTPγS binding is shown for selected peptides in Fig. 3. The EC_{50} values for increased [^{35}S]GTPγS binding ranged from 5 μM for the most potent sequence of peptide *3b* to 69 μM for peptide *3a* (Table 3). For inactive peptides minor effects were detected in millimolar concentrations only (Fig. 3).

In addition to the peptide effect on $G\alpha_{i1}$, several other Gα and heterotrimeric G proteins were tested in order to determine the specificity of the observed effect for G protein subtypes. Fig. 4 reveals that peptide *3b* had a preferential effect on $G\alpha_i$ and $G\alpha_o$ but a much smaller

Table 1
Peptide sequences identified in α_{i1}-binding phages[a]

	Peptide *1*									Peptide *2*								Peptide *3*							
Sequences found		**W**	**P**	H	H	**F**	L	**P**		H	Y	**P**	T	**S**	I	**P**		**L**	**P**	**W**	D	I	N	S	
		W	G	G	Y	**F**	**L**	**P**		**Q**	I	**P**	R	**S**	V	**P**		**L**	T	**V**	Q	H	L	**H**	
				H	Q	**Y**	**H**	**P**	H A	**Q**	P	S	N	**T**	K	**P**	H	**L**	**P**	**L**	W	S	L		
	S	**A**	W	S	H	**V**	**H**			A	W	**P**	P	**T**	P	**P**		**L**	**P**	H	V	S	S	**H**	
		Y	**P**	S	M	**F**	**H**	**P**										**L**	**P**	**A**	L	H	G	**H**	
		I	T	M	H	G	**H**	**P**																	
		V	S	R	H	**V**	**H**	**P**																	
Consensus sequence[a]		**a**	**P**	X	X	**a**	**H**	**P**		**Q**	X	**P**	X	**S**	X	**P**		**L**	**P**	**a**	X	X	X	**H**	
Phage peptide synthesized		**Y**	**P**	S	M	**F**	**H**	**P**	*(1)*	**Q**	I	**P**	R	**S**	V	**P**	*(2)*	**L**	**P**	**A**	L	H	G	**H**	*(3)*
'Protein' peptide synthesized		**Y**	**P**	P	Y	**F**	**H**	**P**	*(1a)*	**Q**	P	**P**	R	**S**	S	**P**	*(2a)*	**L**	**P**	**F**	T	L	M	**H**	*(3a)*

[a] Aliphatic or aromatic amino acid. Identical or similar residues are shown in bold.

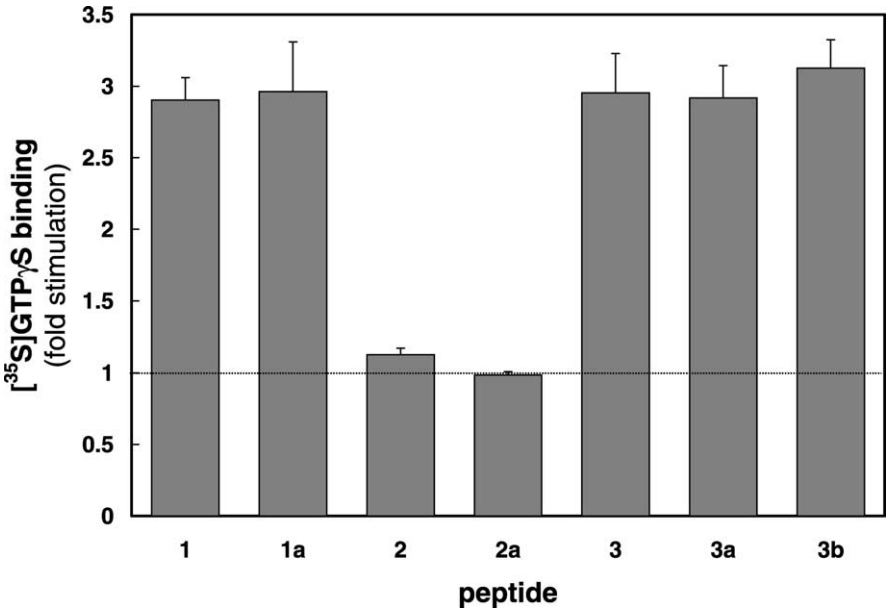

Fig. 1. Peptide effect on [^{35}S]GTPγS binding to $G\alpha_{i1}$. Peptides with consensus sequences identified in phage-display library screening (see Table 1) were tested for their effect on [^{35}S]GTPγS binding. Peptide 'families' *1* and *3* at a concentration of 100 μM caused an about 3-fold increase of [^{35}S]GTPγS binding whereas peptide 'family' *2* was inactive. Shown are means of at least five experiments (±SEM).

