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Modulation of ligand responses by coupling of α_{2A}-adrenoceptors to diverse G_α-proteins

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Abstract

The hypothesis that different signalling may be mediated via a single α_{2A}-adrenoceptor (α_{2A} AR) subtype was investigated by challenging α_2 AR ligands in combination with diverse recombinant wt, mutant, and chimeric G_α-proteins. Possible coupling of α_{2A} AR to endogenous $G_{\alpha i/o}$-proteins in CHO-K1 cells was excluded by measuring pertussis toxin (PTX)-resistant [^{35}S]GTPγS-binding responses as a common functional response to α_{2A} AR activation. (−)-Adrenaline (10 μM) displayed the highest magnitude of [^{35}S]GTPγS-binding response in the co-presence of a PTX-resistant $G_{\alpha o}Cys^{351}Ile$ protein, whereas a decreased response was obtained with the mutant $G_{\alpha i1/2}$-proteins. Replacement of the last six amino acids at the C-terminal portion of the $G_{\alpha o}$-protein by the corresponding amino acid region of either the $G_{\alpha z}$-, $G_{\alpha s}$-, $G_{\alpha q}$-, or $G_{\alpha 15}$-protein and co-expression with the α_{2A} AR resulted in similar maximal (−)-adrenaline-mediated [^{35}S]GTPγS-binding responses with these chimeric $G_{\alpha o}$-proteins. The ligands D-medetomidine, BHT 920 (6-allyl-5,6,7,8-tetrahydro-4*H*-thiazolo[4,5-*d*]azepin-2-ylamine) and (+)-RX 811059 (2-(2-ethoxy-2,3-dihydro-benzo[1,4]dioxin-2-yl)-4,5-dihydro-1*H*-imidazole) were weakly active or virtually inactive at the chimeric $G_{\alpha o/s}$-, $G_{\alpha o/q}$-, and $G_{\alpha o/15}$-proteins in contrast to the $G_{\alpha o/z}$-protein. Furthermore, combining the constitutively active mutant $Thr^{373}Lys$ α_{2A} AR with these chimeric $G_{\alpha o}$-proteins enhanced the apparent intrinsic activity of d-medetomidine and BHT 920. A similar observation was made using the corresponding fusion proteins, where the stoichiometry of the mutant α_{2A} AR to the chimeric $G_{\alpha o}$-protein was fixed at 1.0. These data indicate that a single ligand may display different magnitudes of activation at the α_{2A} AR subtype coupled to chimeric $G_{\alpha o}$ proteins under controlled conditions of α_{2A} AR: $G_{\alpha o}$-protein expression.

Keywords: Recombinant human α_{2A}-adrenoceptor; Wt and chimeric G_α-proteins; Mutagenesis; [^{35}S]GTPγS-binding response; Intrinsic activity

1. Introduction

α_2 ARs mediate many of the physiological effects of the native catecholamines adrenaline and noradrenaline in the central nervous system as well as in the periphery [1,2]. Three distinct α_2 AR subtypes have been described (α_{2A}, α_{2B}, and α_{2C} AR) based on molecular and pharmacological criteria [3]. These receptor subtypes belong to a superfamily of receptors that transmit their signals via guanine nucleotide-binding proteins [(G-proteins); 4]. The α_2 AR subtypes exhibit different cellular and tissue distributions, suggesting that they may be endowed with distinct physiological functions and pharmacological activity profiles. The physiological significance of this diversity is not fully understood, mainly because of the lack of subtype-selective ligands.

Initial studies indicated that α_2 ARs transduce their signal through PTX-sensitive $G_{i/o}$-proteins [5,6]. Whereas the α_{2A} and α_{2B} AR subtypes have been shown to preferentially activate $G_{\alpha i}$-proteins in NIH 3T3 cells, the α_{2C} AR couples to the $G_{\alpha o}$-protein [6,7]. Activation of the three α_2 AR subtypes stably expressed in CHO cells leads to a biphasic regulation of adenylyl cyclase activity with an inhibitory phase mediated by G_i activation at low agonist concentrations and a stimulatory phase mediated by G_s activation at higher agonist concentrations [8,9]. The maximum response to G_s activation largely depends on the agonist's structural features [10], and ligands that act as full agonists for G_i coupling are not necessarily full agonists for G_s coupling.

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Abbreviations: α_2 AR, α_2-adrenoceptor; BHT 920, 6-allyl-5,6,7,8-tetrahydro-4*H*-thiazolo[4,5-*d*]azepin-2-ylamine; PCR, polymerase chain reaction; PTX, *Bordetella pertussis* toxin; RX 811059, 2-(2-ethoxy-2,3-dihydro-benzo[1,4]dioxin-2-yl)-4,5-dihydro-1*H*-imidazole; RX 821002, 2-(2-methoxy-2,3-dihydro-benzo[1,4]dioxin-2-yl)-4,5-dihydro-1*H*-imidazole; and UK 14304, 5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline tartrate.

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Other studies have also suggested that α_2 AR can mediate multiple distinct cellular responses such as activation of K^+ channels, phospholipase (PL) C [5], PLA_2, and PLD besides inhibiting the activation of Ca^{2+} channels [11,12].

Assuming that the α_{2A} AR subtype couples to different effector pathways physiologically [5,13–15], we explored the ligand-mediated responses occurring at the G_α-protein level via a single α_{2A} AR subtype. Therefore, α_2 AR ligands exhibiting a range of intrinsic activities from full agonist to inverse agonist [16] were assayed at this receptor subtype in combination with either PTX-sensitive $G_{\alpha i/o}$- or PTX-insensitive $G_{\alpha z}$-, $G_{\alpha s}$-, $G_{\alpha q}$-, and $G_{\alpha 15}$-proteins in CHO-K1 cells. PTX-resistant, agonist-dependent, and agonist-independent binding of the stable radiolabelled GTP analogue [^{35}S]GTPγS was measured as a common functional parameter. In order to avoid potential coupling of α_{2A} AR to endogenous $G_{\alpha i/o}$-proteins in CHO-K1 cells, the recombinant $G_{\alpha i/o}$-proteins were rendered resistant to PTX by mutation of a cysteine at either position 351 or 352 into an isoleucine or tyrosine [17]. Since the non-$G_{\alpha i/o}$-proteins did not display a measurable [^{35}S]GTPγS-binding response, chimeric $G_{\alpha o}$-proteins were constructed by exchanging the last six amino acids of a rat $G_{\alpha o}$-protein with those of the non-$G_{\alpha i/o}$-protein. This allowed us to monitor the same functional parameter for each mutant and chimeric $G_{\alpha i/o}$-protein. By comparing several α_2 AR ligands, we found similar levels of α_{2A} AR-dependent activation by the chimeric $G_{\alpha o/z}$-protein as by the mutant $G_{\alpha o}Cys^{351}Tyr$ protein. In contrast, almost no stimulation of [^{35}S]GTPγS binding to chimeric $G_{\alpha o/s}$-, $G_{\alpha o/q}$-, and $G_{\alpha o/15}$-proteins was observed with d-medetomidine and BHT 920. Enhanced resolution was observed between the intrinsic activities for both ligands when the chimeric $G_{\alpha o}$-proteins were co-expressed or fused to a constitutively active mutant $Thr^{373}Lys$ α_{2A} AR [18]. These results are discussed in terms of the potential to explore agonist trafficking of α_{2A} AR-mediated responses.

2. Materials and methods

2.1. Cloning of wild-type G-protein α-subunits

The cloning of rat $G_{\alpha o}$-, $G_{\alpha z}$-, and $G_{\alpha s}$ and mouse $G_{\alpha q}$- and $G_{\alpha 15}$-protein genes was performed by PCR using specific primers designed at the start and stop codons of each gene according to the published nucleotide sequence (see Genbank accession numbers in Table 1). For each PCR reaction, the amplification mixture (50 μL) consisted of 250 ng of reverse-transcribed poly(A^+) RNA from rat or mouse total brain, 350 μM of each dNTP, 400 nM of each primer, and 1 μL of Expand long-template DNA polymerase mix in PCR buffer [$(NH_4)_2SO_4$ 16 mM, $MgCl_2$ 1.75 mM, Tris–HCl 50 mM, pH 9.2]. The PCR program consisted of 30 repetitive cycles with a strand separation step at 96° for 30 sec, an annealing step at 60° for 1 min, and an elongation step at 68° for 1.5 min. The PCR fragments were separated by 1% agarose gel electrophoresis, purified using a Geneclean II kit, and subsequently cloned into 50 ng of a pCR3.1 expression vector. Sequencing was performed automatically on an ABI Prism 310 Genetic Analyser using a Big Dye terminator cycle sequencing kit. The wt G_α-protein gene nucleotide sequences were identical to those of the Genbank database.

2.2. Construction of mutant and chimeric $G_{\alpha o}$-proteins

The mutant $G_{\alpha o}Cys^{351}Ile$ and $G_{\alpha o}Cys^{351}Tyr$ and the chimeric $G_{\alpha o}$-protein genes were generated by PCR on a linearised pCR3.1/$G_{\alpha o}$-plasmid using a sense primer containing a *NotI* restriction site and a mutagenic reverse primer carrying the respective mutation; their sequences are indicated in Table 1. The amplification conditions were similar to those described above. The PCR fragments were cloned into a pCR3.1 vector and sequenced to confirm the presence of the respective mutation.

