0026-895X/01/6004-666–673$3.00
MOLECULAR PHARMACOLOGY
Vol. 60, No. 4
746/928176
Mol Pharmacol 60:666–673, 2001
Printed in U.S.A.

Reciprocal Modulation of α_{2A}-Adrenoceptor and $G_{\alpha o}$ Protein States as Determined by Carboxy-Terminal Mutagenesis of a $G_{\alpha o}$ Protein

THIERRY WURCH, JUNKO OKUDA,[1] and PETRUS J. PAUWELS

Department of Cellular and Molecular Biology, Centre de Recherche Pierre Fabre, Castres, France

Received December 6, 2000; accepted June 11, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

The C-terminal portion of G_α proteins plays a key role in their selective activation by cognate receptors. α_{2A}-Adrenoceptors (α_{2A}-ARs) can differentially inhibit or stimulate adenylyl cyclases by the activation of distinct $G_{i/o}$ and G_s protein families. The implication of the C-terminal portion of $G_{\alpha o}$ and $G_{\alpha s}$ proteins in their activation by α_{2A}-ARs was analyzed by constructing mutant $G_{\alpha o}$ proteins in which each of the last five amino acid positions were exchanged for those corresponding to a $G_{\alpha s}$ protein. Agonist-dependent, pertussis toxin-resistant binding of guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) revealed that the degree of positive efficacy of clonidine was highly dependent on the presence of a $G_{\alpha o}$ protein-derived Gly amino acid as the −3 residue at the C-terminal portion of the protein. In contrast, antagonist properties for clonidine were observed for those mutants carrying a $G_{\alpha s}$ protein-derived Glu residue at this position. (−)-Epinephrine yielded almost similar maximal [^{35}S]GTPγS binding responses, but its potency was decreased 22- to 150-fold at the −3 Glu containing mutant $G_{\alpha o}$ proteins compared with those mutants containing a Gly. A 9- to 39-fold increase in the α_{2A}-AR agonist equilibrium dissociation constants further reflected changes in the G_α protein-induced α_{2A}-AR state mediated by the specific Gly to Glu mutation in the C-terminal portion of the $G_{\alpha o}$ protein. The present data emphasize the unique role of the −3 position at the G_α protein C-terminal portion, independent of its surrounding peptidic environment, in constraining a structure favorable for activated receptor interaction and transmission of the mutation-induced conformational change from the $G_{\alpha o}$ protein to the α_{2A}-AR.

Interaction of G protein-coupled receptors (GPCR) with ligands results in conformational changes in the receptor structure that enables its interaction with specific classes of heterotrimeric G proteins (Gudermann et al., 1997; Bockaert and Pin, 1999). The activated G protein subunits α and βγ are then able to modulate the activity of downstream effectors. A single GPCR can interact with several distinct G protein combinations that exist in a given cell, thereby generating divergent signaling through a single receptor subtype (Kenakin, 1995). How this selectivity is achieved in terms of protein-protein interactions is not well understood; this is mainly due to the lack of structural data on receptor-G protein interaction domains. Numerous mutagenesis and biochemical studies (Liu et al., 1995; Kostenis et al., 1997) have shown that the carboxy-terminal portion of G_α subunits is an important determinant of GPCR contact specificity. Synthetic C-terminal peptides of transducin ($G_{\alpha t}$) and monoclonal antibodies specific for the $G_{\alpha t}$ carboxy-terminal portion prevent the interaction between transducin and rhodopsin (Hamm et al., 1988; Mazzoni et al., 1991). Construction of a chimeric $G_{\alpha q}$ protein by substitution of its three C-terminal amino acids by those of a $G_{\alpha i2}$ protein switches GPCR specificity from the adenylyl cyclase to the phospholipase C pathway (Conklin et al., 1993). The use of such chimeric G_α proteins, exchanging up to nine amino acids of their extreme C-terminal portion, has been reported to direct GPCRs differing in their G protein-coupling specificity toward common effector systems, such as the production of inositol phosphates or the mobilization of intracellular Ca^{2+} (Milligan and Rees, 1999).

The α_{2A}-adrenoceptor (α_{2A}-AR; receptor classification, 2.1.ADR.A2A) has been shown to activate multiple and distinct effector pathways, such as inhibition and activation of adenylyl cyclase (Fraser et al., 1989; Eason et al., 1992), activation of phospholipase C (Cotecchia et al., 1990; Dorn et al., 1997), activation of K^+ channels (Fraser et al., 1989), and inhibition of Ca^{2+} channels (Airriess et al., 1997). The dual signaling properties of α_{2A}-AR to the inhibition and activation of adenylyl cyclase is dependent on the ligand structure

[1] Present address: Department of Biotechnology, Tokyo University of Agriculture and Technology, 113-0032 Tokyo, Japan.

ABBREVIATIONS: GPCR, G protein-coupled receptor; α_{2A}-AR, α_{2A}-adrenoceptor; wt, wild-type; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate; PTX, *Bordetella pertussis* toxin; PCR, polymerase chain reaction; RX 821002, 2-(2-methoxy-2,3-dihydro-benzo[1,4]dioxin-2-yl)-4,5-dihydro-1*H*-imidazole; UK 14304, 5-bromo-6-(2-imidazoline-2-ylamino)quinoxaline tartrate.

and is mediated by two distinct G proteins of the $G_{i/o}$ and G_s families (Eason et al., 1994; Eason and Liggett, 1996; Brink et al., 2000). The aim of the current study was to examine the exact contribution of each of the last five carboxy-terminal amino acids of the $G_{\alpha s}$ protein in divergent α_{2A}-AR signaling and their effect on α_{2A}-AR ligand binding properties. Therefore, a collection of mutant $G_{\alpha o}$ proteins in which the last five amino acids positions were systematically exchanged between $G_{\alpha o}$ and $G_{\alpha s}$ proteins was constructed. Functional analysis was performed by co-expression of the mutant $G_{\alpha o}$ proteins with a wt α_{2A}-AR. Agonist-dependent binding of the stable GTP analog [^{35}S]GTPγS to the mutant $G_{\alpha o}$ proteins, and the binding of both ^{3}H-agonist and ^{3}H-antagonist to the α_{2A}-AR co-expressed in COS-7 cellular membranes were measured. Because none of the mutant $G_{\alpha o}$ proteins contained an ADP-ribosylation site by *Bordetella pertussis* toxin (PTX), cells were treated with PTX to avoid α_{2A}-AR coupling to endogenous $G_{i/o}$ proteins in COS-7 cells. The $G_{\alpha o}$ protein-derived Gly residue at the −3 position away from the protein C-terminal extremity demonstrated a pivotal role in decreasing the efficacy of the partial agonist clonidine. Moreover, a similar mutation induced a decrease in agonist, but not antagonist, binding affinity to the α_{2A}-AR. These results are discussed in view of structural conformation data on G_α protein C-terminal portion and receptor interactions.