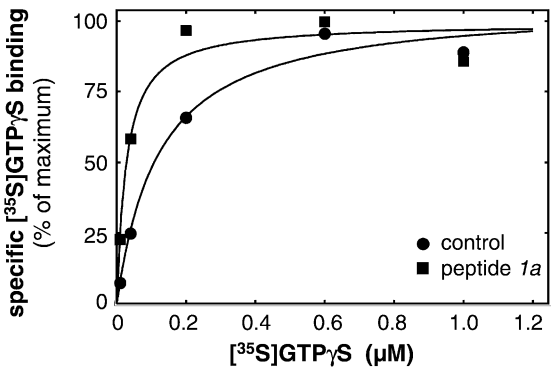

Fig. 2. Saturation binding with [^{35}S]GTPγS. $G\alpha_{i1}$ was incubated with increasing concentrations of [^{35}S]GTPγS in the presence and absence of peptide *1a*. The data are from a representative experiment showing an increase of the apparent [^{35}S]GTPγS affinity in the presence of peptides. For data in detail see Table 2.

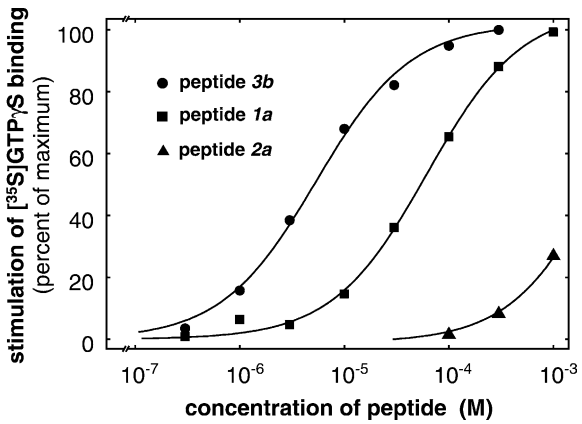

Fig. 3. Concentration dependence of the peptide effect on [^{35}S]GTPγS binding (10 nM). Shown is an example of each of the three peptide 'families'. Peptide *3b* (●) is the most potent sequence found whereas peptide *2a* (▲) is inactive in concentrations up to 100 μM. The figure shows representative experiments, for results in detail see Table 3.

Table 2
Apparent [^{35}S]GTPγS affinity for purified $G\alpha_{i1}$[a]

Peptide		K_D (nM)	95% confidence interval
Control		139	124–155
1	Y P S M F H P	32	29–35
1a	Y P P Y F H P	36	31–40
2	Q I P R S V P	144	123–169
2a	Q P P R S S P	156	148–165
3	L P A L H G H	29	27–31
3a	L P F T L M H	33	29–38
3b	L P F L H G H	30	27–33

[a] [^{35}S]GTPγS binding (10 nM) to his-tagged $G\alpha_{i1}$ in saturation experiments was measured as described in Section 2. K_D values are geometric means from 3–4 independent experiments (control N = 8). All peptides were used at a concentration of 100 μM.

Table 3
Peptide effect on [^{35}S]GTPγS binding to $G\alpha_{i1}$[a]

Peptide		EC_{50} (μM)	95% confidence interval
1	Y P S M F H P	16	14–18
1a	Y P P Y F H P	35	22–56
2	Q I P R S V P	>1000	–
2a	Q P P R S S P	>1000	–
3	L P A L H G H	17	15–19
3a	L P F T L M H	69	54–87
3b	L P F L H G H	4.9	3.6–6.7

[a] [^{35}S]GTPγS binding (10 nM) was measured in the presence of increasing concentrations of peptide as described in Section 2. EC_{50} values are geometric means from 3–5 independent experiments.

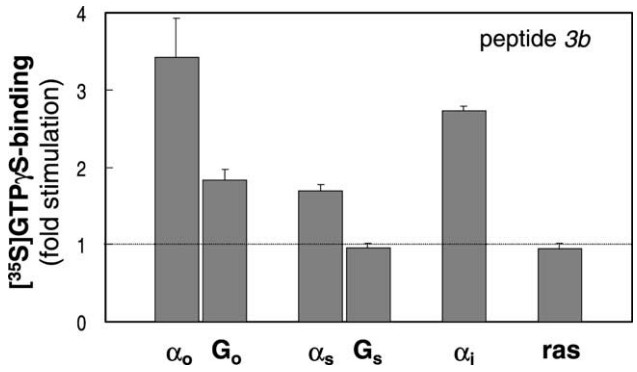

Fig. 4. Specificity of peptide effect for different G proteins. The potentiating effect of peptide *3b* on [^{35}S]GTPγS binding (10 nM) to different G proteins is shown. The largest increase in [^{35}S]GTPγS binding was observed for recombinant $G\alpha_i$ and bovine brain $G\alpha_o$ whereas no effect was found on binding to heterotrimeric G_s (rabbit liver) or the small recombinant G protein ras. Shown are means of at least three experiments (±SEM).

effect on $G\alpha_s$ and the heterotrimeric G_o. The peptide had essentially no effect on ras and heterotrimeric G_s.