2.3. Construction of $Thr^{373}Lys$ α_{2A} AR: $G_{\alpha o}$-fusion proteins

The mutant $Thr^{373}Lys$ α_{2A} AR was modified by PCR by mutating its stop codon into an alanine and simultaneously adding a *NotI* restriction site in frame with the α_{2A} AR coding sequence. The fusion of the $Thr^{373}Lys$ α_{2A} AR with the above described mutant and chimeric $G_{\alpha o}$-proteins was achieved after *NotI* digestion of both plasmids and subsequent ligation. The resulting constructs consisted of the mutant $Thr^{373}Lys$ α_{2A} AR, in which the stop codon was mutated into an alanine followed by two additional alanine residues generated by the *NotI* site, and by the entire mutant or chimeric $G_{\alpha o}$-protein gene sequence. Each fusion product was fully sequenced, confirming the respective nucleotide sequences.

2.4. Transient expression of human α_{2A} AR with wt, mutant G_α-, and chimeric $G_{\alpha o}$-proteins

The CHO-K1 cell line (ATCC, CCL 61) was cultured in Petri dishes (50 cm^2) with Ham's F12 nutrient mixture supplemented with 10% heat-inactivated foetal bovine serum. Cells grown to 60–80% confluency were used for transfection using a lipofectamine plus kit. Three micrograms of pCR3.1 plasmid containing either the wt (RC: 2.1.ADR.A2A) or mutant $Thr^{373}Lys$ α_{2A} AR gene [19] supplemented with three micrograms of pCR3.1 plasmid, or three micrograms of wt or mutant $Thr^{373}Lys$ α_{2A} AR gene and three micrograms of either wt, mutant, or chimeric G_α-protein gene was mixed with 10 μL lipofectamine plus reagent in 0.2 mL of Opti-MEM and incubated at room temperature for 15 min. Fusion protein genes were transfected at six micrograms. Subsequently, twenty microliters of lipofectamine reagent diluted in 0.2 mL of Opti-MEM was added for 15 min and exposed with 5 mL of Opti-MEM

Table 1
Sequence characteristics of the C-terminal portion of the wild-type, mutant, and chimeric $G_{\alpha o}$-proteins

G_{α}-protein	Reverse primer	C-terminal last six amino acids	Genbank accession number
wt $G_{\alpha o}$ Cys^{351}	5′ TCAGTACAAGCCACAGCCCCGGAGATT 3′	RGCGLY	M17526
$G_{\alpha o}$ Cys^{351}Ile	5′ TCAGTACAAGCC**AAT**GCCCCGGAGATT 3′	RG**I**GLY	
$G_{\alpha o}$ Cys^{351}Tyr	5′ TCAGTACAAGCC**ATA**GCCCCGGAGATT 3′	RG**Y**GLY	
$G_{\alpha o/z}$	5′ TCA**GCAAAGGCCAATGTACTT**GAGATTGTTGGCAATGATG 3′	**KYI**GL**C**	J03773
$G_{\alpha o/s}$	5′ **TTAGAGCAGCTCGTAAAGGCG**GAGATTGTTGGCAATGATG 3′	R**QYE**L**L**	M12676
$G_{\alpha o/q}$	5′ **TTAGACCAGATTGTACTCCTT**GAGATTGTTGGCAATGATG 3′	**KEYN**L**V**	M55412
$G_{\alpha o/15}$	5′ TCA**CAGCAGGTTGATCTCGTC**GAGATTGTTGGCAATGATG 3′	**DEIN**L**L**	M80632
$G_{\alpha o}$ [truncated]	5′ TCAGAGATTGTTGGCAATGATGATGTC 3′	- - - - - -	
$G_{\alpha o}$/$[Ala]_6$	5′ TCA**GGCGGCGGCGGCGGCGGC**GAGATTGTTGGCAATGATG 3′	**AAAAAA**	
$G_{\alpha o}$/$[Glu\text{-}Ala\text{-}Tyr\text{-}(Ala)_3]$	5′ TCA**GGCGGCGGCGTAGGCCTC**GAGATTGTTGGCAATGATG 3′	**EAYAAA**	

The last six C-terminal amino acids of the rat $G_{\alpha o}$-protein (Arg^{349} to Tyr^{354}) were exchanged with the equivalent residues of either rat $G_{\alpha z}$-rat $G_{\alpha s}$-mouse $G_{\alpha q}$-, or mouse $G_{\alpha 15}$-proteins. This rat $G_{\alpha o}$ portion was also fully deleted ($G_{\alpha o}$[truncated]), exchanged for six alanine residues ($G_{\alpha o}$/$[Ala]_6$) and for arbitrary amino acid insertion ($G_{\alpha o}$/$[Glu\text{-}Ala\text{-}Tyr\text{-}(Ala)_3]$). The arrow indicates the position of the PTX-mediated ADP-ribosylation site. The mutant $G_{\alpha o}Cys^{351}$Ile, $G_{\alpha o}Cys^{351}$Tyr, and the chimeric $G_{\alpha o}$-proteins were constructed as described in Methods using the indicated mutagenic reverse primers. The nucleotides or amino acids indicated in bold are those that are modified according to the wt rat $G_{\alpha o}$-protein gene. The respective Genbank accession number for the wt rat $G_{\alpha z}$-, rat $G_{\alpha s}$-, mouse $G_{\alpha q}$-, and mouse $G_{\alpha 15}$-protein genes is indicated.

to CHO-K1 cells for 3 hrs at 37°. Thereafter, cells were further incubated with 10 mL of complete growth medium and harvested 48 hr after transfection. Treatment with PTX (20 ng/mL) was performed overnight before membranes were prepared.

2.5. Membrane preparation and radioligand-binding experiments

Membrane preparation steps were performed at 4°. Cells were washed twice with PBS and stored at −80°. Cells were then scraped mechanically in Tris–HCl 10 mM supplemented with EDTA 0.1 mM (pH 7.5) and centrifuged for 10 min at 45,000 *g*. The pellet was homogenised in the same buffer using a Polytron and recentrifuged. The final pellet was dispersed in aliquots of 0.5 mL of Tris/EDTA buffer (0.5 to 1.5 mg/mL of protein) and stored at −80° until used. Membrane preparations were diluted in Tris–HCl 50 mM (pH 7.7) containing $CaCl_2$ 4 mM, pargyline 10 μM, and ascorbic acid 0.1%, and used for [^{3}H]RX 821002 (2 nM)-binding experiments as described previously [19]. Ten micromolar of phentolamine was used to determine non-specific radioligand binding. Saturation [^{3}H]RX 821002-binding experiments and Scatchard analysis were performed as described [19].

2.6. [^{35}S]GTPγS-binding responses

Agonist-independent (basal) and agonist-dependent [^{35}S]GTPγS-binding [16] were also measured using the above-described membrane preparation in HEPES 20 mM (pH 7.4) supplemented with GDP 30 μM, NaCl 100 mM, $MgCl_2$ 3 mM, and ascorbic acid 0.2 mM. Steady-state [^{35}S]GTPγS binding was achieved within the 30-min period of incubation [16]. Maximal stimulation of [^{35}S]GTPγS binding was defined in the presence of 10 μM (−)-adrenaline and calculated versus basal [^{35}S]GTPγS binding, unless otherwise indicated. Each of the compounds was investigated at a maximally effective concentration: UK 14304, 10 μM; d-medetomidine, 10 μM; BHT 920, 10 μM; (+)-RX 811059, 1 μM; see [16]. Saturation [^{35}S]GTPγS binding was determined as previously described [20] to quantify the amount of (−)-adrenaline-mediated G_{α}-protein activation.

2.7. Immunological detection of G_{α}-protein expression

Membrane fractions of CHO-K1 cells transiently co-expressing the α_{2A} AR in the presence of wt, mutant, or chimeric $G_{\alpha o}$-proteins were prepared as described above. Total proteins were separated by using SDS, 12.5% (w/v) polyacrylamide gel electrophoresis [SDS–PAGE; 21]. After electrophoresis, the proteins were blotted onto a nylon membrane by semi-dry electrotransfer (23 V, 45 min) in Towbin buffer (glycine 190 mM, methanol 20% (v/v), Tris–HCl 25 mM, pH 8.3). Proteins were probed using a monoclonal antibody raised against a peptide corresponding to amino acids 18 to 33 of the $G_{\alpha o}$-protein. The incubation was performed in PBS buffer containing 0.1% Tween 20 (w/v), 5% dry non-fat milk, and the antibody at a dilution of 1:1000. Proteins were visualised with an anti-mouse immunoglobulin G antibody coupled to alkaline phosphatase using a colourimetric reaction (4-nitroblue tetrazolium chloride monohydrate 0.12 mM, 5-bromo 4-chloro 3-indolylphosphate *p*-toluidine salt 0.12 mM, $MgCl_2$ 5 mM in diethanolamine 100 mM, pH 9.6). Densitometric analysis was performed using a computer-based image analysis system (Imagena 2000).

2.8. Protein content

Membrane protein concentrations were estimated using a Bio-Rad dye-binding kit. BSA was used as a standard [22].

2.9. Statistical analysis

Statistical analysis of the ligand's maximal [^{35}S]GTPγS-binding responses was performed by comparing the values for the wt or mutant Thr^{373}Lys α_{2A} AR and $G_{\alpha o}$Cys^{351}Ile protein (either co-expression or fusion condition) versus the indicated mutant and chimeric $G_{\alpha o}$-protein conditions using a one-way ANOVA, followed by an all pairwise multiple comparison procedure (Tukey's test).