Experimental Procedures

Construction of Mutant $G_{\alpha o}$ Proteins. The investigated mutant $G_{\alpha o}$ proteins were generated by PCR on linearized pCR3.1/$G_{\alpha o}$ cDNA plasmid (Pauwels et al., 2001) using a sense primer designed according to the rat $G_{\alpha o}$ cDNA nucleotide sequence (GenBank accession number M17526) and a mutagenic reverse primer carrying the respective mutation; their sequences are indicated in Table 1. The amplification conditions were similar, as previously described (Pauwels et al., 2001). PCR products were cloned into a pCR3.1 expression vector (Invitrogen, San Diego, CA) and fully sequenced on an ABI 310 Prism genetic analyzer (PerkinElmer Life Science Products, Foster City, CA) using a Big Dye Terminator cycle sequencing ready reaction kit (PerkinElmer Life Science Products), confirming the presence of the respective mutations.

Cell Culture and Transfection Procedures. The COS-7 cell line (ATCC: CRL 1651; American Type Culture Collection, Manassas, VA) was cultured in Petri dishes (50 cm^2) with Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. Cells grown to 60 to 80% confluence were used for transfection using a LipofectAMINE plus kit (Invitrogen, Paisley, UK). pCR3.1 plasmid (3–0.03 μg) containing the wt human α_{2A}-AR gene (receptor classification, 2.1.ADR.A2A; GenBank accession number M23533) and 3 μg of either empty plasmid or indicated mutant $G_{\alpha o}$ protein plasmid were mixed with 10 μl of LipofectAMINE plus reagent diluted in 0.2 ml of Opti-MEM and incubated at room temperature for 15 min. Subsequently, 20 μl of LipofectAMINE reagent diluted 20 times in 0.2 ml of Opti-MEM was added and incubated for 15 min. COS-7 cells were exposed to the plasmid/LipofectAMINE mixture with 5 ml of Opti-MEM for 3 h at 37°C. Thereafter, cells were incubated with 10 ml of complete growth medium and harvested 48 h after transfection. Treatment with PTX (20 ng/ml) was performed overnight before membranes were prepared.

Membrane Preparation and Radioligand Binding Experiments. Membrane preparation steps were performed at 4°C. Cells were washed with phosphate-buffered saline and stored at −80°C. Cells were scraped mechanically in 10 mM Tris-HCl, 0.1 M EDTA, pH 7.5, and centrifuged for 10 min at 45,000*g*. The pellet was homogenized in the same buffer and centrifuged under similar conditions. The final pellet was distributed at 0.5 to 1.5 mg of protein/ml in Tris-EDTA buffer and stored at −80°C. Membrane preparations were diluted in 20 mM Hepes, 100 mM NaCl, 3 mM $MgCl_2$, and 0.2 mM ascorbic acid, pH 7.4, and used for the binding study with [^{3}H]2-(2-ethoxy-2,3-dihydro-benzo[1,4]dioxin-2-yl)-4,5-dihydro-1*H*-imidazole ([^{3}H]RX 821002), [^{3}H]5-bromo-6-(2-imidazoline-2-ylamino)quinoxaline tartrate ([^{3}H]UK 14304), and [^{3}H]clonidine, as described (Wurch et al., 1999). Nonspecific radioligand binding was determined in the presence of 10 μM phentolamine. Scatchard analysis was performed as described (Pauwels et al., 1996) using concentrations of radioligand ranging from 0.3 to 10 nM for [^{3}H]RX 821002, 0.2 to 100 nM for [^{3}H]clonidine, and 0.04 to 40 nM for [^{3}H]UK 14304. Data were analyzed by the nonlinear square curve-fitting program, Ligand version 4.0 (Biosoft, Cambridge, UK; Rovati et al., 1989)

[^{35}S]GTPγS Binding Responses. Agonist-independent (basal) and agonist-dependent [^{35}S]GTPγS binding responses were per-

TABLE 1

Sequence characteristics of the C-terminal portion of the mutant $G_{\alpha o}$ proteins

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 the rat $G_{\alpha s}$ or the mouse $G_{\alpha 15}$ protein. Systematic mutations of the four residues, diverging between the C-terminal portions of $G_{\alpha o}$ and $G_{\alpha s}$ proteins, were realized as described under *Experimental Procedures* using the indicated mutagenic reverse primers. The arrow indicates the position of the PTX-mediated ADP ribosylation site in the wt $G_{\alpha o}$ protein. The nucleotides or amino acids indicated in bold are those that are modified according to the $G_{\alpha o/s}$ cDNA sequence. The −6 residue is identical for the $G_{\alpha o}$ and $G_{\alpha s}$ proteins (Arg) but different for the $G_{\alpha 15}$ protein (Asp). It will only be indicated for mutants involving this latter G_α protein.

