Differential regulation of G protein α-subunit GTPase activity by peptides derived from the third cytoplasmic loop of the α_2-adrenergic receptor

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Abstract The effect of peptides homologous to segments of a G protein-coupled receptor on the GTPase activity of recombinant $G_o\alpha$ ($rG_o\alpha$) and $G_s\alpha$ ($rG_s\alpha$) has been tested. These peptides contain overlapping sequences spanning from amino acid 212 of the putative fifth transmembrane domain to amino acid 229 of the third cytoplasmic loop of the α2 adrenergic receptor. Interestingly, two peptides (comprising residues 212–227 and 214–227) strongly inhibit the basal GTPase activity of both $rG_o\alpha$ and $rG_s\alpha$. Instead, a C-terminally extended peptide (residues 216–229) stimulates $rG_o\alpha$ but slightly inhibits $rG_s\alpha$. Circular dichroism spectroscopy of the peptides reveals that an a helical structure is more easily inducible in the inhibitory ones. These findings constitute an example of peptides representing cytoplasmic receptor sequences that differentially modulate the GTPase activity of recombinant G protein α-subunits.

Key words: Receptorial peptide; CD spectroscopy; G protein α-subunit; GTPase activity

1. Introduction

Cell surface receptors with seven transmembrane domains transduce signals to ion channels or to intracellular effectors (enzymes involved in the synthesis of second messengers such as cyclic nucleotides, phosphoinositols and diacylglycerols) through heterotrimeric (consisting of α, β and γ subunits) guanine nucleotide binding proteins (G proteins). Upon receptor-ligand interaction, GDP is exchanged for GTP on the G protein's α-subunit, which subsequently dissociates from the receptor and the βγ dimer and modulates the activity of ion channels or effectors. Following GTP hydrolysis, Gα·GDP reassociates with βγ on the inner surface of the cell membrane [1–4].

There is convincing evidence that the receptors regulate G proteins through their intracellular loops. It has been shown for several receptors that one of these regions corresponds to the N-terminal segment of the third cytoplasmic loop [5]. However, in the case of the α_2A-adrenergic receptor it is at present unclear whether or not this receptor's respective segment participates in the interaction with G proteins. Switching the sequence in this region of the β_2-adrenergic receptor to that of the α_2-adrenergic receptor does not change the specificity of interaction with the G protein. Such mutants of the β_2-adrenergic receptor still couple to G_s as judged by the agonist induced stimulation of adenylate cyclase activity [6–8]. It has also been reported that a dodecapeptide homologous to the N-terminal portion of the third intracellular loop of the α_2-adrenergic receptor (residues 218–229) does not interfere with the activation of G proteins. In the presence of ligand, receptor-stimulated GTP hydrolysis in platelet membranes is unaffected by the peptide [9]. On the other hand, it has been shown that an analogous undecapeptide, differing only by the lack of the C-terminal amino acid, markedly stimulates GTPγS binding to purified G_i, G_o, and G_s [10].

We have attempted to further elucidate the possible role of the α_2-adrenergic receptor's N-terminal segment of its third intracellular region in coupling to G proteins. To this end we have tested the effect of peptides, derived from that segment and part of the adjacent fifth transmembrane domain, on the basal GTPase activity of recombinant α subunits of G_o and G_s ($rG_o\alpha$ and $rG_s\alpha$). The peptides have a common core sequence with different C- or N-terminal extensions that include amino acid residues 218–229 of the receptor. Depending on the extension, we observe different effects (stimulation or inhibition) on the GTPase activity of $rG_o\alpha$. The GTP hydrolysis by $rG_s\alpha$, instead, is not affected by one of these peptides and is inhibited by the others.

2. Experimental

2.1. Recombinant Gα subunits

The *E. coli* strain BL21(DE3) harboring the $G_o\alpha$ expression vector NpT7-5/$G_o\alpha$ was provided by Dr. Maurine Linder (University of Texas Southwestern Medical Center, Dallas, Texas). Cell growth and induction of protein expression were obtained as described [11]. The expression of recombinant $G_o\alpha$ was verified by SDS/PAGE of induced lysates on 12.5% slab gels according to Laemmli [12] after Coomassie blue staining, as well as after immunoblot analysis using a 1 : 10,000 dilution of a rabbit antiserum specific for $G_o\alpha$ [13], provided by Dr. Graeme Milligan (University of Glasgow, Scotland). The purification of the recombinant $G_o\alpha$ from bacterial lysates was carried out as previously reported [14]. The recombinant $G_s\alpha$ subunit ($G_s\alpha$-s, 45 kDa form), purified according to a slightly modified protocol [15], was a gift from Drs. Christian Nanoff and Michael Freissmuth (University of Vienna, Austria). Overall protein concentrations were determined by the method of Bradford [16], whereas the concentrations of active G protein α subunits were determined by [γ-^{35}S]GTP binding [17].

