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Specific activation of G_s by synthetic peptides corresponding to an intracellular loop of the β-adrenergic receptor

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Peptides corresponding to the amino acid sequence of the hamster β_1-adrenergic receptor (β_1AR) were synthesized and their ability to activate purified G-proteins determined. Two peptides, comprising the N- and C-terminal 15 amino acids of the putative third intracellular loop region of the β_2AR were found to activate the G-protein G_s but not to activate a preparation of G_i/G_o. Other peptides corresponding to the internal portions of this loop and the C-terminal tail region failed to activate either G-protein. The presence of phospholipid vesicles was required for this activation. The observation that peptides with sequences corresponding to the ends of the third intracellular loop of the βAR can specifically activate G_s confirms the results of previous mutagenesis studies on the receptor and demonstrates that the secondary structure conferred by the amino acid sequences in these regions is sufficient for the activation of G-proteins.

Receptor; G-protein; Peptide; GTP; Amphiphilic sequence

1. INTRODUCTION

Many hormone receptors promote their cellular responses by interacting with one of a family of guanine nucleotide binding regulatory proteins (G-proteins). Activation of G-proteins by receptors is agonist-dependent and procedes by a mechanism involving the exchange of G-protein complexed GDP for GTP [1]. The GTP-bound G-protein species interact with specific effector systems in the plasma membrane, propagating second messenger signals within the cell. This activation cycle is terminated by the intrinsic GTPase activity of the G-protein.

G-protein-coupled receptors share a structural motif which is characterized by 7 hydrophobic domains, thought to represent membrane-spanning helices, connected by more hydrophilic extracellular and intracellular loops [2]. Genetic and biochemical analysis of several of these receptors suggests that the intracellular loop domains mediate the coupling of the receptors with G-proteins. Deletion mutagenesis and hybrid receptor analysis have implicated regions at the N- and C-terminal ends of the third intracellular loop (loop i3) as the major, but not the sole, determinants of G-protein coupling [3–9].

Loop i3 represents the most divergent domain of G-protein coupled receptors, consistent with its postulated involvement in directing G-protein selectivity. Secondary structure predictions suggest that the regions at the N- and C-terminal ends of this loop may be α-helical in nature, forming amphipathic cytoplasmic extensions of transmembrane helices 5 and 6. However, there is no consensus amino acid sequence in this region from which coupling to a specific G-protein can be predicted. The lack of primary sequence homology in this region among receptors which couple to the same G-protein has led to the hypothesis that it is the amphiphilic nature of these α-helical regions that is the main determinant in the interaction of receptors with G-proteins [10,11]. Indeed, mastoparan, a bee venom peptide which has been demonstrated to form an amphiphilic α-helix in solution, has been shown to be an activator of G-proteins [11].

In the present study, peptides corresponding to amino acid sequences in the intracellular loops of the β-adrenergic receptor (βAR) were synthesized and assayed for their ability to activate recombinant G_s and a preparation of G_i and G_o. The data support the hypothesis that the secondary structure of the regions at the N- and C-termini of i3 is a critical determinant of both G-protein selectivity and activation.

2. EXPERIMENTAL PROCEDURES

2.1. *Materials*

Phosphatidyl serine from bovine brain and dioleolphosphatidyl choline were purchased from Sigma. GTPγS was purchased from Sigma and purified as described. [^{35}S]GTPγS and [γ-^{32}P]GTP were purchased from New England Nuclear. Mastoparan was purchased from Sigma.

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2.2. *Peptide synthesis and purification*

Peptides were synthesized by Multiple Peptide Systems (San Diego, CA) as C-terminal amides, and were purified by reverse-phase HPLC on C18 columns. All purified peptides represent single peaks of UV absorption at 215 nm in the chromatograms.

2.3. *Preparation of G-proteins*

The long form of recombinant $G_{i\alpha}$ ($rG_{i\alpha}$) was expressed in *E. coli* and purified as described previously [12]. Oligomeric G-protein (a mixture of G_i and G_o) was purified from bovine brain by the method of Sternweis and Robishaw [13] and resolution of $\beta\gamma$ from the purified oligomer was achieved by methods previously described [14].