G_α Protein Nomenclature	Reverse PCR Primer	C-Terminal Last Six Amino Acids
$G_{\alpha o}$Cys^{351}Ile	5′-C TTA GAG CAA CTC GTA **GCC** GCG GAG ATT GTT GGC AAT GAT-3′	R**GIG**L**Y**
$G_{\alpha o}$Cys^{351}Tyr	5′-C TTA GAG CAA CTC GTA **ATA** GCG GAG ATT GTT GGC AAT GAT-3′	R**GY**G**L**Y
$G_{\alpha o}$/QYELL (= $G_{\alpha o/s}$)	5′-**T TAG AGC AGC TCG TAA AGG CG**G AGA TTG TTG GCA ATG ATG-3′	RQYELL
$G_{\alpha o}$/GYELL	5′-C TTA GAG CAA CTC GTA **GCC** GCG GAG ATT GTT GGC AAT GAT-3′	R**G**YELL
$G_{\alpha o}$/QIELL	5′-C TTA GAG CAA CTC **GAT** TTG GCG GAG ATT GTT GGC AAT GAT-3′	RQ**I**ELL
$G_{\alpha o}$/QYGLL	5′-C TTA GAG CAA **GCC** GTA TTG GCG GAG ATT GTT GGC AAT GAT-3′	RQY**G**LL
$G_{\alpha o}$/QYELY	5′-C TTA **GTA** CAA GCC GTA TTG GCG GAG ATT GTT GGC AAT GAT-3′	RQYEL**Y**
$G_{\alpha o}$/GIELL	5′-C TTA GAG CAA CTC **GAT GCC** GCG GAG ATT GTT GGC AAT GAT-3′	R**GI**ELL
$G_{\alpha o}$/QYGLY	5′-C TTA **GTA** CAA **GCC** GTA TTG GCG GAG ATT GTT GGC AAT GAT-3′	RQY**GLY**
$G_{\alpha o}$/GYGLL	5′-C TTA GAG CAA **GCC** GTA **GCC** GCG GAG ATT GTT GGC AAT GAT-3′	R**GYG**LL
$G_{\alpha o}$/QIGLL	5′-C TTA GAG CAA **GCC GAT** TTG GCG GAG ATT GTT GGC AAT GAT-3′	RQ**IG**LL
$G_{\alpha o}$/QIELY	5′-C TTA **GTA** CAA CTC **GAT** TTG GCG GAG ATT GTT GGC AAT GAT-3′	RQ**I**EL**Y**
$G_{\alpha o}$/GYELY	5′-C TTA **GTA** CAA CTC GTA **GCC** GCG GAG ATT GTT GGC AAT GAT-3′	R**G**YEL**Y**
$G_{\alpha o}$/GIGLL	5′-C TTA GAG CAA **GCC GAT GCC** GCG GAG ATT GTT GGC AAT GAT-3′	R**GIG**LL
$G_{\alpha o}$/GIELY	5′-C TTA **GTA** CAA CTC **GAT GCC** GCG GAG ATT GTT GGC AAT GAT-3′	R**GI**EL**Y**
$G_{\alpha o}$/QIGLY	5′-C TTA **GTA** CAA **GCC GAT** TTG GCG GAG ATT GTT GGC AAT GAT-3′	RQ**IGLY**
$G_{\alpha o}$/DEINLL (= $G_{\alpha o/15}$)	5′-T TCT TAG ACC AG**A CC**G TAC ACC TTG AGA TTG TTG GCA ATG AT-3′	**DEIN**LL
$G_{\alpha o}$/DEIGLL	5′-G CGT CAC AGC AG**G CC**G ATC TCG TCG AGA TTG TTG GCA ATG AT-3′	**DEIG**LL ↑

formed to the membrane preparations described above in 20 mM Hepes, 30 μM GDP, 100 mM NaCl, 3 mM $MgCl_2$, and 0.2 mM ascorbic acid, pH 7.4. Maximal stimulation of [^{35}S]GTPγS binding was defined in the presence of 10 μM (−)-epinephrine and calculated versus basal [^{35}S]GTPγS binding, unless otherwise indicated. The maximal capacity of recombinant mutant $G_{\alpha o}$ protein agonist-mediated activation was determined by saturation [^{35}S]GTPγS binding on the same membrane preparations in GDP 30 μM, 0.5 nM [^{35}S]GTPγS, and 0 to 300 nM unlabeled GTPγS in 20 mM Hepes, 100 mM NaCl, 3 mM $MgCl_2$, and 0.2 mM ascorbic acid, pH 7.4. The binding reaction was terminated by rapid filtration through Whatman GF/B glass fiber filters (Brandel, Gaithersburg, MD) treated as described (Pauwels et al., 2001). EC_{50} values were derived graphically as the concentration of compound yielding 50% of its own maximal [^{35}S]GTPγS binding response. The potency of clonidine to antagonize (−)-epinephrine-mediated [^{35}S]GTPγS binding responses was calculated according to the equation $K_B = (B)/[(A')/(A) - 1]$, where B is the clonidine concentration, and A and A' are the EC_{50} values of (−)-epinephrine in the absence and presence of clonidine, respectively.

Immunological Detection of Mutant $G_{\alpha o}$ Protein Expression. Membrane fractions of COS-7 cells transiently co-expressing the α_{2A}-AR and mutant $G_{\alpha o}$ proteins were prepared as described above. Total proteins were separated by denaturing 12.5% (w/v) SDS-polyacrylamide gel electrophoresis, as described (Laemmli, 1970). After electrophoresis, proteins were blotted onto a nylon membrane by semidry electrotransfer (23 V, 45 min) in 25 mM Tris-HCl, pH 8.3, 190 mM glycine, 20% (v/v) methanol. 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 phosphate-buffered saline buffer containing 0.1% (w/v) Tween 20, 5% (w/v) dry nonfat milk, and the antibody at a dilution of 1:1000. Proteins were visualized with an anti-mouse IgG antibody coupled to horseradish peroxidase using a chemiluminescence reaction. Quantification of the immunodetected signal was performed using a computer-based image analysis system (Imagena 2000 software; Biocom, Les Ulis, France).

Protein Content. The protein level of membrane preparations was estimated with a dye-binding assay using a Bio-Rad kit (Bio-Rad, Hercules, CA); bovine serum albumin was used as a standard (Bradford, 1976).

Statistical Analysis. Statistical analyses were performed on K_D and B_{max} values of the radioligands by a one-way analysis of variance, followed by an all pairwise multiple comparison procedure (method of Tukey) between $G_{\alpha o}$/GYGLY (= $G_{\alpha o}Cys^{351}Tyr$) and the other mutant $G_{\alpha o}$ proteins.

Materials. The ABI Prism 310 genetic analyzer and big dye terminator cycle sequencing ready reaction kit were obtained from PerkinElmer Life Science Products. The Imagena 2000 software was obtained from Biocom. The pCR3.1 expression was purchased from Invitrogen. COS-7 cells were obtained from the American Type Culture Collection. The LipofectAMINE plus kit, cell culture medium, fetal calf serum, and *B. pertussis* toxin (50 μg/ml) were purchased from Invitrogen. [^{3}H]RX 821002 (67 Ci/mmol), [^{3}H]UK 14304 (74 Ci/mmol), and [^{3}H]clonidine (70.2 Ci/mmol) were obtained from PerkinElmer Life Science Products. [^{35}S]GTPγS (1035–1163 Ci/mmol) and the ECL chemiluminescence reaction kit were obtained from Amersham Pharmacia Biotech (Les Ulis, France). (−)-Epinephrine and clonidine were from Sigma (St. Louis, MO).