2.2. GTP hydrolysis

Reconstitution of $rG_o\alpha$ into phospholipid vesicles and determination of GTP hydrolysis by this protein in the absence or in the presence of peptides, were performed as previously described [14]. Alternatively, $rG_o\alpha$ or $rG_s\alpha$ were incubated for 5 min at 20°C or 30°C respectively, in 50 μl of a reaction mixture containing 50 mM HEPES-KOH (pH 8), 1mM EDTA, 1 mM DTT and 0.1% Lubrol (HEDL) and in the case of $rG_o\alpha$, 1.1 mM $MgCl_2$ and 0.4 μM [γ-^{32}P]GTP; while in the case of

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$rG_s\alpha$, 2 mM $MgCl_2$ and 1 μM [γ-^{32}P]GTP (10,000–30,000 cpm/pmol). Each experiment was repeated at least twice and each point shown represents the average of duplicate determinations.

2.3. Peptides

Peptides 1 and 4 were synthesized on an ABIMED AMS 422 multiple peptide synthesizer using fluorenylmethoxycarbonyl (Fmoc) chemistry [18] for the coupling reactions. Instead, in the synthesis of peptides 2 and 3, tertiary butyloxycarbonyl (tboc) chemistry was employed using the method developed by Houghten [19]. The peptides (about 5 mg each of the raw product) were purified by HPLC on a Merck LiChrospher 300 RP 18 column (10 × 250 mm) applying varying linear gradients of acetonitrile (typically 20–30% in 40 min) in 0.1% trifluoroacetic acid at a flow rate of 2 ml/min. The purified peptides were concentrated to dryness in a Speedvac concentrator, redissolved in 0.2 ml of bidestilled water, concentrated to dryness again and finally dissolved in 0.1 ml of bidestilled water. Their purity was assessed by analytical RP-HPLC and their concentrations were determined as described [20]

2.4. Circular dichroism spectroscopy

CD spectra were measured at room temperature in a 1 mm cell (total volume 1.1 ml) using a JASCO 600 spectropolarimeter. The final concentration of the peptides was 50 μM. Trifluoroethanol or lysophosphatidylcholine were added as indicated. Each spectrum is the average of five scans at 10 nm/min.

3. Results

3.1. Effects of receptorial peptides on the GTPase activity of $rG_o\alpha$

Four peptides corresponding to sequences spanning from residue 212 to residue 229 of the human α_2A-adrenergic receptor were synthesized and purified by RP-HPLC. These peptides comprised overlapping sequences of the N-terminal region of the third cytoplasmic loop and part of the adjacent fifth transmembrane domain (Table 1). Their effect on the basal GTPase activity of $rG_o\alpha$ was first determined with protein that had been reconstituted into phospholipid vesicles (Fig. 1). Under these conditions, peptide 1 (amino acids 218–228) and peptide 3 (amino acids 216–229) stimulated the GTP hydrolysis by $rG_o\alpha$ in a concentration-dependent manner (Fig. 1A), the former enhancing the activity nearly 1.5-fold and the latter 2-fold at 1 mM peptide. Instead, peptide 4 (amino acids 212–227) and peptide 2 (amino acids 214–227) strongly inhibited the basal GTPase activity of $rG_o\alpha$ (Fig. 1B). In the case of peptide 4 the inhibition was virtually complete, reaching over 90% at a concentration of 320 μM. Peptide 2 was less potent, with 80% inhibition at 1 mM peptide.