2.10. Materials

The ABI Prism 310 Genetic Analyser and the Big Dye terminator cycle sequencing kit were from Perkin Elmer. The pCR3.1 expression vector was from Invitrogen. The Geneclean II kit was from Bio 101 Inc. The Expand long-template polymerase mix was from Boehringer Mannheim. The Imagena 2000 software was from Biocom. CHO-K1 cells were obtained from ATCC. The monoclonal anti-$G_{\alpha o}$ (mono3E7) antibody and [^{3}H]RX 821002 (50 Ci/mmol) were obtained from New England Nuclear. [^{35}S]GTPγS (1035–1163 Ci/mmol) was obtained from Amersham. The lipofectamine plus kit, cell culture media, foetal bovine serum, culture plates, and *Bordetella pertussis* toxin (50 μg/mL) were obtained from GIBCO Biocult Laboratories. The Emulsifier-Safe was obtained from Packard. (−)-Adrenaline and the substrates for the immunological colourimetric reaction were from RBI-Sigma. d-Medetomidine was purchased from Smith Kline Beecham. UK 14304 and (+)-RX 811059 were prepared *intramuros*. BHT 920 was a gift from Boehringer Ingelheim. Stock solutions of ligands were prepared at 10^{-3} M. Serial dilutions were made in the respective incubation buffers.

3. Results

Wild-type α_{2A} ARs displayed a weak [^{35}S]GTPγS-binding response to 10 μM (−)-adrenaline (47% versus basal) upon transient expression in CHO-K1 cells. This response was fully blocked by PTX (20 ng/mL) and therefore is likely to be mediated by endogenous $G_{\alpha i/o}$-proteins (Fig. 1). Co-expression of the α_{2A} AR with recombinant $G_{\alpha i/o}$-protein subtypes mutated at their cysteine residue in either position 351 or 352 into an isoleucine yielded PTX-resistant [^{35}S]GTPγS-binding responses to (−)-adrenaline. This response was highest in the co-presence of a $G_{\alpha o}$Cys^{351}Ile protein and was significantly lower with the $G_{\alpha i/2}$Cys$^{351/352}$Ile proteins. The (−)-adrenaline-mediated $G_{\alpha i3}$Cys^{351}Ile protein-dependent [^{35}S]GTPγS-binding response was not statistically different from that observed in the absence of

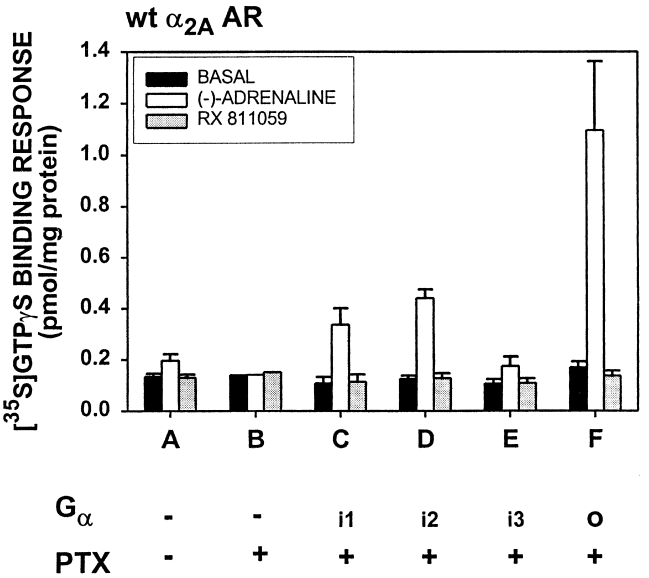

Fig. 1. Coupling of wt α_{2A} AR to $G_{\alpha i/o}$-proteins. CHO-K1 cells were transfected with either empty plasmid, $G_{\alpha i1}$Cys^{351}Ile, $G_{\alpha i2}$Cys^{352}Ile, $G_{\alpha i3}$Cys^{351}Ile, or $G_{\alpha o}$Cys^{351}Ile protein and treated or not with PTX (20 ng/mL) as indicated. [^{35}S]GTPγS-binding responses were measured in the absence of ligand (basal), 10 μM (−)-adrenaline, and 10 μM (+)-RX 811059. Bar graphs were constructed using means ± SEM values of 2–6 independent transfection experiments, each one performed in duplicate. A: wt α_{2A} AR; B: wt α_{2A} AR + PTX; C: wt α_{2A} AR + $G_{\alpha i1}$Cys^{351}Ile + PTX; D: wt α_{2A} AR + $G_{\alpha i2}$Cys^{352}Ile + PTX; E: wt α_{2A} AR + $G_{\alpha i3}$Cys^{351}Ile + PTX; F: wt α_{2A} AR + $G_{\alpha o}$Cys^{351}Ile + PTX.

recombinant G_α-protein. The basal [^{35}S]GTPγS-binding response was slightly (27%) enhanced upon co-expression of the $G_{\alpha o}$Cys^{351}Ile protein; this enhancement could be reversed by 10 μM (+)-RX 811059. Further analysis of the maximum responses to α_2 AR agonists showed that in the co-presence of the $G_{\alpha o}$Cys^{351}Ile protein, the partial agonists d-medetomidine and BHT 920 could be made to behave as full agonists with a maximal response similar to that of (−)-adrenaline. This was in contrast to the mutant forms of either the $G_{\alpha i1}$- or $G_{\alpha i2}$-protein or upon activation of endogenous $G_{\alpha i/o}$-proteins in CHO-K1 cells (Fig. 2).

Co-expression of the α_{2A} AR with either a wt $G_{\alpha z}$-, $G_{\alpha s}$-, $G_{\alpha q}$-, or $G_{\alpha 15}$-protein did not produce a significant modification in [^{35}S]GTPγS-binding by either 10 μM (−)-adrenaline or (+)-RX 811059 as compared to the basal [^{35}S]GTPγS-binding level (Table 2). Replacement of the last six amino acids at the C-terminal portion of the $G_{\alpha o}$-protein by the corresponding amino acid region of either the $G_{\alpha z}$-, $G_{\alpha s}$-, $G_{\alpha q}$-, or $G_{\alpha 15}$-protein yielded (−)-adrenaline-induced [^{35}S]GTPγS-binding responses that were of a similar magnitude (Table 3). These responses were weaker as compared to those mediated by mutant $G_{\alpha o}$Cys^{351}Ile and $G_{\alpha o}$Cys^{351}Tyr proteins, which correspond to the amino acid at the −4 position away from the C-terminal extremity of $G_{\alpha z}$- or $G_{\alpha 15}$- and of $G_{\alpha s}$- or $G_{\alpha q}$-proteins, respectively. The wt α_{2A} AR and chimeric $G_{\alpha o}$-protein expression levels (not shown) were similar in these various G_α-protein constructions. A trend toward an enhanced basal [^{35}S]GTPγS-bind-

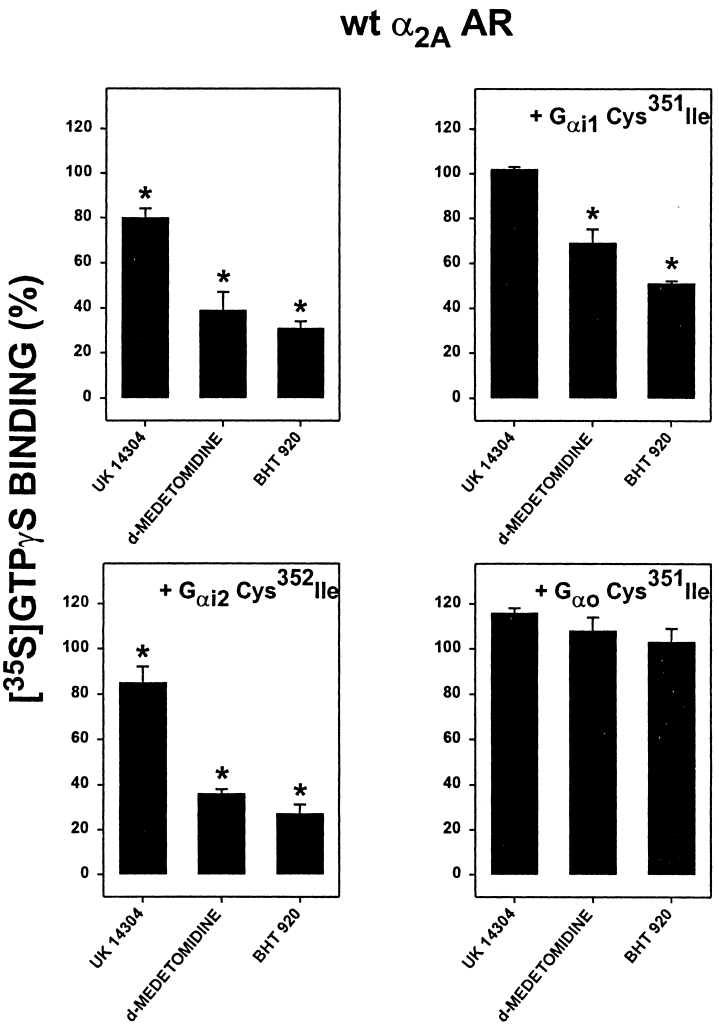

Fig. 2. Ligand-mediated [^{35}S]GTPγS-binding responses by wt α_{2A} AR in either the absence or presence of recombinant $G_{\alpha i/o}$-proteins. CHO-K1 cells were transfected with the wt α_{2A} AR and recombinant PTX-resistant $G_{\alpha i/o}$-proteins and treated with PTX (20 ng/mL) except for the co-expression with empty plasmid, as indicated in the legend to Fig. 1. [^{35}S]GTPγS-binding data were expressed in percentage of the respective maximal [^{35}S]GTPγS-binding responses (absolute values in fmol/mg protein are shown in Fig. 1) as obtained with 10 μM (−)-adrenaline. Bar graphs were constructed using means ± SEM values of 3–6 independent transfection experiments, each one performed in duplicate. Statistical analysis was performed as described in section 2 by comparing the ligand's maximal responses in the presence of a $G_{\alpha o}Cys^{351}Ile$ protein vs empty plasmid or $G_{\alpha i1/2}Cys^{351/352}Ile$ proteins. *$P < 0.05$.