Results

[^{35}S]GTPγS Binding Responses as Mediated by Mutant $G_{\alpha o}$ Proteins in the Co-Presence of α_{2A}-AR. Neither wt α_{2A}-AR nor $G_{\alpha o}Cys^{351}Tyr$ protein, expressed independently in COS-7 cells, displayed a detectable [^{35}S]GTPγS binding response upon stimulation by 10 μM (−)-epinephrine (not shown). Co-expression of the α_{2A}-AR with a chimeric $G_{\alpha o}$ protein in which the last five amino acids of the wt $G_{\alpha o}$ protein were replaced by the equivalent portion of the $G_{\alpha s}$ protein ($G_{\alpha o}$/QYELL) resulted in a low-magnitude clonidine-mediated [^{35}S]GTPγS binding response (14% stimulation versus (−)-epinephrine; Table 2) compared with the PTX-resistant $G_{\alpha o}Cys^{351}Ile$ protein (73% stimulation versus (−)-epinephrine; Table 2). The (−)-epinephrine-mediated [^{35}S]GTPγS binding response was only decreased 2-fold compared with its basal [^{35}S]GTPγS binding level for these two mutant $G_{\alpha o}$ proteins (Table 2). Both $G_{\alpha o}Cys^{351}Ile$ and $G_{\alpha o}$/QYELL proteins differ at four amino acid positions ($Gln^{350}Gly$, $Tyr^{351}Ile$, $Glu^{352}Gly$, and $Leu^{354}Tyr$). A gain-of-function approach to investigate which of these four amino acids may be involved in the low-magnitude profile of clonidine at the $G_{\alpha o}$/QYELL protein was conducted by measuring [^{35}S]GTPγS binding responses; the data are sum-

TABLE 2

[^{35}S]GTPγS binding responses of α_{2A}-AR co-expressed with a series of mutant $G_{\alpha o}$ proteins

Classification was performed according to the maximal [^{35}S]GTPγS binding response of clonidine calculated in percentage vs. (−)-epinephrine. Co-expression of α_{2A}-AR and respective mutant $G_{\alpha o}$ protein was performed as described under *Experimental Procedures*. All conditions were treated with PTX (20 ng/ml). Basal, 10 μM (−)-epinephrine, and 10 μM clonidine-stimulated [^{35}S]GTPγS binding responses, mediated by the α_{2A}-AR, were performed as described under *Experimental Procedures*. Data represent mean values ± S.E.M. of three to seven independent transfection experiments, each performed in duplicate. The bold amino acids correspond to those that are different between $G_{\alpha o/s}$ ($G_{\alpha o}$/QYELL) and the various mutant $G_{\alpha o}$ proteins.

G_α Protein	[^{35}S]GTPγS Binding Responses				
	Basal	(−)-Epinephrine		Clonidine	
	fmol/mg protein	*fmol/mg protein*	*% above basal*	*% above basal*	*% vs. (−)-epinephrine*
$G_{\alpha o}$/Q**IG**LL	240 ± 7	1031 ± 72	333 ± 44	361 ± 48	110 ± 7
$G_{\alpha o}$/Q**IGLY**	283 ± 52	967 ± 28	262 ± 56	275 ± 76	107 ± 7
$G_{\alpha o}$/QY**G**LL	190 ± 2	995 ± 79	424 ± 47	360 ± 86	85 ± 1
$G_{\alpha o}$/QY**GLY**	168 ± 5	953 ± 47	469 ± 45	388 ± 57	83 ± 2
$G_{\alpha o}$/**GIG**LL	170 ± 4	1005 ± 6	491 ± 8	370 ± 35	75 ± 2
$G_{\alpha o}$/***GI***GLY$G_{\alpha o}Cys^{351}$*Ile*	170 ± 22	1095 ± 267	534 ± 59	375 ± 27	73 ± 12
$G_{\alpha o}$/G**Y**G**L**Y$G_{\alpha o}Cys^{351}$*Tyr*	168 ± 14	912 ± 42	463 ± 48	278 ± 44	60 ± 2
$G_{\alpha o}$/G**Y**G**L**L	148 ± 7	923 ± 12	527 ± 20	298 ± 22	57 ± 3
$G_{\alpha o}$/Q**IEL**Y	108 ± 6	548 ± 9	411 ± 22	55 ± 8	14 ± 1
$G_{\alpha o}$/QYELL(= $G_{\alpha o}$/s)	166 ± 16	542 ± 64	231 ± 30	33 ± 14	14 ± 2
$G_{\alpha o}$/QYEL**Y**	142 ± 2	735 ± 82	418 ± 52	59 ± 14	14 ± 1
$G_{\alpha o}$/Q**IE**LL	137 ± 9	675 ± 25	399 ± 52	41 ± 4	11 ± 1
$G_{\alpha o}$/**G**YEL**Y**	105 ± 4	380 ± 23	264 ± 34	19 ± 5	7 ± 0
$G_{\alpha o}$/**G**YELL	132 ± 4	358 ± 35	175 ± 35	8 ± 6	5 ± 1
$G_{\alpha o}$/**GI**ELL	115 ± 8	372 ± 13	230 ± 36	11 ± 3	5 ± 0
$G_{\alpha o}$/**GIEL**Y	112 ± 6	445 ± 11	302 ± 34	15 ± 1	5 ± 1