3.2. Inhibition of the GTPase activity of $rG_s\alpha$ by receptorial peptides

The effect of the same four peptides (see above and Table 1) on the basal GTPase activity of $rG_s\alpha$ was measured in the presence of a detergent (0.1% Lubrol) according to Graziano et al. [21]. While peptide 1 had no effect (up to a concentration of 1 mM), peptides 2, 3 and 4 inhibited the GTP hydrolysis by this protein to varying extents and with different potencies (Fig. 2). The inhibition by peptide 2 leveled off at a concentration of 560 μM but having reached only about 50%. Similarly, at the same concentration peptide 3 decreased the basal GTPase activity of $rG_s\alpha$ by a maximum 60%. However, as opposed to peptide 2, peptide 3 started to have an effect only above 200 μM. In the presence of peptide 4, the GTP hydrolysis was already inhibited by more than 75% at 10 μM and was abolished at 100 μM peptide.

Table 1
Synthesized peptides

Peptide	Sequence	Residue numbers
1	R-I-Y-Q-I-A-K-R-R-T-R	218–228
2	L-V-Y-V-R-I-Y-Q-I-A-K-R-R-T	214–227
3	Y-V-R-I-Y-Q-I-A-K-R-R-T-R-V	216–229
4	M-I-L-V-Y-V-R-I-Y-Q-I-A-K-R-R-T	212–227

These peptides represent sequences present in the mammalian α_2A-adrenergic receptor. The underlined amino acid residues are part of the putative 5th transmembrane domain, the others constitute the N-terminal segment of the third cytoplasmic loop.

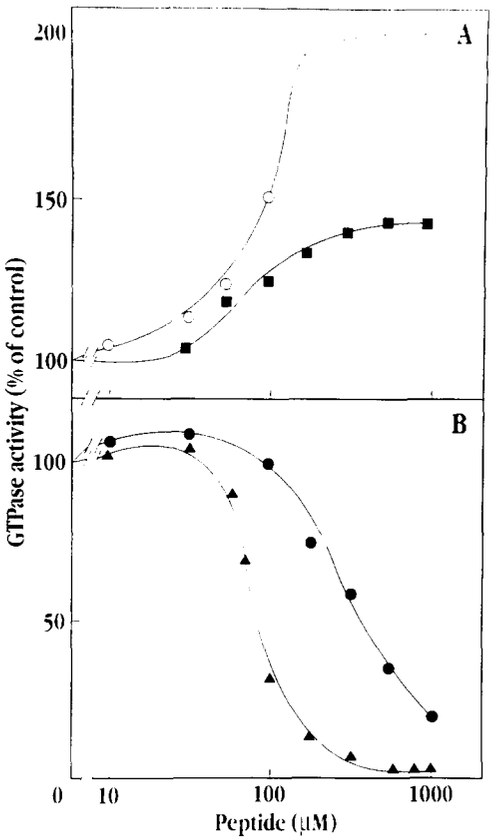

Fig. 1. Opposite effects on the GTPase activity of $rG_o\alpha$ reconstituted into phospholipid vesicles. The $rG_o\alpha$ was preincubated in a mixture of phospholipids at 4°C overnight and subsequently the GTP hydrolysis was assayed at 20°C for 5 minutes in a final volume of 50 μl in the presence of peptide 3 (○) or peptide 1 (■) (panel A) and peptide 2 (●) or peptide 4 (▲) (panel B). Under these conditions, 100% GTPase activity corresponds to a molar turnover number of 0.028 min^{-1}. The final protein concentration was 60 nM as determined by [γ-^{35}S]GTP binding. The data represent the mean of duplicate measurements of three separate experiments.

3.3. Inhibition of the GTPase activity of $rG_s\alpha$ and $rG_o\alpha$ by peptide 4

For the purpose of assessing the actual potency of the inhibitory peptide 4, it was also tested at concentrations below 10 μM (Fig. 3). The GTPase activity of $rG_s\alpha$ declined sharply in the rather narrow concentration range of 3–10 μM peptide. When the GTP hydrolysis by $rG_o\alpha$ was assayed under similar