A functional consequence of the peptide effect on G proteins is shown by agonist binding to human A_1 adenosine receptors in membranes from stably transfected CHO cells. Fig. 5 illustrates inhibition of high affinity binding of the A_1 selective agonist [^{3}H]CCPA by GTP in the presence and absence of 50 μM peptide *3b*. The inhibition curve is shifted to the left by a factor of 3 in the presence of the peptide documenting an increased sensitivity of agonist binding for GTP. Similar effects were found for the other active peptides whereas the inactive peptide *2* caused no shift (not shown). Table 4 summarizes the results from eight experiments with peptide *3b*. Although the effect is relatively small it was highly reproducible in all experiments. The shift in individual experiments ranged from

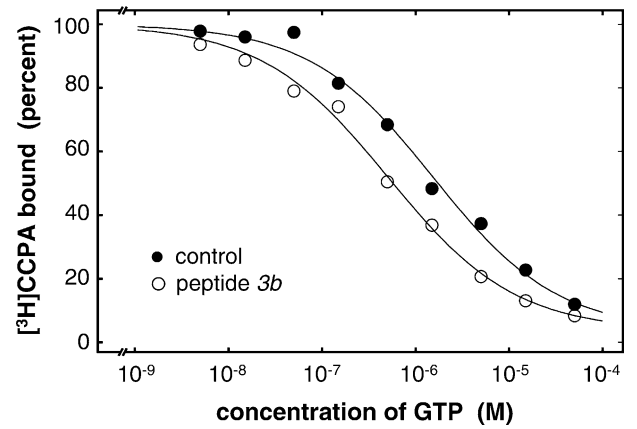

Fig. 5. Active peptides increase GTP sensitivity of high-affinity agonist binding. Shown is the GTP-induced inhibition of high-affinity binding of the agonist [^{3}H]CCPA (1 nM) to human A_1 adenosine receptors stably expressed in CHO cells. Peptide *3b* (50 μM) shifts the inhibition curve to the left. In the representative experiment shown here the IC_{50}-values for GTP are 1.6 μM (●) and 0.51 μM (○) in the absence and presence of peptide, respectively. The Hill slopes of the curves are 0.65 and 0.64, respectively. For data in detail see Table 4.

Table 4
IC_{50}-values for GTP-induced inhibition of agonist binding to A_1 adenosine receptors[a]

	IC_{50}-value	Slope
Control	2.19 (1.60–3.01)	0.64 ± 0.05
Peptide *3b*	0.91 (0.61–1.35)	0.65 ± 0.04

[a] [^{3}H]CCPA binding to A_1 receptors was measured in the presence of increasing concentrations of GTP with and without 50 μM peptide *3b* as described in Section 2. IC_{50} values for GTP (μM) are geometric means from eight independent experiments with 95% confidence intervals in parentheses. Hill slopes are given ±SEM.

1.6–3-fold resulting in an average value of 2.5 ± 0.2 (SEM).

4. Discussion

GPCR are among the most important targets for modern drugs. Typically, the compounds targeting this class of signaling proteins are antagonists. Although activation of a signaling pathway may be desirable in many pathophysiological conditions agonists are less common drugs. One of the main reasons for this might be the fact that receptor function is a carefully regulated process which results in tachyphylaxis upon sustained receptor activation in long-term treatment. On a molecular level this is the result of a process called desensitization which is operative on the receptor level [7,8]. Consequently, it is conceivable to pharmacologically activate a GPCR-regulated signaling pathway on the level of the G protein in order to circumvent the counterregulation that is triggered in the case of receptor stimulation. The functional diversity of G proteins would provide an additional benefit of such a drug target as it would allow for an alternative signal specificity compared to targeting receptors.

The transduction of an extracellular signal from a membrane-bound receptor to an effector protein which will then generate an intracellular second messenger requires a G_α subunit to interact with a large number of proteins. A G_α subunit will be in contact with receptor, βγ-subunits, various effector proteins and in addition with a number of additional proteins which function to modulate the GPCR-initiated signal [18]. Therefore, one would expect many functional domains on G_α that could be targeted to modulate G protein function. Identification of peptides binding to such functional G protein domains might be a first step towards lead structures for pharmacological modification of G protein-mediated signaling.