marized in Table 2. Basal [^{35}S]GTPγS binding responses for most of the mutant $G_{\alpha o}$ proteins were between 105 and 190 fmol/mg of protein; a trend for an elevated basal level was observed for the mutant $G_{\alpha o}$/QIGLL and $G_{\alpha o}$/QIGLY proteins. (−)-Epinephrine (10 μM) stimulated the binding of [^{35}S]GTPγS from 175 to 534%. Clonidine yielded two types of responses; it acted as a partial to efficacious agonist with maximal responses between 60 and 110% of that mediated by (−)-epinephrine or as a weak agonist with a maximal response below 15% compared with (−)-epinephrine. Both responses could be associated with a single amino acid position in the C-terminal portion of the $G_{\alpha o}$ protein. Clonidine behaved as an efficacious agonist, with a maximal response as high as that of (−)-epinephrine when a Gly residue is present at the −3 position of the mutant $G_{\alpha o}$ protein (Table 2). In contrast, when the −3 position was a Glu, the maximal stimulation of [^{35}S]GTPγS binding by clonidine was below 15% compared with (−)-epinephrine (Table 2). The amino acid at the −5 position also influenced, but to a lesser extent, the level of mutant $G_{\alpha o}$ protein activation by the clonidine-occupied α_{2A}-AR; the −5 Gln/−3 Gly combination generated the highest activation level ($G_{\alpha o}$/QIGLL, $G_{\alpha o}$/QIGLY, $G_{\alpha o}$/QYGLL, and $G_{\alpha o}$/QYGLY proteins, 83 to 110%; Table 2) compared with (−)-epinephrine, whereas the mutants carrying the −5 Gly/−3 Glu combination yielded almost no stimulation with clonidine ($G_{\alpha o}$/GYELY, $G_{\alpha o}$/GYELL, $G_{\alpha o}$/GIELL, and $G_{\alpha o}$/GIELY proteins, 5 to 7%; Table 2). The −1 position (Leu or Tyr) did not influence the G_{α} protein activation level independently of the other amino acid positions. Thus, four different classes based on the (−5)/(−3) amino acid positions in the mutant $G_{\alpha o}$ proteins could be differentiated according to the rank order of their clonidine-mediated [^{35}S]GTPγS binding response: $G_{\alpha o}$/**Q**(I/Y)**G**L(L/Y) > $G_{\alpha o}$/**G**(I/Y)**G**L(L/Y) ≫ $G_{\alpha o}$/**Q**(I/Y)**E**L(L/Y) > $G_{\alpha o}$/**G**(I/Y)**E**L(L/Y), as depicted in Table 2 (in bold the −5 Gly/Gln and the −3 Gly/Glu positions).

Analysis of Agonist-Occupied α_{2A}-AR-Mediated Maximal [^{35}S]GTPγS Binding Capacity to Mutant $G_{\alpha o}$ Proteins. To further analyze the influence of the −5/−3 amino acid composition of the $G_{\alpha o}$ protein on ligand-dependent α_{2A}-AR activation, mutant $G_{\alpha o}$/GYELL, $G_{\alpha o}$/QYGLL, $G_{\alpha o}$/GYGLL, $G_{\alpha o}$/QYELL (= $G_{\alpha o/s}$), and $G_{\alpha o}$/GYGLY (= $G_{\alpha o}Cys^{351}Tyr$) proteins were selected to perform agonist-specific [^{35}S]GTPγS binding analyses. To exclude putative differences in functional responses due to variation in the expression of the mutant $G_{\alpha o}$ proteins, immunological detection indicated the expression level of the mutant $G_{\alpha o}$ proteins varied between 53 and 163% compared with that of the mutant $G_{\alpha o}Cys^{351}Tyr$ protein (Fig. 1). No relation between the mutant $G_{\alpha o}$ protein expression level and the clonidine-mediated maximal [^{35}S]GTPγS binding response was apparent. (−)-Epinephrine (10 μM)-mediated saturation [^{35}S]GTPγS binding indicated a single population of high-affinity [^{35}S]GTPγS binding sites for each of the investigated mutant $G_{\alpha o}$ proteins. The apparent dissociation constant of [^{35}S]GTPγS was not statistically different, with the exception of the $G_{\alpha o}$/GYELL protein, which yielded about a 5-fold increased K_D value (Table 3). The maximal (−)-epinephrine-mediated [^{35}S]GTPγS binding capacity varied between 3.97 and 11.37 pmol/mg of protein for these mutant $G_{\alpha o}$ proteins. Clonidine (10 μM) stimulated [^{35}S]GTPγS binding to the mutant $G_{\alpha o}$/GYGLY, $G_{\alpha o}$/QYGLL, and $G_{\alpha o}$/GYGLL proteins to the same extent as (−)-epinephrine, but the mutant $G_{\alpha o}$/QYELL and $G_{\alpha o}$/GYELL proteins were only weakly stimulated, and consequently saturation analysis was not performed. Dose-dependent [^{35}S]GTPγS binding response curves for (−)-epinephrine yielded a 24- and 54-fold decreased potency at the α_{2A}-AR in the co-presence of the mutant $G_{\alpha o}$/QYELL and $G_{\alpha o}$/GYELL proteins, respectively, compared with the $G_{\alpha o}Cys^{351}Tyr$ protein (Fig. 2). Clonidine potently (EC_{50}, 13.0 to 32.0 nM) stimulated [^{35}S]GTPγS binding responses at the mutant $G_{\alpha o}$ proteins carrying a −3 glycine residue, but it acted as a competitive antagonist of the (−)-epinephrine-mediated [^{35}S]GTPγS binding response at the α_{2A}-AR in the co-presence of those mutant $G_{\alpha o}$ proteins with a −3 Glu residue (Fig. 2). To evaluate the influence of putative spare α_{2A}-ARs on mutant $G_{\alpha o}$/QYGLL and $G_{\alpha o}$/GYELL protein activation, [^{35}S]GTPγS binding responses were monitored in the presence of decreasing amounts of α_{2A}-ARs (21.1 to 0.29 pmol/mg of protein; Table 4). Decreasing the α_{2A}-AR expression by about 50-times yielded only a slight decrease (2- to 3-fold) in potency for both (−)-epinephrine and clonidine at the mutant $G_{\alpha o}$/QYGLL protein, without altering the maximal response of clonidine (Table 4). The degree of basal [^{35}S]GTPγS binding was not affected by the expression level of α_{2A}-AR (Table 4).

Saturation Radioligand Binding Responses at α_{2A}-AR in the Co-Presence of Mutant $G_{\alpha o}$ Proteins. Saturation binding experiments using an α_2 AR antagonist [^{3}H]RX 821002, an efficacious α_2 AR agonist [^{3}H]UK 14304 (Jasper et al., 1998), and an α_2 AR partial agonist [^{3}H]clonidine (Jasper et al., 1998) were performed (Table 5) to assess their binding properties to the α_{2A}-AR in either the absence or in the co-presence of the mutant $G_{\alpha o}$/GYGLY, $G_{\alpha o}$/QYELL, $G_{\alpha o}$/GYELL, $G_{\alpha o}$/QYGLL, and $G_{\alpha o}$/GYGLL proteins. The equilibrium dissociation constant and maximal binding capacity of [^{3}H]RX 821002 at the α_{2A}-AR were not statistically different with each of the co-expressed mutant $G_{\alpha o}$ proteins, although a slightly higher amount of α_{2A}-AR binding sites in the co-presence of the mutant $G_{\alpha o}$/QYELL protein was observed (Table 5). In contrast, the K_D values for the labeled agonists were highly dependent on the co-expressed mutant $G_{\alpha o}$ protein; a 12- to 39-fold and a 9- to 33-fold increased ($P < 0.05$) dissociation constant value for [^{3}H]clonidine and [^{3}H]UK 14304, respectively, was observed for the α_{2A}-AR in the presence of either a $G_{\alpha o}$/QYELL or $G_{\alpha o}$/GYELL protein compared with the mutant $G_{\alpha o}$ proteins carrying a glycine as the −3 amino acid residue. The maximal radioligand binding capacity at the α_{2A}-AR sites was