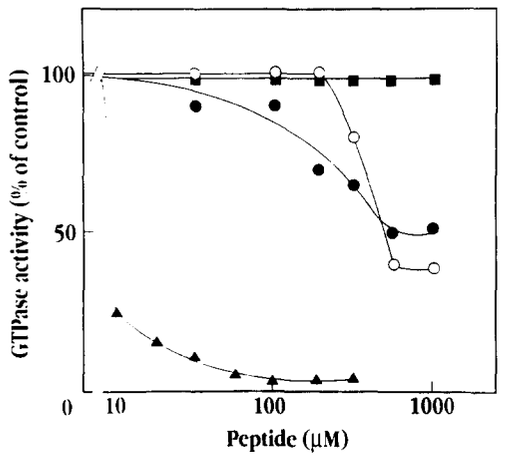

Fig. 2. Inhibition of the basal GTPase activity of $rG_s\alpha$. The $rG_s\alpha$ was incubated in the presence of 0.1% Lubrol, at a final protein concentration of 40 nM, as described in the experimental section. 100% GTPase activity corresponds to a molar turnover number of 0.095 min^{-1}. Peptide 1 (■), peptide 2 (●), peptide 3 (○) and peptide 4 (▲).

conditions (i.e. in the presence of 0.1% Lubrol) the inhibitory effect of peptide 4 was less pronounced than with $rG_s\alpha$ (Fig. 3). However, the concentration of peptide 4 needed to inhibit the GTPase activity of $rG_o\alpha$ by 90% or more was much lower (100 μM) in the presence of Lubrol than in the case where the protein had been reconstituted into phospholipid vesicles (320 μM, compare Fig. 1).

3.4. Circular dichroism spectroscopy of receptorial peptides

In order to obtain information about possible structures adopted in solution, the four peptides were analysed by CD spectroscopy in the 190–240 nm range. In aqueous solution, in the absence or presence of a detergent (0.1% Lubrol), these peptides do not form ordered secondary structures (data not shown). Structural transitions can be induced by gradually increasing the concentration of trifluoroethanol, solvent used to create a hydrophobic environment. For example, in the spectra obtained with peptides 2 and 3 (Fig. 4) the addition of trifluoroethanol causes the negative ellipticity to shift towards longer wavelengths, which is characteristic for an increasing content of α-helical structure. The shift occurs at a relatively low concentration of trifluoroethanol (20%) in the case of peptide 2, whereas peptide 3 starts to adopt α-helical structure above 40% of the solvent (Fig. 4). In the presence of the same increasing percentages of trifluoroethanol, peptide 4 behaves exactly like peptide 2 (spectra not shown). The spectra obtained with peptide 1 do not change as the percentage of trifluoroethanol in the solution is increased, indicating that it remains in the random coil form. These results reveal the propensity of the peptides with elongated N-termini (which include a part of the putative transmembrane domain) to form ordered structures. The same structural transitions can also be observed with peptides 4 and 2 in the presence of lysophosphatidylcholine, a phospholipid used to emulate a membrane environment (Fig. 5). As judged by the spectra obtained under these conditions, peptide 3 is much less structured as compared to the former two, while peptide 1 is a random coil also in the presence of the phospholipid.

4. Discussion

Cell surface receptors featuring seven transmembrane domains interact with G proteins through portions of their intracellular loops [4,5]. A segment of the third cytoplasmic loop (comprising the N-terminal 11 amino acids) of the mammalian α_2A-adrenergic receptor has been implicated in the activation of G proteins [10]. We have further investigated the involvement in G protein coupling of the N-terminal region of this receptor's third cytoplasmic loop using corresponding synthetic peptides that also contain some amino acids of the adjacent fifth transmembrane domain. The four peptides, spanning from residue 212 in the fifth transmembrane domain to residue 229 in the third intracellular loop, have a common core sequence of 10 amino acids and varying C- or N-terminal extensions (Table 1).

In current models of signal transduction the interaction between agonist-bound receptors and G proteins induces the exchange of GDP for GTP on the G protein α-subunit, leading to effector activation and ultimately to an enhancement of the intrinsic GTPase activity of the α-subunit [1–4]. However, it was recently shown that the activated prostaglandin E receptor EP_{3C} couples to two different G proteins, G_s and G_o, but differentially regulates them, stimulating the GTPase activity of G_s and inhibiting that of G_o [22]. Evidence indicating receptor-mediated inhibition of the GTP hydrolysis by G_i1 and G_i2 but not G_o, was also found with reconstituted membranes from guinea pig cerebellum in the presence of an opiod κ-agonist [23].