2.4. *Assay of GTPase activity*

Phospholipid vesicles were prepared by sonicating a mixture of 1.3 mg/ml of phosphatidylcholine and 1.3 mg/ml of phosphatidylserine in buffer D (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT and 100 mM NaCl), rG_i was mixed with a preparation of $\beta\gamma$ in an approximate 1:1 molar ratio before reconstituting into the preparation of vesicles while oligomeric G_i/G_o was reconstituted directly [11]. GTPase activity was measured by the method of Higashijima et al. [15]. Peptides were first incubated with the reconstituted G-protein/phospholipid mixture on ice for 10 min. The GTPase assay was carried out with 1 μM [γ-^{32}P]GTP (5000–10 000 cpm/pmol) in buffer D with 2 mM $MgCl_2$ at 30°C for 15 min. Aliquots were taken for the determination of free P_i as described [15]. [^{35}S]GTPγS binding was quantitated as previously described [16].

3. RESULTS AND DISCUSSION

To determine whether peptide fragments of the βAR were able to directly activate G-proteins, a series of peptides 14–15 amino acids in length corresponding to sequences within intracellular regions of the βAR were synthesized (Fig. 1). Homology searches of the sequences of other G-protein coupled receptors showed residues 220–229 in loop i3 of the human M1 muscarinic receptor to have the greatest similarity to mastoparan; therefore, a peptide from this region was also synthesized (Fig. 1). Like mastoparan, all of the peptides used in the present study were C-terminal amides. The ability of these peptides to stimulate the GTPase activity of $rG_{i\alpha}$ reconstituted with $\beta\gamma$ or of oligomeric G_i/G_o was measured, with the results shown in Table I.

Mastoparan, in the presence of phospholipid vesicles, stimualted the GTPase activity of both rG_i and G_i/G_o by approximately 3-fold during the 15 min assay (Table I). The rate of GTP hydrolysis was linear during the time of the assay in both the presence and absence of mastoparan (data not shown). Peptide P6, from the N-terminus of βAR i3, also stimulated the GTPase activity of G_i, by 3.3-fold, but activated G_i/G_o only slightly (Table I). The activation was completely inhibited in the presence of 10 μM GDP, suggesting that the peptide activates G_i by increasing the rate of GDP-GTP exchange on the G-protein. By this criterion, the activation by peptide P6 mimics stimulation by agonist-occupied receptor. As would be expected for stimulation by an agonist-occupied receptor, the peptide also promoted an increase in the rate of GTPγS binding to G_i, without affecting the maximal extent of the binding (Fig. 2).

Peptide P1, which overlaps with P6 but is shifted 5 residues towards the C-terminus, was much less effective than P6 in activating G_i (Table I), although the EC_{50} values for activation by the two peptides were similar (P6 = 30 μM, P1 = 25 μM). Thus, peptide P1 may mimic the conformation of the receptor in the presence of a partial agonist. Peptide P2, shifted yet five more residues into i3, was inactive in this assay. Likewise, peptide P5, which represents approximately the analogous region from the M1 muscarinic receptor and was selected because of its homology to mastoparan, failed to activate either G_s or G_i/G_o. Peptide P4, corresponding to the N-terminal 15 residues of the cytoplasmic tail of the βAR, was inactive against