ing response was observed upon co-expression with a $G_{\alpha o/z}$-protein, as was also the case with the $G_{\alpha o}Cys^{351}Ile$ and $G_{\alpha o}Cys^{351}Tyr$ proteins. Control experiments performed with a truncated $G_{\alpha o}$-protein deleted of its last six amino acids, a $G_{\alpha o}$-protein containing six alanine residues at its C-terminal portion, or a $G_{\alpha o}$-protein with an arbitrary sequence of the last six amino acids generated either no or a weak (<36%) (−)-adrenaline response (Table 3). In Fig. 3, a comparison between the ligands' maximal [^{35}S]GTPγS-binding responses is summarised for each of the chimeric $G_{\alpha o}$-proteins along with the $G_{\alpha o}Cys^{351}Ile$ and $G_{\alpha o}Cys^{351}Tyr$ proteins. Co-expression of α_{2A} AR with a chimeric $G_{\alpha o/z}$-protein yielded an agonist and inverse agonist pattern of [^{35}S]GTPγS-binding responses not statistically different from that obtained with a $G_{\alpha o}Cys^{351}Ile$ protein. Otherwise, none of the ligands, with the exception of (−)-adrenaline and UK 14304, displayed more than 20% positive intrinsic activity upon co-expression of the α_{2A} AR with either a $G_{\alpha o/15}$-, $G_{\alpha o/s}$-, or $G_{\alpha o/q}$-protein. UK 14304 attained only 60% of the intrinsic activity of (−)-adrenaline at these last three chimeric $G_{\alpha o}$-proteins, whereas it acted as a full agonist at $G_{\alpha o/z}$ and $G_{\alpha o}Cys^{351}Ile/Tyr$ proteins. The fact that we could not differentiate between these three chimeric $G_{\alpha o}$-proteins led us to set up similar experiments with a mutant $Thr^{373}Lys$ α_{2A} AR that had previously been shown to be constitutively active [18]. Fig. 4 illustrates the amount of wt, mutant, and chimeric $G_{\alpha o}$-protein expression in the co-presence of the $Thr^{373}Lys$ α_{2A} AR. Though a small variation was apparent in the G_{α}-protein expression level, the magnitude of the (−)-adrenaline-mediated [^{35}S]GTPγS-binding responses of the different chimeric and mutant $G_{\alpha o}$-proteins appeared to be only slightly affected when the wt α_{2A} AR (Table 3) and $Thr^{373}Lys$ α_{2A} AR

Table 2
Receptor amount and [^{35}S]GTPγS-binding responses of wt α_{2A} AR co-expressed with various wt G_{α}-proteins in CHO-K1 cells

Co-expression	[^{3}H]RX 821002 binding (pmol/mg protein)	[^{35}S]GTPγS-binding response (fmol/mg protein)		
		basal	(−)-adrenaline	(+)-RX 811059
wt α_{2A} AR + plasmid	5.28 ± 0.77	134 ± 11	196 ± 25	130 ± 12
wt α_{2A} AR + $G_{\alpha z}$[Lys-Tyr-Ile-Gly-Leu-Cys]	3.95 ± 0.65	145 ± 10	155 ± 6	139 ± 12
wt α_{2A} AR + $G_{\alpha s}$[Arg-Gln-Tyr-Glu-Leu-Leu]	2.73 ± 0.51	106 ± 29	137 ± 24	139 ± 37
wt α_{2A} AR + $G_{\alpha q}$[Lys-Glu-Tyr-Asn-Leu-Val]	3.72 ± 0.12	153	141	156
wt α_{2A} AR + $G_{\alpha 15}$[Asp-Glu-Ile-Asn-Leu-Leu]	5.13 ± 0.82	94 ± 26	110 ± 23	119 ± 28
wt α_{2A} AR + $G_{\alpha o}$[Arg-Gly-Cys-Gly-Leu-Tyr]	5.39 ± 0.83	148 ± 19	692 ± 93	138 ± 18

Co-expression of the wt α_{2A} AR and respective G_{α}-protein was performed as described in Methods. All conditions except the $G_{\alpha o}$-protein and the empty plasmid were treated with PTX (20 ng/mL). The α_{2A} AR receptor amount was estimated by measuring specific [^{3}H]RX 821002 binding. Basal, 10 μM (−)-adrenaline-, and 10 μM (+)-RX 811059-mediated [^{35}S]GTPγS-binding responses were performed with 0.5 nM [^{35}S]GTPγS. Data represent mean values ($G_{\alpha q}$) or mean values ± SEM of 3 to 6 independent transfection experiments, each one performed in duplicate. The underlined amino acid corresponds to the fourth last amino acid at the PTX-mediated ADP-ribosylation site of the C-terminal portion of the G_{α}-protein.

(Table 4) were compared. The basal response was increased in the case of the $G_{\alpha o/z}$, $G_{\alpha o}$Cys^{351}Ile, and $G_{\alpha o}$Cys^{351}Tyr proteins and was statistically different from the basal [^{35}S]GTPγS-binding level at the chimeric $G_{\alpha o/15}$-, $G_{\alpha o/q}$-, and $G_{\alpha o/s}$-proteins. The pattern for most of the ligands' responses at the mutant Thr^{373}Lys α_2 AR was very similar for the $G_{\alpha o/z}$, $G_{\alpha o}$Cys^{351}Ile, and $G_{\alpha o}$Cys^{351}Tyr proteins. The inverse agonist activity of (+)-RX 811059 was highest at the mutant Thr^{373}Lys α_2 AR in the co-presence of a $G_{\alpha o}$Cys^{351}Ile protein. A trend toward a (+)-RX 811059-mediated decrease (not significant) in the basal [^{35}S]GTPγS-binding level was observed in the case of the $G_{\alpha o/z}$ and $G_{\alpha o}$Cys^{351}Tyr proteins. It was free of intrinsic activity in the presence of the chimeric $G_{\alpha o/q}$-, $G_{\alpha o/s}$-, and $G_{\alpha o/15}$-proteins. UK 14304 yielded a maximal response that was not statistically different in the six mutant $G_{\alpha o}$-proteins. Its potency was 12- to 54-fold decreased at the chimeric $G_{\alpha o/s}$-, $G_{\alpha o/q}$-, and $G_{\alpha o/15}$-proteins as compared to the $G_{\alpha o/z}$- and mutant $G_{\alpha o}$Cys^{351}Ile/Tyr proteins (Fig. 5) BHT 920 and d-medetomidine displayed similar partial agonist properties inferior to those of the $G_{\alpha o}$Cys^{351}Ile protein, except for d-medetomidine in the case of the $G_{\alpha o/z}$ protein. The potency of these two ligands was decreased 5- to 37-fold in a similar manner as that observed for UK 14304 (Fig. 5).

Because the co-expression experiments could not exclude differences in receptor: G_{α}-protein ratios, another set of experiments was performed with fusion proteins composed of the Thr^{373}Lys α_{2A} AR and each of the mutant or chimeric $G_{\alpha o}$-proteins. Our aim was to verify the ligand-mediated responses under controlled expression conditions at a receptor: G_{α}-protein stoichiometry of 1.0. Analysis of (−)-adrenaline-specific saturation [^{35}S]GTPγS binding indicated a single class of high-affinity [^{35}S]GTPγS-binding sites for each of the investigated G_{α}-proteins (Table 5), with the exception of the Thr^{373}Lys α_{2A} AR: $G_{\alpha o/q}$-fusion protein, which could not be evaluated. A 4-fold attenuation in the [^{35}S]GTPγS dissociation constant was observed with the chimeric $G_{\alpha o/15}$- and $G_{\alpha o/s}$-proteins. Otherwise, the maximal (−)-adrenaline-mediated [^{35}S]GTPγS-binding capacity of each of these fusion proteins was very similar; it varied between 2.66 and 4.02 pmol/mg protein. In addition, these values were virtually similar to the maximal Thr^{373}Lys α_{2A} AR binding capacity as estimated by [^{3}H]RX

Table 3
Receptor amount and [^{35}S]GTPγS-binding responses of wt α_{2A} AR co-expressed with either mutant or chimeric $G_{\alpha o}$-proteins in CHO-K1 cells

Co-expression	[^{3}H]RX 821002 binding (pmol/mg protein)	[^{35}S]GTPγS-binding response (fmol/mg protein)		
		basal	(−)-adrenaline	(+)-RX 811059
wt α_{2A} AR + $G_{\alpha o/z}$ [Lys-Tyr-Ile-Gly-Leu-Cys]	5.06 ± 1.06	139 ± 26	627 ± 132	120 ± 25
wt α_{2A} AR + $G_{\alpha o/15}$ [Asp-Glu-Ile-Asn-Leu-Leu]	4.68 ± 0.95	117 ± 19	517 ± 117	116 ± 19
wt α_{2A} AR + $G_{\alpha o}$Cys^{351}Ile	5.28 ± 0.77	170 ± 22	1095 ± 267	137 ± 19
wt α_{2A} AR + $G_{\alpha o/s}$ [Arg-Gln-Tyr-Glu-Leu-Leu]	4.26 ± 0.60	108 ± 19	481 ± 118	126 ± 21
wt α_{2A} AR + $G_{\alpha o/q}$ [Lys-Glu-Tyr-Asn-Leu-Val]	4.34 ± 0.59	105 ± 20	499 ± 101	115 ± 18
wt α_{2A} AR + $G_{\alpha o}$ Cys^{351}Tyr	5.03 ± 0.56	158 ± 12	848 ± 134	137 ± 9
wt α_{2A} AR + $G_{\alpha o}$ [truncated]	3.33 ± 0.53	115 ± 8	128 ± 1	127 ± 11
wt α_{2A} AR + $G_{\alpha o}$ [Ala_6]	3.15 ± 0.88	112 ± 2	135 ± 19	109 ± 5
wt α_{2A} AR + $G_{\alpha o}$ [Glu-Ala-Tyr-$(Ala)_3$]	2.96 ± 0.80	129 ± 7	175 ± 13	135 ± 7

Co-expression of the wt α_{2A} AR and respective modified $G_{\alpha o}$-proteins was performed as described in Methods. All conditions were treated with PTX (20 ng/mL). The α_{2A} AR receptor amount and basal, 10 μM (−)-adrenaline-, and 10 μM (+)-RX 811059-mediated [^{35}S]GTPγS-binding responses were performed as described in the legend to Table 2. Data represent mean values ± SEM of 4 to 6 independent transfection experiments, each one performed in duplicate. The underlined amino acid corresponds to the fourth last amino acid of the C-terminal portion of the modified $G_{\alpha o}$-protein.