Here we have shown that synthetic peptides, representing overlapping sequences of the α_2A-adrenergic receptor's N-terminal segment of the third cytoplasmic loop and part of the adjacent fifth transmembrane domain, inhibit the GTP hydrolysis by $rG_o\alpha$ and $rG_s\alpha$. Peptide 4, which contains 16 amino acids, is the most potent inhibitor of both GTPase activities. Among the two tetradecapeptides 2 and 3, peptide 2 inhibits the GTP hydrolysis by both proteins, while peptide 3 exerts its inhibitory effect only on $rG_s\alpha$. These results further suggest that receptors can negatively regulate G protein activity.

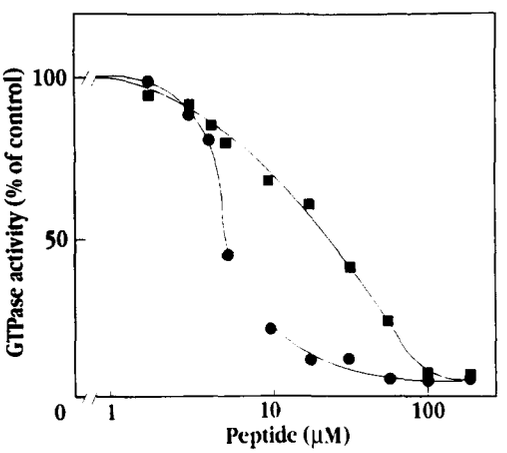

Fig. 3. Inhibition of the basal GTPase activity of $rG_o\alpha$ and $rG_s\alpha$ by peptide 4. The GTP hydrolysis by $rG_o\alpha$ or $rG_s\alpha$ was determined as described in the legend to Fig. 2, but in the presence of lower concentrations of peptide 4. Under these conditions, 100% GTPase activities correspond to molar turnover numbers for $rG_o\alpha$ and for $rG_s\alpha$ of 0.099 and of 0.095 min^{-1}, respectively. The final protein concentrations were 60 nM for $rG_o\alpha$ (■) and 40 nM for $rG_s\alpha$ (●).

Peptide 1 already has been shown to stimulate GTPγ S binding to heterotrimeric G_i, G_o and G_s [10]. This peptide and peptide 3 stimulate the GTPase activity of $rG_o\alpha$, while peptides 2 and 4 are inhibitory. Peptide 3 is 'frame shifted' by two amino acids as compared to peptide 2, whereas peptide 1 is a truncated version of peptide 3 (see Table 1). It is remarkable that such subtle differences give rise to opposite effects on the enzymatic activity of $rG_o\alpha$. This suggests that the molecular mechanism of G protein regulation may involve only a few critical amino acids of the receptor.

Recently it has been reported that only a few charged amino acids at the membrane/cytoplasm boundary of the m3 muscarinic acetylcholine receptor's third intracellular loop appear to be important for signal transduction [24]. In this context it is noteworthy that an arginine residue is present at the extended C-terminus of both stimulatory peptides, but is absent in the inhibitory ones. This arginine, which corresponds to residue 228 of the α_2-adrenergic receptor, may thus be relevant for the receptor's ability to activate $G_o\alpha$.