One of the obvious strategies to find lead peptides interacting with G proteins is to identify sequences in GPCR domains involved in receptor-G protein coupling [19,20]. A strategy independent of sequence knowledge of interacting protein domains which would not restrict the search to one given target domain is the use of an epitope library [21]. Such libraries have primarily been used to

map antigen-binding epitopes in monoclonal antibodies [22–24] where a high affinity protein–protein interaction is confined to a small peptide sequence. This strategy has also been successfully employed to identify sites of interaction in protein complexes like the phagocyte NADPH oxidase [25]. In our study we have utilized a commercially available phage-display library to screen for peptides interacting with the G protein subunit $G\alpha_{i1}$. With this method we found a number of peptide sequences that were bound to the immobilized G protein. From these, three groups of peptides with different consensus sequences were identified. In a GenBank search with these consensus sequences their potential occurrence in proteins was checked. Although they were found in a few proteins relevant to signal transduction they turned out to be rather common sequences found in several hundred to thousand proteins. This suggests that the sequences found may not play a specific role in the proteins relevant in signal transduction. Further support for this notion stems from the fact that the sequences found in the M1 muscarinic acetylcholine receptor (peptide 'family' 2) and in human β-arrestin 1 (peptide 'family' 3) are not conserved in closely related proteins like the M2 muscarinic acetylcholine receptor and in human β-arrestin 2, respectively. Nevertheless, for each of the peptide groups one peptide with a 'phage' sequence and another one with a 'protein' sequence were synthesized and analyzed for their effect on G protein function. The binding of [^{35}S]GTPγS to the G protein subunit α_{i1} was used as a readout to identify such an effect. Peptide families *1* and *3* increased [^{35}S]GTPγS binding whereas peptides *2* were ineffective. The increase in [^{35}S]GTPγS binding was caused by a 5-fold increase in the apparent [^{35}S]GTPγS affinity (Table 2). The potency of the protein-derived peptides *1a* and *3a* was lower than the corresponding phage peptides *1* and *3*, again suggesting that these sequences do not play a functional role in these proteins. It appears that the consensus sequences deduced from the phage-display library screen are coincidentially found in a large number of proteins suggesting that the functional interaction of these two peptide 'families' with G proteins is unrelated to an occurrence in a natural protein. With the most potent peptide *3b* G protein selectivity was demonstrated. With 100 μM of peptide *3b* the binding of [^{35}S]GTPγS to $G\alpha_{i1}$ and $G\alpha_o$ was stimulated about 3-fold while binding to $G\alpha_s$ showed a much smaller effect. [^{35}S]GTPγS binding to the small GTP-binding protein ras was unaffected by the peptide. In the case of G_o and G_s the peptide effect was also tested with the holotrimeric proteins and turned out to be considerably less pronounced compared to the monomeric a subunits.

The most important data demonstrating a functional significance of the peptide effect is the shift in the IC_{50}-value of the GTP-induced inhibition of agonist binding shown here for A_1 adenosine receptors. Agonist binding to the inhibitory A_1 subtype and other G_i-coupled GPCR is shifted to a low-affinity state in the presence of guanine nucleotides [16,26]. As shown in this study the inhibition curve for such a GTP effect is shifted to the left making the R-G complex more sensitive towards guanine nucleotides. Although this reproducible effect is not dramatic it might be of significance for modulatory intervention in respective signaling cascades. Small but functionally relevant effects have been found in the case of receptor–receptor interactions, e.g. between D_2 dopamine and A_{2A} adenosine receptors [27,28]. It is conceivable that such a small modulatory effect may be very useful in terms of a pharmacological intervention. The effect of benzodiazepines on GABA binding [29] may serve as evidence for a useful drug effect which is dependent on the modulation of an endogenous signal resulting in a self-limiting effect.

In this study we have successfully applied a phage-display library to identify peptides which activate G proteins. Such peptides may serve as leads for the development of nonpeptide analogs with similar activity. The peptides found have no sequence similarity to known peptide modulators of G protein function like mastoparan [11] or substance P-derived peptide antagonists [30]. The potential relevance of this functional interaction of peptides with G proteins is evidenced by an effect on GTP-dependent modulation of agonist binding to A_1 adenosine receptors. Such an effect may be of general interest as a mode to pharmacologically modify specific signaling pathways *via* defined G proteins.

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