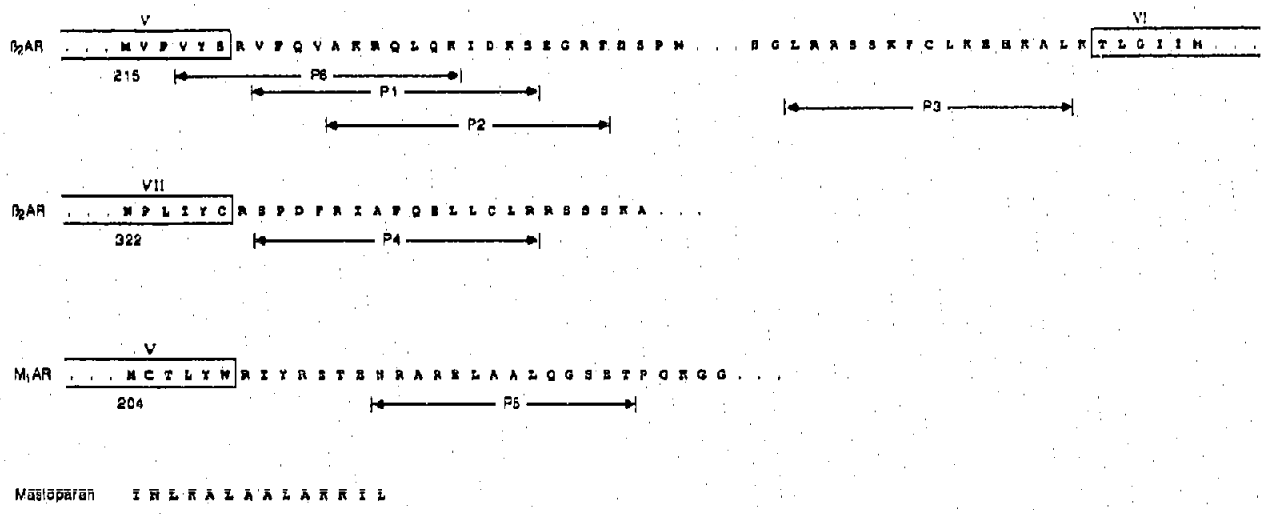

Fig. 1. Sequences of synthetic peptides. Partial sequences of intracellular regions of the hamster β_2AR or human M_1 muscarinic receptor (M_1AR) are given. Boxed residues represent transmembrane regions of the indicated receptors, with the transmembrane helix numbers designated by Roman numerals. The sequences of peptides P1–P6 are indicated with arrows below the corresponding region of the protein. The sequence of mastoporan is also given. All peptides were synthesized as C-terminal amides.

Table I

Effect of different peptides on the GTPase activities of G_s and G_i/G_o

	GTPase (fold stimulation over basal activity)			
	G_s	n	G_i/G_o	n
With Vesicles				
Mastoparan	3.26 ± 0.65	7	2.95 ± 0.33	6
Peptide 1	1.62 ± 0.33	7	1.18 ± 0.07	5
2	1.05 ± 0.05	2	-	-
3	2.54 ± 0.47	8	1.31 ± 0.20	5
4	1.33 ± 0.28	6	1.12 ± 0.07	5
6	3.30 ± 0.65	8	1.30 ± 0.19	4
5	1.05 ± 0.05	2	1.02 ± 0.04	5
Without Vesicles				
Peptide 3	0.88 ± 0.13	3	-	-
6	0.99 ± 0.08	3	-	-
5	1.02 ± 0.16	3	-	-

Activation of G-proteins by synthetic peptides. GTPase activities of G-proteins were assayed in the presence of 100 μM of each peptide in the presence or absence of phospholipid vesicles using 1 μM GTP, as described in section 2. Basal GTPase activities of G_s and G_i/G_o were 0.039 min^{-1} and 0.046 min^{-1} respectively. Free [Mg^{+2}] was approximately 1 mM.

both G_s and G_i/G_o. This is in agreement with the results of Palm et al., who found that the analogous peptide (338–353) of the turkey βAR was inactive in inhibiting hormone-stimulated adenylyl cyclase activity in turkey erythrocyte membranes [17].