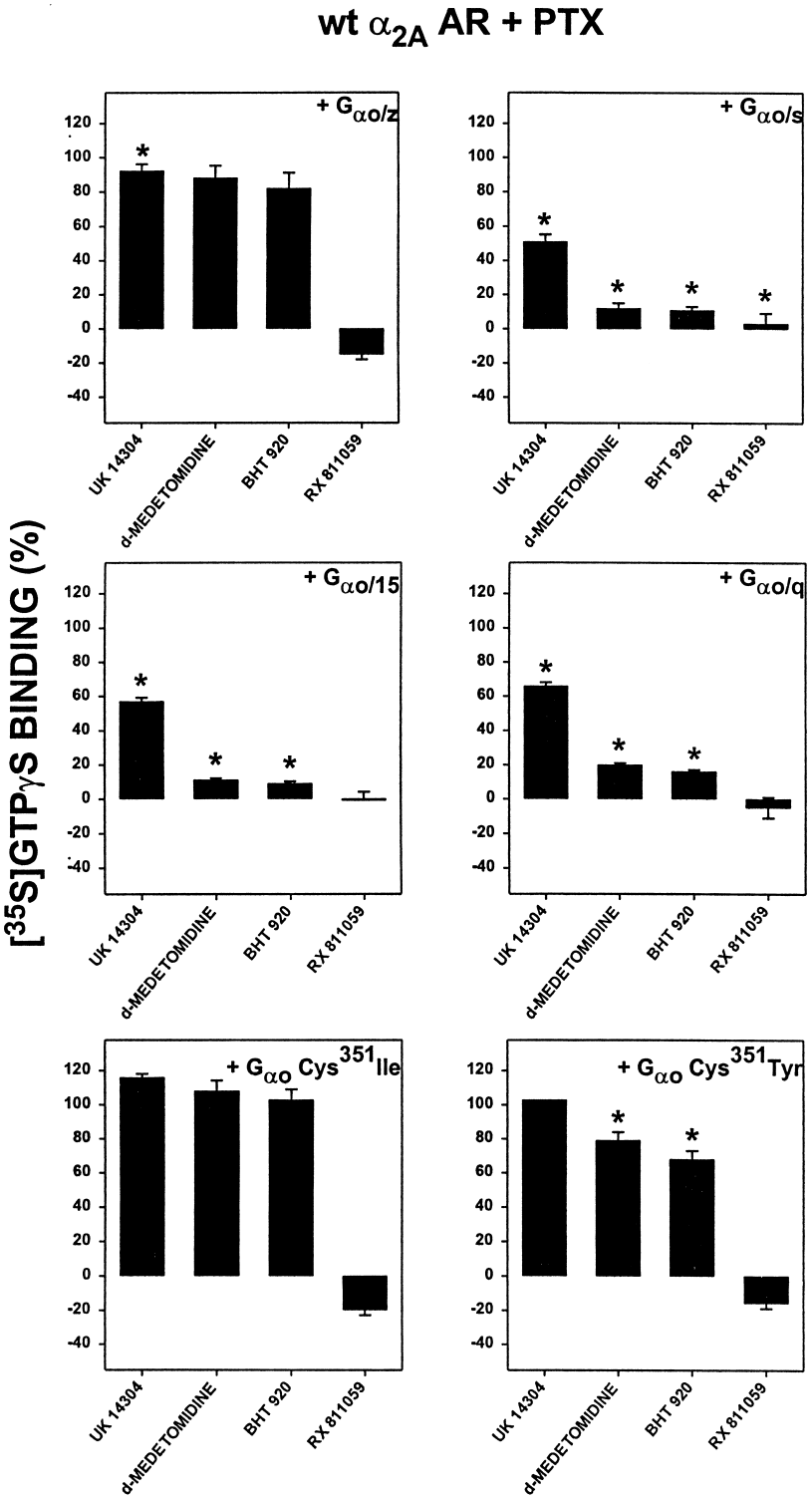

Fig. 3. Ligand-mediated [^{35}S]GTPγS-binding responses by wt α_{2A} AR in the presence of chimeric and mutant $G_{\alpha o}$-proteins. CHO-K1 cells were transfected with the wt α_{2A} AR and chimeric or mutant $G_{\alpha o}$-proteins and treated with PTX (20 ng/mL) as indicated in the legend to Fig. 1. [^{35}S]GTPγS-binding data were expressed as percentage of the respective maximal [^{35}S]GTPγS-binding responses (absolute values in fmol/mg protein are summarised in Table 3) as obtained with 10 μM (−)-adrenaline except for (+)-RX 811059, which was expressed versus its respective basal [^{35}S]GTPγS-binding value. pEC_{50} values (means ± SD) were determined for the α_{2A} AR in the co-presence of a chimeric $G_{\alpha o/z}$-protein: UK14304 (8.44 ± 0.12), d-medetomidine (8.34 ± 0.26), and BHT 920 (7.29 ± 0.15). Bar graphs were constructed using means ± SEM values of 4–6 independent transfection experiments, each one performed in duplicate. Statistical analysis was performed as described in Methods by comparing the ligand's maximal response in the presence of a $G_{\alpha o}Cys^{351}Ile$ protein versus the other mutant and chimeric $G_{\alpha o}$-proteins. *$P < 0.05$.

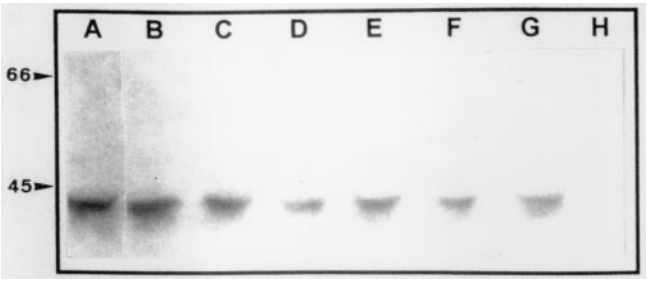

Fig. 4. Immunological detection of G_{α}-protein expression. CHO-K1 cells were transfected with $Thr^{373}Lys$ α_{2A} AR and either wt, mutant, or chimeric $G_{\alpha o}$-proteins. Two hundred micrograms of total cellular membrane proteins of CHO-K1 cells co-expressing $Thr^{373}Lys$ α_{2A} AR and either wt $G_{\alpha o}$-(A), mutant $G_{\alpha o}Cys^{351}Ile$ (B), mutant $G_{\alpha o}Cys^{351}Tyr$ (C), chimeric $G_{\alpha o/z}$-(D), $G_{\alpha o/s}$-(E), $G_{\alpha o/q}$-(F), $G_{\alpha o/15}$-(G) proteins, or empty plasmid (H) were separated by 12.5% SDS-PAGE, blotted onto a nylon membrane, and immunodetection performed as described in Methods using a selective anti-$G_{\alpha o}$-antibody. Molecular weight markers are indicated in the left margin. Quantification (percentage versus lane A, upon subtraction of lane H) of the immunodetected signal was: 100, 114, 96, 65, 94, 76, 88 for lanes A to G, respectively. A rectangle covering the signal in lane A was identically reproduced as surface template for the quantification of the other lanes.

821002 saturation binding. This observation strongly indicates a receptor: G_{α}-protein ratio close to 1.0 in accordance with what would be expected for a fusion protein. Ligand-mediated [^{35}S]GTPγS-binding responses at the fusion proteins illustrated a pharmacological profile both for the potency and maximal response (Fig. 6) which was very similar to that found with the corresponding co-expression experiments (Fig. 5). The maximal [^{35}S]GTPγS-binding responses of BHT 920 and d-medetomidine were statistically inferior at the fusion proteins involving a chimeric $G_{\alpha o/s}$- and $G_{\alpha o/15}$-protein.