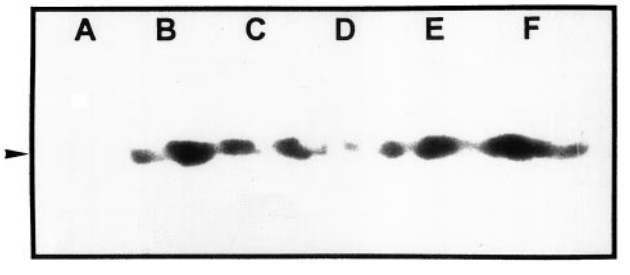

Fig. 1. Immunological detection of mutant $G_{\alpha o}$ protein expression in COS-7 cells in the co-presence of α_{2A}-AR. One hundred micrograms of total cellular membrane proteins of COS-7 cells co-expressing α_{2A}-AR and empty plasmid (A), mutant $G_{\alpha o}Cys^{351}Tyr$ (B), $G_{\alpha o}$/QYELL (C), $G_{\alpha o}$/GYELL (D), $G_{\alpha o}$/QYGLL (E), and $G_{\alpha o}$/GYGLL (F) proteins were separated by 12.5% SDS- polyacrylamide gel electrophoresis, blotted onto a nylon membrane, and the immunodetection was performed, as described under *Experimental Procedures*, using a selective anti-$G_{\alpha o}$ antibody. The arrow indicates a signal corresponding to the mutant $G_{\alpha o}$ proteins. Quantification (percentage versus mutant $G_{\alpha o}Cys^{351}Tyr$ protein) of the immunodetected signal was 78, 53, 92, and 167 for lane C to F, respectively.

either slightly increased ($P < 0.05$, [^{3}H]clonidine) or unaffected ($P > 0.05$, [^{3}H]UK 14304) by the presence of the mutant $G_{\alpha o}$ proteins but was lower for both radiolabeled agonists compared with [^{3}H]RX 821002. The absence of a difference in maximal [^{3}H]UK 14304 binding sites for the $G_{\alpha o}$/QYELL and $G_{\alpha o}$/GYELL proteins compared with the other mutant $G_{\alpha o}$ proteins containing a −3 Gly residue suggests that both of them can also exist in an α_{2A}-AR-coupled state (Table 5). In the absence of recombinant G_α proteins, the binding parameters of both [^{3}H]clonidine and [^{3}H]UK 14304 were close to those observed for α_{2A}-ARs in the copresence of a mutant $G_{\alpha o}$/GYELL protein (Table 5).

Another set of experiments was performed to evaluate the influence of the −3 C-terminal residue in a different amino acid context. A mutant $G_{\alpha o}$ protein, which corresponds to the exchange of the six last amino acids of the $G_{\alpha 15}$ protein (Wilkie et al., 1991) into the $G_{\alpha o}$ protein ($G_{\alpha o}$/DEINLL = $G_{\alpha o/15}$), and the corresponding Asn to Gly mutation in its −3 position ($G_{\alpha o}$/DEIGLL) were constructed. [^{35}S]GTPγS binding response of the mutant $G_{\alpha o}$/DEIGLL protein resembled that of the $G_{\alpha o}$Cys^{351}Tyr protein; it was strongly activated to the same extent by clonidine (10 μM) and by (−)-epinephrine (10 μM) (Table 6). The maximal [^{35}S]GTPγS binding capacity of clonidine at the $G_{\alpha o}$/DEINLL protein decreased to an almost undetectable level (Table 6). Saturation binding experiments with α_{2A}-AR and $G_{\alpha o}$/DEINLL protein indicated a 47- and 51-fold ($P < 0.05$) decrease in affinity for the agonists [^{3}H]UK 14304 and [^{3}H]clonidine, respectively, without affecting the binding properties of [^{3}H]RX 821002 compared with the mutant $G_{\alpha o}$/DEIGLL protein (Table 6).

Discussion

This study demonstrates reciprocal interactions between a wt α_{2A}-AR and a $G_{\alpha o}$ protein mutated in its five carboxy-terminal amino acid residues. Analysis was conducted using saturation binding experiments of either a labeled, nonhydrolyzable analog of guanine nucleotides, [^{35}S]GTPγS, as well as labeled radioligands being either efficacious or partial agonists or an antagonist. Maximal agonist-mediated saturation [^{35}S]GTPγS binding responses for the various mutant $G_{\alpha o}$ proteins and its comparison with the maximal antagonist [^{3}H]RX 821002 binding capacity gives an appropriate approximation of the ratio between total α_{2A}-AR amount and activated $G_{\alpha o}$ protein capacity. Among the various mutant $G_{\alpha o}$ proteins, the most significant effect on the modulation of the magnitude of maximal agonist-mediated [^{35}S]GTPγS binding response was the exchange, at the carboxy-terminal end of the protein, of a −3 glutamate or asparagine residue, as derived from a $G_{\alpha s}$ or $G_{\alpha 15}$ protein, respectively, for a $G_{\alpha i/o}$ protein-derived glycine. When a glycine, this position, independent of the surrounding peptidic sequence corresponding either to that of a $G_{\alpha s}$ or a $G_{\alpha 15}$ protein, yielded an enhanced maximal response for the partial agonist clonidine. A single mutation at this critical position not only modulated the ligand-occupied α_{2A}-AR-mediated [^{35}S]GTPγS binding response but also reciprocally altered the agonist binding pocket at the α_{2A}-AR because agonist equilibrium dissociation constants were decreased. This also indicates that interaction of the −3 Gly containing mutant $G_{\alpha o}$ proteins stabilized an activated α_{2A}-AR conformational state, as suggested by the increased potency of (−)-epinephrine and the enhanced dissociation constants of the labeled agonists. α_{2A}-ARs may possess an enhanced affinity for the −3 Gly containing mutant $G_{\alpha o}$ proteins, as predicted by the extended ternary complex model (Lefkowitz et al., 1993). Remarkably, mutation of the −3 residue into a negatively charged Glu residue produced an effect that is opposite to that obtained at the GPCR third intracellular loop distal portion, where the mutation of a noncharged residue by either an acidic (i.e., mutant Ala^{293}Glu α_{1B} AR) or basic (i.e., mutant Thr^{373}Lys α_{2A}-AR) amino acid generated constitutive activation by constraining a G protein-coupled state of the receptor (Pauwels and Wurch, 1998). Thus, although the GPCR third intracellular loop distal portion has been postulated to interact with a G_α protein C-terminal end (Kostenis et al., 1997), the exact contribution of the −3 Gly versus Glu residue cannot be foreseen.