Thus, of the 3 peptides corresponding to segments of the N-terminal region of loop i3 of the βAR, only the most N-terminal (P6) was able to activate the GTPase activity of G_s to the same extent as mastoparan. This comparison defines the peptide sequence in this region which, when incorporated into vesicles, can best mimic the conformation of the activated receptor. Peptide P6 incorporates 3 amino acids which would be predicted to lie within the transmembrane hydrophobic core of the receptor, at the C-terminus of transmembrane helix 5, and which are absent from peptide P1. Since P1 stimulates G_s to a markedly lower extent than peptide P6, the addition of these hydrophobic amino acids to P6 may be significant in stabilizing the active conformation of the peptide.

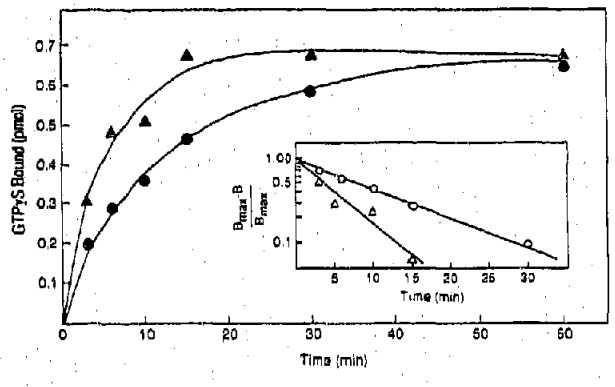

Fig. 2. Peptide 6-stimualted guanine nucleotide exchange. [^{35}S]GTPγS binding was assayed at 20°C using G_s reconstituted in phospholipid vesicles as described in section 2, in the absence (•) or presence (▲) of 30 μM peptide 6. Inset shows calculated rates of GTPγS binding of the two curves.

Peptide P3, corresponding to the C-terminal 15 amino acids of βAR i3, activated G_s to a slightly lesser extent than P6 (2.5-fold), while failing to significantly activate G_i/G_o (Table I). Since this peptide does not incorporate any hydrophobic residues from the putative transmembrane helix 6, the activation of G_s by peptide P3 may not represent the maximum stimulation by this region of the receptor. The EC_{50} for G_s activation by P3 (50 μM) was similar to the values determined for P1 and P6 (see above). As has previously been observed for mastoparan [11,17], the stimulation of GTPase activity by peptides P3 and P6 requires the presence of phospholipid vesicles (Table I), also consistent with a contribution of hydrophobic interactions to the active conformation of the peptides. In agreement with these results, a recent analysis of G_i and G_o activation by a series of mastoparan analogs of varying potency revealed the hydrophobic moment of the peptide to be a critical component of its ability to stimulate G-proteins [18]. In that study, mastoparan preferentially activated G_i and G_o over G_s. The differences seen in the present study may be due to different amounts of βγ incorporated with the different G-protein preparations [19].

Unlike mastoparan, which activated G_s and G_i/G_o to the same extent, peptides P6 and P3 were significantly more effective in activating G_s than G_i/G_o. Thus, these amino acid sequences contribute directly to the determination of the selectivity for G_s over G_i or G_o. Mutagenesis studies have implicated the analogous regions of several receptors in mediating the activation of G-proteins. Deletion analysis revealed that the regions at the N- and C-termini of βAR i3 are required for G-protein coupling, although deletions of similar

size from the middle of the loop have no effect on this parameter [4]. In addition, experiments utilizing chimeric receptors have demonstrated that the substitution of regions at the N-terminus of loop i3 from one receptor to another is sufficient to alter the G-protein selectivity [5-8]. These data indicate that the regions at the ends of loop i3 are critical for promoting G-protein activation and determining specificity. However, mutagenesis studies, which utilize the intact receptor protein, cannot distinguish between a direct or a conformational effect of these regions of the receptor inmediating G-protein interactions.

The direct activation of G_s by the peptides in the present study indicates that the structural information contained within the amino acid sequences at the N- and C-termini of the third intracellular loop of the βAR is important for both directing the recognition of a specific G-protein and for promoting its activation. Further structural and functional analysis of these and related peptides should permit the delineation of specific molecular interactions which contribute to the stimulation of G-proteins by agonist-occupied receptors.

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