4. Discussion

A broad range of data demonstrates that α_2 ARs modulate various effector systems such as inhibition and stimulation of adenylyl cyclase, inhibition of voltage-gated Ca^{2+} channels, activation of K^+ channels, and stimulation of phospholipases A_2, C, and D [23]. The exact nature of the G-protein subunits that are activated by α_{2A} AR following stimulation by a given ligand and that subsequently regulate the effector activity remains largely unknown. Though the ubiquitous coupling of α_{2A} AR to the inhibition of adenylyl cyclase is mediated by PTX-sensitive $G_{\alpha i/o}$-proteins [14], controversy exists concerning the G_{α}- and/or $G_{\beta\gamma}$-subunits involved in the PLC pathway and the resulting effects on Ca^{2+} mobilisation [24–26]. The $G_{\alpha s}$-protein has been shown to couple to α_{2A} AR in addition to α_{2B} and α_{2C} AR. This activation is dependent on the agonist's structural features and occurs with a 100-fold decrease in ligand potency compared to the activation of $G_{\alpha i/o}$-protein subtypes [10]. In the present study, modulation of ligand-mediated responses at α_{2A} AR by diverse mutant and chimeric $G_{\alpha o}$-proteins was investigated. [^{35}S]GTPγS-binding responses as elicited by the activation of a series of PTX-resistant, mutant $G_{\alpha i/o}$-, and chimeric $G_{\alpha o}$-proteins in the co-presence of either a wt α_{2A} AR or its constitutively active mutant $Thr^{373}Lys$ α_{2A} AR [18] were analysed. The mutant $G_{\alpha i/o}$- and chimeric $G_{\alpha o}$-protein constructs exhibited PTX resistance because of the modification of their ADP-ribosylation site four amino acids away from the C-terminal extremity of the G_{α}-protein into either an isoleucine or tyrosine residue. Hence, coupling of the α_{2A} AR to endogenous $G_{\alpha i/o}$-proteins present in

Table 4
Receptor amount and [^{35}S]GTPγS-binding responses of mutant $Thr^{373}Lys$ α_{2A} AR co-expressed with either mutant or chimeric $G_{\alpha o}$-proteins in CHO-K1 cells

Co-expression	[^{3}H]RX 821002 binding (pmol/mg protein)	[^{35}S]GTPγS-binding response (fmol/mg protein)		
		basal	(−)-adrenaline	(+)-RX 811059
$Thr^{373}Lys$ α_{2A} AR + $G_{\alpha o/z}$	0.96 ± 0.37	199 ± 52	670 ± 115	150 ± 32
$Thr^{373}Lys$ α_{2A} AR + $G_{\alpha o/15}$	1.50 ± 0.59	112 ± 26	552 ± 171	121 ± 35
$Thr^{373}Lys$ α_{2A} AR + $G_{\alpha o}Cys^{351}Ile$	1.81 ± 0.42	405 ± 51	1286 ± 234	189 ± 17
$Thr^{373}Lys$ α_{2A} AR + $G_{\alpha o/s}$	1.34 ± 0.80	125 ± 36	532 ± 251	118 ± 37
$Thr^{373}Lys$ α_{2A} AR + $G_{\alpha o/q}$	1.59 ± 0.72	124 ± 31	657 ± 235	120 ± 31
$Thr^{373}Lys$ α_{2A} AR + $G_{\alpha o}$ $Cys^{351}Tyr$	1.74 ± 0.60	192 ± 19	881 ± 126	154 ± 3

Co-expression of the mutant $Thr^{373}Lys$ α_{2A} AR and respective modified $G_{\alpha o}$-proteins was performed as described in Methods. All conditions were treated with PTX (20 ng/mL). The α_{2A} AR receptor amount and basal, 10 μM (−)-adrenaline-, and 10 μM (+)-RX 811059-mediated [^{35}S]GTPγS-binding responses were performed as described in the legend to Table 2. Data represent mean values ± SEM of 3 to 9 independent transfection experiments, each one performed in duplicate. No stimulation of [^{35}S]GTPγS-binding was obtained by 10 μM (−)-adrenaline with wt $G_{\alpha z}$-, $G_{\alpha 15}$-, $G_{\alpha s}$-, or $G_{\alpha q}$-proteins, $G_{\alpha o}$[truncated], $G_{\alpha o}$/[Ala_6] or $G_{\alpha o}$/[Glu-Ala-Tyr-$(Ala)_3$] proteins.

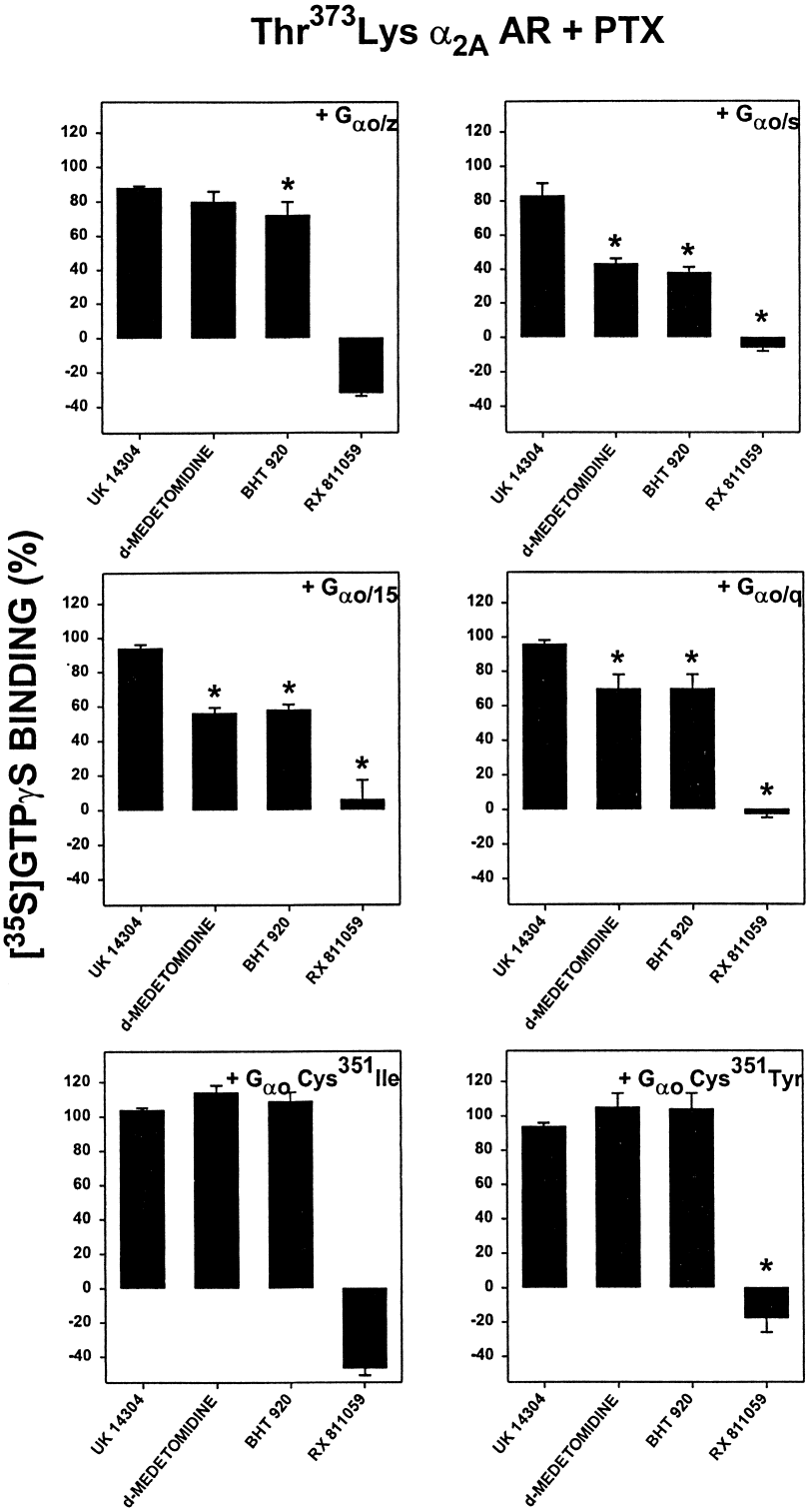

Fig. 5. Ligand-mediated [^{35}S]GTPγS-binding responses by mutant Thr^{373}Lys α_{2A} AR in the presence of chimeric and mutant $G_{\alpha o}$-proteins. CHO-K1 cells were transfected with the Thr^{373}Lys α_{2A} AR and chimeric or mutant $G_{\alpha o}$-proteins and treated with PTX (20 ng/mL) as indicated in the legend to Fig. 1. [^{35}S]GTPγS-binding data were expressed as percentage of the respective maximal [^{35}S]GTPγS-binding responses (absolute values in fmol/mg protein are summarised in Table 4) as obtained with 10 μM (−)-adrenaline except for (+)-RX 811059, which was expressed versus its respective basal [^{35}S]GTPγS-binding value. Mean pEC_{50} values were determined for the mutant Thr^{373}Lys α_{2A} AR in the co-presence of a chimeric $G_{\alpha o/z}$-, $G_{\alpha o/15}$-, $G_{\alpha o}$Cys^{351}Ile, $G_{\alpha o/s}$-, $G_{\alpha o/q}$-, and $G_{\alpha o}$Cys^{351}Tyr protein, respectively: UK 14304 (9.11, 7.59, 9.06, 7.38, 7.66, and 8.70), d-medetomidine (9.20, 7.91, 9.19, 7.77, 8.00, and 8.72), BHT 920 (8.08, 6.64, 7.91, 6.51, 6.64, and 7.43). Bar graphs were constructed using means ± SEM values of 3–11 independent transfection experiments, each one performed in duplicate. Statistical analysis was performed as described in Methods by comparing the ligand's maximal response in the presence of a $G_{\alpha o}$Cys^{351}Ile protein versus the other mutant and chimeric $G_{\alpha o}$-proteins. *$P < 0.05$.