The systematic mutation of each of the last five C-terminal amino acids of the $G_{\alpha o}$ protein, either alone or in combination, emphasized a pivotal role of the −3 residue. It can be either a Gly for the $G_{\alpha o}$ protein studied here or for the closely related $G_{\alpha i1/2/3}$ and $G_{\alpha z}$ proteins, a charged Glu in the case of $G_{\alpha s}$, or a polar Asn for the $G_{\alpha q/11/15/16}$ proteins. The nature of this peculiar residue is such that it modulates on its own the

TABLE 3

Dissociation constants and B_{max} values for binding of [^{35}S]GTPγS to membrane preparations of COS-7 cells expressing α_{2A}-AR and various mutant $G_{\alpha o}$ proteins

Coexpression of α_{2A}-AR and respective mutant $G_{\alpha o}$ protein was performed, as described under *Experimental Procedures*. All conditions were treated with PTX (20 ng/ml). Saturation [^{35}S]GTPγS binding responses mediated by the α_{2A}-AR were performed as described under *Experimental Procedures*. Membranes were incubated with 0.5 nM [^{35}S]GTPγS, 30 μM GDP, and either without or with 0.1 to 300 nM unlabeled GTPγS. K_D (nM) and B_{max} (pmol/mg of protein) values were deduced from saturation analysis for specific (−)-epinephrine (10 μM) and/or clonidine (10 μM)-stimulated [^{35}S]GTPγS binding. Data represent mean values ± S.E.M. of four independent transfection experiments, each performed in duplicate. The bold amino acids correspond to those that are different between $G_{\alpha o/s}$ ($G_{\alpha o}$/QYELL) and the various mutant $G_{\alpha o}$ proteins. Statistical analysis was performed on K_D and B_{max} values between $G_{\alpha o}$/GYGLY and the other mutant $G_{\alpha o}$ proteins.

G_α Protein	[^{35}S]GTPγS Binding			
	(−)-Epinephrine		Clonidine	
	K_D	B_{max}	K_D	B_{max}
	nM	*pmol/mg protein*	*nM*	*pmol/mg protein*
$G_{\alpha o}$/**GYGLY***	4.44 ± 0.46	4.28 ± 0.84	7.33 ± 1.01	5.43 ± 1.11
$G_{\alpha o}$/QYELL**	5.44 ± 0.78	3.97 ± 0.57	weak stimulation (not determined)	
$G_{\alpha o}$/**G**YELL	23.93 ± 3.55[a]	11.37 ± 2.44[a]	weak stimulation (not determined)	
$G_{\alpha o}$/Q**YG**LL	3.36 ± 0.27	4.56 ± 0.27	4.76 ± 0.77[a]	6.17 ± 1.40
$G_{\alpha o}$/**GYG**LL	4.29 ± 0.29	5.66 ± 0.91	8.14 ± 0.51	7.24 ± 1.25

*, $G_{\alpha o}$Cys^{351}Tyr protein; **, $G_{\alpha o/s}$ protein.
[a] $P < 0.05$.

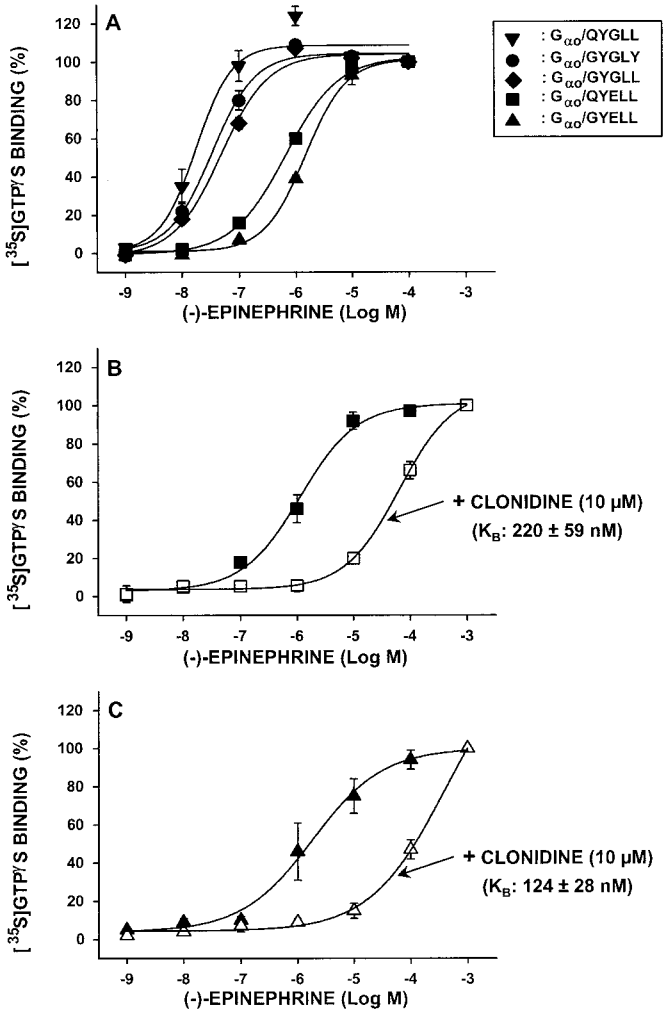

Fig. 2. (−)-Epinephrine dose-dependent [^{35}S]GTPγS binding response curves at α_{2A}-AR in the co-presence of various mutant $G_{\alpha o}$ proteins in COS-7 cells. Cultures were treated overnight with PTX (20 ng/ml) and assayed for [^{35}S]GTPγS binding, as described under *Experimental Procedures*. (−)-Epinephrine dose-dependent response curves (A) are shown for the various mutant $G_{\alpha o}$ proteins (EC_{50}, nM): $G_{\alpha o}$/GYGLY (37 ± 9.2; ●), $G_{\alpha o}$/QYELL (900 $_{\alpha o}$ ± 151; ■), $G_{\alpha o}$/GYELL (2000 ± 94; ▲), $G_{\alpha o}$/QYGLL (13.5 ± 2.5; ▼), $G_{\alpha o}$/GYGLL (40.0 ± 3.5; ♦). Antagonism of (−)-epinephrine response curves by clonidine is presented for the mutant $G_{\alpha o}$/QYELL (B) and $G_{\alpha o}$/GYELL proteins (C) in either the absence (closed symbols) or presence (open symbols) of clonidine (10 μM). Data are presented in percentage versus the maximal (−)-epinephrine-mediated [^{35}S]GTPγS binding response for each mutant $G_{\alpha o}$ protein. Concentration binding curves are constructed using mean values ± S.E.M. from four independent transfection experiments, each performed in duplicate.