The N-termini of peptides 3, 2 and 4 comprise, respectively,

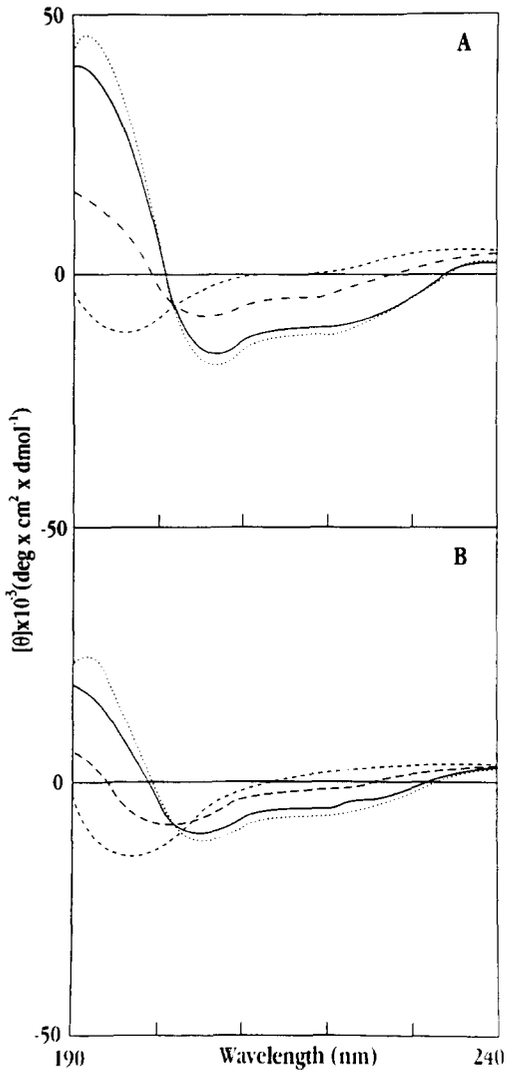

Fig. 4. CD spectra of peptides 2 and 3 in the presence of trifluoroethanol. The spectra were recorded as described in the experimental section for peptides 2 (panel A) and 3 (panel B) in aqueous solutions containing 10% (- - - -), 20% (– – –), 30% (——) or 40% (·····) trifluoroethanol.

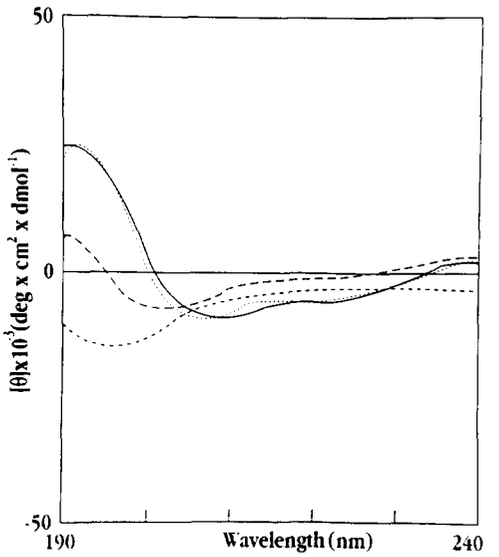

Fig. 5. CD spectra of peptides in the presence of phospholipid. The aqueous solutions of peptides 1 (- - - -), 2 (·····), 3 (– – –) and 4 (——) contained L-α-lysophosphatidyl choline at 1 mg/ml.

two, four and six residues from the receptor's fifth transmembrane domain in addition to the contiguous intracellular segment of the third cytoplasmic loop represented by peptide 1. As the length of this hydrophobic tail increases, the peptides become more potent inhibitors of the GTPase activities (in the case of $rG_o\alpha$ this applies only to peptides 2 and 4). The peptides with hydrophobic tails indeed have a propensity to become partly α-helical, but only in a lipidic environment as shown by CD spectroscopic analysis. It is therefore likely that the hydrophobic residues interact directly with the G protein α-subunits rather than inducing structural conformations that facilitate their mode of action. The varying effects that a receptor may exert on the same G protein α-subunit could reflect different extents of hydrophobic amino acid exposure in the receptor's G protein-coupling sites.

It has been proposed that the intracellular regions of receptors interacting with G proteins possess amphiphilic α-helical properties [25,26]. Contrary to this model, Voss et al. reported that a peptide derived from the third cytoplasmic loop's N-terminal portion of the dopamine D_2 receptor specifically interfered with signal transduction by this receptor, but did not form an amphiphilic α-helix [27]. CD spectroscopy of the peptides studied here revealed that peptides 2, 3 and 4 could adopt partial α-helical structures in a phospholipidic environment, where their differential effects on the activity of $rG_o\alpha$ are most pronounced. However, peptide 4 is a much more potent inhibitor of the GTPase activities of both G protein α-subunits in the presence of detergent, where this peptide is not structured at all as judged by spectroscopic analysis. In addition, none of the four peptides is amphiphilic. Therefore, our observations add further doubt to the concept of an amphiphilic α-helix as the G protein interacting entity in receptors featuring seven transmembrane domains.

In conclusion, here we have reported studies on peptides homologous to sequences of a cell surface receptor with seven transmembrane domains that negatively modulate the activity of recombinant G protein a subunits. Furthermore, we have

provided evidence suggesting that the α_2A-adrenergic receptor can oppositely regulate the GTPase activity of $G_o\alpha$ and that such opposite effects can be the result of a shifted exposure of a few amino acids at the boundary of the receptor's fifth transmembrane domain and its third cytoplasmic loop.

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