Table 5
Comparison of α_{2A} AR binding capacity and (−)-adrenaline-activated G_{α}-protein amount by $Thr^{373}Lys$ α_{2A} AR fused to mutant and chimeric $G_{\alpha o}$-proteins

Fusion protein	[^{3}H]RX 821002 binding		[^{35}S]GTPγS-binding responses	
	pK_d	B_{max} (pmol/mg protein)	pK_d	B_{max} (pmol/mg protein)
$Thr^{373}Lys$ α_{2A} AR: $G_{\alpha o/z}$	8.97	4.07	8.50	2.66
$Thr^{373}Lys$ α_{2A} AR: $G_{\alpha o/15}$	9.04	3.96	7.86	3.45
$Thr^{373}Lys$ α_{2A} AR: $G_{\alpha o}Cys^{351}Ile$	8.95	3.79	8.53	4.02
$Thr^{373}Lys$ α_{2A} AR: $G_{\alpha o/s}$	9.00	4.16	7.83	4.02
$Thr^{373}Lys$ α_{2A} AR: $G_{\alpha o}Cys^{351}Tyr$	9.04	3.89	8.55	3.62

Membranes of CHO-K1 cells transfected with 6 μg of fusion protein were analysed for [^{3}H]RX 821002 binding as described in Methods. Homologous displacement and analysis of [^{35}S]GTPγS-binding was performed with 0.5 nM [^{35}S]GTPγS, 30 μM GDP, and either without or with 0.1 to 3 nM unlabelled GTPγS in the absence or presence of 10 μM (−)-adrenaline. Basal [^{35}S]GTPγS binding was performed in the co-presence of 10 μM (+)-RX 811059. Analysis of saturation [^{3}H]RX 821002 and (−)-adrenaline-specific saturation [^{35}S]GTPγS binding is shown for a representative experiment out of 2 to 4 independent experiments.

CHO-K1 cells could be blocked to assure α_{2A} AR activation of the co-expressed recombinant G_{α}-protein. The largest (−)-adrenaline-induced [^{35}S]GTPγS-binding response was observed with the $G_{\alpha o}Cys^{351}Ile$ protein, whereas the $G_{\alpha i3}Cys^{351}Ile$ protein yielded almost no GDP/GTP exchange. The $G_{\alpha i3}$-protein also seems not to contribute to the inhibition of adenylyl cyclase activity by activation of α_{2A} AR expressed in Rat-1 fibroblasts, whereas the $G_{\alpha i2}$-protein does [27]. d-Medetomidine and BHT 920 behaved as partial agonists when the α_{2A} AR was co-expressed with the $G_{\alpha i1}Cys^{351}Ile$ and $G_{\alpha i2}Cys^{352}Ile$ proteins, but yielded enhanced efficacy in combination with the $G_{\alpha o}Cys^{351}Ile$ protein. A similar effect on efficacy for the partial agonists oxymetazoline and clonidine was obtained in NIH 3T3 cells stably co-expressing the α_{2A} AR and the wt $G_{\alpha o}$-protein instead of the $G_{\alpha i}$-protein family [28]. The increased ligand efficacy at the $G_{\alpha o}Cys^{351}Ile$ protein is unlikely to be due only to the $Cys^{351}Ile$ mutation, but also to a higher activation level of this G_{α} protein by the α_{2A} AR as compared to the $G_{\alpha i}$-proteins. The amino acid sequence of the three $G_{\alpha i}$-protein subtypes shares between 85 and 94% identity, dropping to 69% identity for the $G_{\alpha o}$-protein. Nevertheless, restricted $G_{\alpha i/o}$-protein domains such as their C-terminal portion may specifically interact with the α_{2A} AR [29]. This portion is fully identical for the $G_{\alpha i1}$- and $G_{\alpha i2}$-proteins, and most divergent between the $G_{\alpha i1/2}$- and $G_{\alpha o}$-protein subtypes. The $G_{\alpha i3}$-protein is almost identical to the $G_{\alpha i1/2}$-proteins with only two conservative differences, $Glu^{350}Asp$ and $Tyr^{354}Phe$, in their last six amino acids. These amino acid differences may be related to a reduced ability of the $G_{\alpha i3}$-protein to be activated by the wt α_{2A} AR or may result in distinct guanine nucleotide exchange properties [30], resulting in a weak (−)-adrenaline-elicited [^{35}S]GTPγS-binding response.

The α_{2A} AR-mediated [^{35}S]GTPγS-binding response was further investigated through its interaction with non-$G_{\alpha i/o}$-protein subtypes. None of the wt $G_{\alpha z}$-, $G_{\alpha s}$-, $G_{\alpha q}$-, and $G_{\alpha 15}$-proteins induced agonist-independent or agonist-dependent stimulation of [^{35}S]GTPγS-binding via either the wt or mutant $Thr^{373}Lys$ α_{2A} AR. Several reports indicate that the wt α_{2A} AR can activate these G_{α}-proteins: the $G_{\alpha z}$-protein has been shown to inhibit adenylyl cyclase upon stimulation of α_{2A} AR by UK 14304 (10 nM) in a PTX-resistant manner [31]. The α_{2A} AR is able to stimulate adenylyl cyclase in a PTX-resistant and CTX-sensitive way via its interaction with a $G_{\alpha s}$-protein [8]. Co-expression of wt and mutant $Thr^{373}Lys$ α_{2A} AR with either a $G_{\alpha q}$- or $G_{\alpha 15}$-protein stimulates the production of inositol phosphates [19,24]. The inability to measure enhanced [^{35}S]GTPγS-binding responses in our assay system is probably due to a slow rate of GTP/GDP exchange for the naturally PTX-resistant G_{α}-proteins as compared to the $G_{\alpha i/o}$-proteins [32] rather than to a lack of interaction between the α_{2A} AR and these G_{α}-protein subunits. To evaluate a potential interaction between the α_{2A} AR and these G_{α}-protein subunits, chimeric proteins between the $G_{\alpha o}$-protein and the last six C-terminal amino acids of either $G_{\alpha z}$-, $G_{\alpha s}$-, $G_{\alpha q}$-, or $G_{\alpha 15}$-protein subunits were constructed. This strategy has already been successfully employed to switch the functional response of various G-protein-coupled receptors (i.e. metabotropic glutamate, muscarinic and opioid receptors) to a common effector system such as phospholipase C [33]. The involvement of other G_{α}-protein domains such as the N-terminal portion, in modulation of the receptor: G-protein selectivity as shown for a $G_{\alpha q}$- [34] and $G_{\alpha z}$- [35] protein, was not evaluated.

The chimeric $G_{\alpha o/z}$-, $G_{\alpha o/s}$-, $G_{\alpha o/q}$-, and $G_{\alpha o/15}$-proteins were able to strongly enhance the [^{35}S]GTPγS-binding response to a similar level (380 to 520% over basal) by the native agonist (−)-adrenaline using membranes with the wt α_{2A} AR. The imidazoline derivative UK 14304 was a partial agonist at the wt α_{2A} AR when co-expressed with the chimeric $G_{\alpha o/s}$-, $G_{\alpha o/q}$-, and $G_{\alpha o/15}$-proteins, but displays full agonist properties in combination with the chimeric $G_{\alpha o/z}$-protein as is also the case when α_{2A} ARs are transiently or stably expressed in HEK 293 or CHO cells [this study, 36,37]. Another imidazoline derivative, d-medetomidine, being a partial agonist at $G_{\alpha i/o}$-proteins, yielded vir-

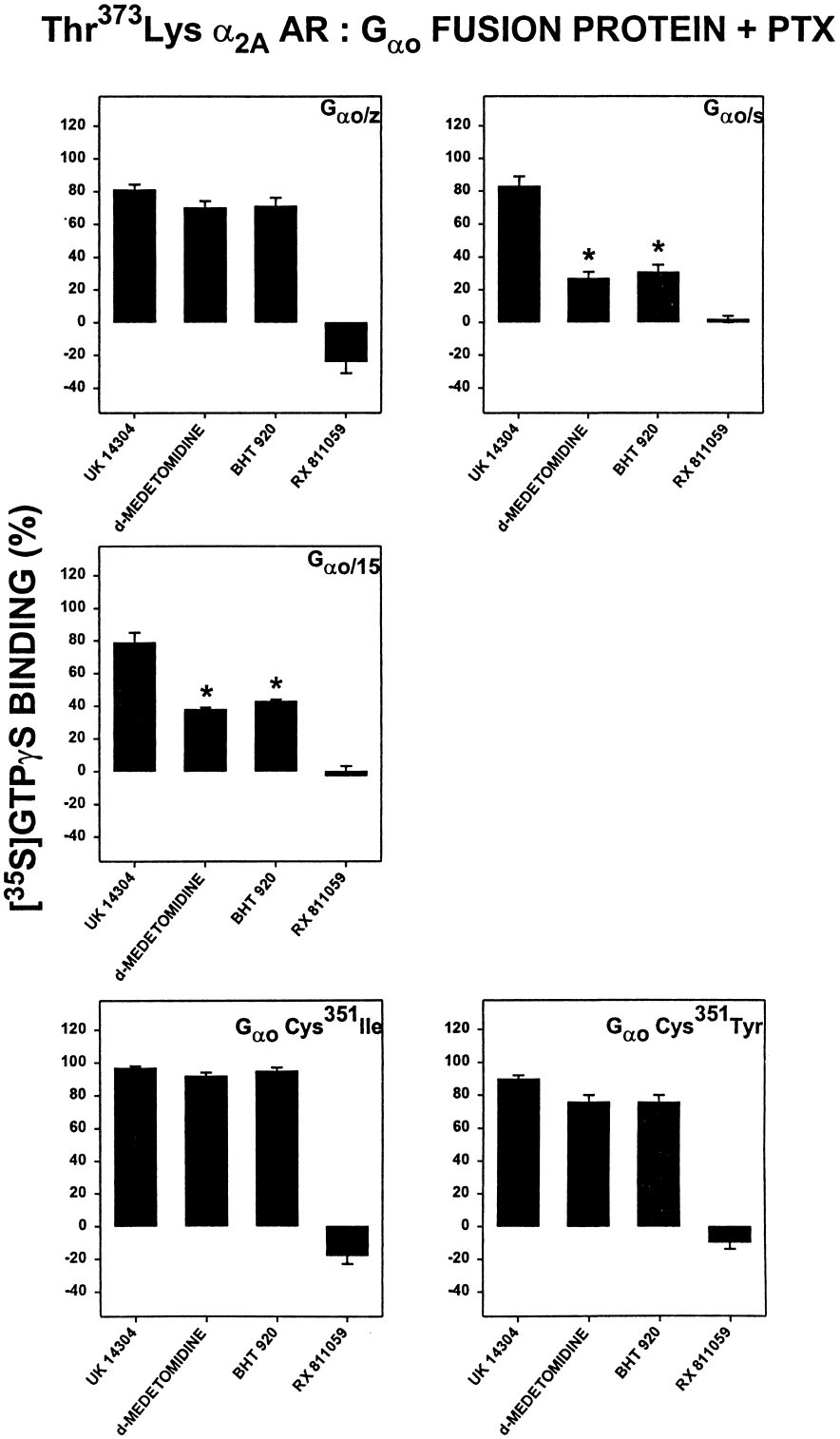

Fig. 6. Ligand-mediated [^{35}S]GTPγS-binding responses by mutant Thr^{373}Lys α_{2A} AR fused to either a chimeric or mutant $G_{\alpha o}$-protein. CHO-K1 cells were transfected with 6 μg of the indicated Thr^{373}Lys α_{2A} AR: $G_{\alpha o}$-fusion protein and treated with PTX (20 ng/mL) as indicated in the legend to Fig. 1. [^{35}S]GTPγS-binding data were expressed as percentage of the respective maximal [^{35}S]GTPγS-binding responses as obtained with 10 μM (−)-adrenaline except for (+)-RX 811059, which was expressed versus its respective basal [^{35}S]GTPγS-binding value. Mean pEC_{50} values were determined for the mutant Thr^{373}Lys α_{2A} AR fused to a chimeric $G_{\alpha o/z}$-, $G_{\alpha o/15}$-, $G_{\alpha o}$Cys^{351}Ile, $G_{\alpha o/s}$-, and $G_{\alpha o}$Cys^{351}Tyr protein, respectively: UK 14304 (8.76, 7.62, 9.10, 7.22, and 8.39), d-medetomidine (8.91, 8.07, 9.20, 7.34, and 8.51), BHT 920 (7.75, 6.85, 8.03, 6.82, and 7.54). Bar graphs were constructed using means ± SEM values of 2–4 independent transfection experiments, each one performed in duplicate. The Thr^{373}Lys α_{2A} AR: $G_{\alpha o/q}$-fusion protein did not yield a detectable (−)-adrenaline-mediated [^{35}S]GTPγS-binding response. Statistical analysis was performed as described in section 2 by comparing the ligand's maximal response in the presence of a Thr^{373}Lys α_{2A} AR: $G_{\alpha o}$Cys^{351}Ile fusion protein vs the other mutant and chimeric $G_{\alpha o}$-derived fusion proteins. *$P < 0.05$.

tually no response with the chimeric $G_{\alpha o}$-proteins, with the exception of the $G_{\alpha o/z}$-protein. Similar data were obtained with the azepine derivative BHT 920. The putative α_2 AR antagonist (+)-RX 811059 was unable to attenuate the basal [^{35}S]GTPγS-binding response by the chimeric $G_{\alpha o/s}$-, $G_{\alpha o/q}$-, and $G_{\alpha o/15}$-proteins in contrast to the $G_{\alpha o/z}$-protein. This compound has also been described as a silent antagonist at wt and mutant Thr^{373}Lys α_{2A} AR co-expressed with a wt $G_{\alpha 15}$-protein by measuring the formation of inositol phosphates [19]. It is likely that the partial agonists d-medetomidine and BHT 920 are unable to stabilise an α_{2A} AR conformation that allows it to interact productively with the chimeric $G_{\alpha o/s}$-, $G_{\alpha o/q}$-, and $G_{\alpha o/15}$-proteins. The partial agonists displayed higher intrinsic activity at the mutant Thr^{373}Lys α_{2A} AR when co-expressed or fused with either the chimeric $G_{\alpha o/s}$-, $G_{\alpha o/q}$-, or $G_{\alpha o/15}$-protein. Almost no gain in the ligands' intrinsic activity was observed for the chimeric $G_{\alpha o/z}$-protein; agonists were already highly efficacious at the combination of wt α_{2A} AR with the $G_{\alpha o/z}$-protein. Otherwise, the magnitude of the inverse agonist response of (+)-RX 811059 was increased at the mutant Thr^{373}Lys α_{2A} AR co-expressed or fused with the $G_{\alpha o/z}$-protein. It remained a neutral antagonist in combination with the $G_{\alpha o/s}$-, $G_{\alpha o/q}$-, and $G_{\alpha o/15}$-proteins. These results point to a different ability of certain α_{2A} AR ligands to stabilise a wt and mutant Thr^{373}Lys α_{2A} AR conformation that preferentially interacts with a given and specific chimeric $G_{\alpha o}$-protein. We emphasise that the herein-described G_α-protein-dependent α_2 AR ligand effects were observed under similar experimental conditions, at a receptor: G_α-protein density ratio of 1.0 by using the fusion protein approach. Hence, different activation of G_α-proteins by partial and full agonists may occur. Partial agonists will activate one set of G_α-proteins submaximally, while full agonists will do this more efficaciously and with multiple, distinct G_α-proteins. Consequently, partial agonists may yield a more selective response, as they will only activate a single effector pathway as opposed to full agonists, which may mediate diverse signalling responses. This scenario argues that diverse signalling by ligands at a single receptor subtype can occur in more ways than simple ligand:receptor:G-protein channelling [38].

The nature of the last six C-terminal amino acid residues of the G_α-subunit, which have been exchanged in the different chimeric $G_{\alpha o}$-proteins, seems to favour a specific interaction between one particular conformation of the wt and mutant Thr^{373}Lys α_{2A} AR when activated by a given ligand. Molecular genetic and biochemical studies have shown that the precise positions of the C-terminal amino acids of the G_α-protein subunits are critical for determining the specificity of receptor: G-protein interactions [39,40]. These last six amino acid positions differ between the different functional classes of G_α-protein subunits except for the -2 residue, which corresponds to a leucine in each of the reported mammalian G_α-protein subunits. The -6 residue is a basic amino acid (Lys or Arg) except for the $G_{\alpha 15}$-protein, which possesses an acidic aspartate. The most divergent residue is the -5 position: it can be an acidic Glu as for $G_{\alpha q}$- and $G_{\alpha 15}$-proteins, a polar Gln as for the $G_{\alpha s}$-protein, or a hydrophobic Tyr residue as for the $G_{\alpha z}$-protein. The -4 residue (the PTX-mediated ADP-ribosylation site in $G_{\alpha i/o}$-proteins) is always a hydrophobic residue (Tyr or Ile) at the naturally PTX-resistant G_α-protein subunits, and their [^{35}S]GTPγS-binding responses were compared to those of the mutant $G_{\alpha o}$Cys^{351}Ile/Tyr proteins. The facilitation of α_{2A} AR:$G_{\alpha i1}$-protein and 5-HT$_{1A}$ receptor:$G_{\alpha o}$-protein interactions by a non-polar amino acid at this particular position has already been suggested [18,41,42]. Nevertheless, the presence of either a Tyr or Ile -4 residue in the chimeric $G_{\alpha o}$-proteins is not sufficient to enhance the efficacy of partial agonists at the wt α_{2A} AR with the exception of the $G_{\alpha o/z}$-protein. The observed modulation of ligand responses is unlikely to be influenced only by this particular amino acid. Kostenis *et al*. [43] reported on the role of the -3 residue for muscarinic m_3, V_{1a} vasopressin, and gastrin-releasing peptide receptors: G_α-subunit interactions. None of these receptors was able to interact with a wt $G_{\alpha s}$-protein, whereas the three receptors productively couple to a mutant $G_{\alpha s}$-protein containing a Glu to Asn mutation at position -3 (Asn occurs in the wt $G_{\alpha q}$-protein that preferentially couples to these three receptors). The amino acid residue present at the -3 position seems conserved within individual G_α-protein subtypes: a Gly residue for the $G_{\alpha i/o/z}$-proteins, an Asn residue for the $G_{\alpha q/11/15}$-proteins, and a Glu residue for the $G_{\alpha s}$-protein. It can, according to Kostenis *et al*. [43], predict favourable interactions between a wt, mutant, or chimeric G_α-protein subunit and a given receptor subtype. The last C-terminal amino acid of the G_α protein always seems to be a hydrophobic residue (Cys, Leu, Val), but no clear link between the different classes of G_α-subunits can be extrapolated. Further mutational analysis of the herein-described chimeric $G_{\alpha o}$-proteins may reveal more subtle contact points between both the wt and mutant Thr^{373}Lys α_{2A} AR and a given G_α-protein subunit. The relative importance of the G_α-protein subunit C-terminal portion in permitting coupling to a given receptor may also be influenced by the type of receptor with which it is paired; certain combinations, for instance the β_2 AR and the chimeric G_{qs5}-protein, are unable to signal via the inositol phosphate pathway in contrast to the G_s-coupled V_2 vasopressin receptor [44].

In conclusion, our results suggest that certain α_2 AR ligands may display a different G_α-protein activation profile at a single α_{2A} AR subtype. Thus, it is likely that pharmacological diversity may not only be achieved between different receptor subtypes, but even occurs for a single receptor subtype.

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