activation level of the G_{α} protein, as mediated by efficacious and partial α_2 AR agonists, without modifying its basal activation level. Similarly, a chimeric $G_{\alpha o}$ protein exchanging its last six amino acids for those of a $G_{\alpha z}$ protein yielded an enhanced maximal [^{35}S]GTPγS binding response for the partial agonist *d*-medetomidine, whereas almost no stimulation of [^{35}S]GTPγS binding was obtained with a chimeric $G_{\alpha o/q}$ protein (Pauwels et al., 2001). Clonidine (10 μM)-occupied α_{2A}-ARs activated a number of high-affinity [^{35}S]GTPγS binding sites similar to that of the native α_2 AR agonist (−)-epinephrine in the co-presence of mutant $G_{\alpha o}$ proteins containing a −3 Gly residue (i.e., $G_{\alpha o}$Cys^{351}Tyr, $G_{\alpha o}$/QYGLL, and $G_{\alpha o}$/GYGLL proteins). Therefore, clonidine and (−)-epinephrine can be considered as agonists with a similar maximal response under these experimental conditions. On the other hand, when the α_{2A}-AR was expressed with mutant $G_{\alpha o}$ proteins containing a −3 Glu residue (i.e., $G_{\alpha o}$/QYELL and $G_{\alpha o}$/GYELL proteins), clonidine at saturating concentrations (10 μM) acted not only as a very weak agonist, but it also competitively antagonized the (−)-epinephrine-mediated [^{35}S]GTPγS binding response. Clonidine has been reported to display a comparable antagonist potency of the (−)-epinephrine-mediated [^{35}S]GTPγS binding response at α_{2A}-ARs stably expressed in HEK 293 cells (Jasper et al., 1998). This shows that, depending on the co-presence of a particular mutant $G_{\alpha o}$ protein, the clonidine-occupied α_{2A}-AR is able or not to activate the $G_{\alpha o}$ protein. In the absence of efficacious $G_{\alpha o}$ protein activation, clonidine can antagonize the functional response of (−)-epinephrine. Our data extend the implication of this residue, which has previously been involved in the selectivity of $G_{\alpha q}$ protein coupling to α_{2A}-ARs; a single mutation (Asn^{357}Gly) at the −3 position of the C-terminal portion of a $G_{\alpha q}$ protein renders it responsive to an agonist-activated α_{2A}-AR (Conklin et al., 1996). Similarly, the mutant $G_{\alpha q}$ Asn^{357}Gly protein efficiently coupled the $G_{i/o}$-coupled muscarinic m_2 receptor to the inositol phosphate pathway, without modification of the potency of the agonist carbachol (Liu et al., 1995; Kostenis et al., 1997).

The importance of the −3 residue has also been reported on a structural basis; NMR studies on an 11-amino-acid-long peptide corresponding to the C-terminal portion of the rod cell $G_{\alpha t}$ protein (the α subunit of transducin) suggests that its disordered conformation is shifted upon light activation of rhodopsin to a highly structured helical turn, followed by an open reverse turn centered at the −3 glycine residue (Kisselev et al., 1998). Fluorescence studies also revealed that the $G_{\alpha t}$ protein activation leads to a conformational

TABLE 4

Influence of the expression level of α_{2A}-AR on the [^{35}S]GTPγS binding response of mutant $G_{\alpha o}$/QYGLL and $G_{\alpha o}$/GYELL proteins

Co-transfection with 3 and 0.03 μg of α_{2A}-AR plasmid and 3 μg of indicated mutant $G_{\alpha o}$ protein plasmid was performed as described under *Experimental Procedures*. All conditions were treated with PTX (20 ng/ml). [^{3}H]RX 821002 (saturating concentration, 4.0 nM) and [^{35}S]GTPγS (0.5 nM) binding responses were performed as described under *Experimental Procedures*. Data represent mean values + S.E.M. of three independent transfection experiments, each performed in duplicate.

	[^{3}H]RX 821002 Binding	[^{35}S]GTPγS Binding				
		Basal	(−)-Epinephrine		Clonidine	
			EC_{50}	E_{max}	EC_{50}	E_{max}
	pmol/mg protein	*fmol/mg protein*	*nM*	*% above basal*	*nM*	*% vs. (−)-epinephrine*
$G_{\alpha o}$/QYGLL	21.11 ± 4.25	164 ± 25	15.23 ± 2.81	510 ± 35	8.05 ± 0.92	80 ± 4
	0.41 ± 0.08	117 ± 36	40.15 ± 6.26	253 ± 29	26.47 ± 3.15	76 ± 7
$G_{\alpha o}$/GYELL	14.69 ± 2.12	124 ± 48	1400 ± 210	180 ± 14	N.D.	2 ± 1
	0.29 ± 0.35	135 ± 29	2000 ± 340	107 ± 8	N.D.	3 ± 1

N.D., not determined.

change at its C-terminal portion, which may provide a structural basis for communication between a $G_{\alpha t}$ protein and light-activated rhodopsin (Yang et al., 1999). The formation of a highly structured motif at the C-terminal portion of the mutant $G_{\alpha o}$ proteins may favor specific interactions with the α_{2A}-AR in which conformation has been modified upon activation by an agonist. The presence of the −3 Gly residue is likely to be necessary for an optimal protein structure because 98% of the mutant $G_{\alpha o}$ proteins corresponds to the native $G_{\alpha o}$ protein. The flexibility of the C-terminal portion might be affected by the $Glu^{352}Gly$ mutation because of the loss of a negative charge, which may be stabilized by intramolecular interactions otherwise existing in the wt $G_{\alpha s}$ protein. A similar effect of the −3 Asn to Gly mutation in the chimeric $G_{\alpha o/15}$ protein and the loss of the noncharged polar moiety may suggest an unique role of the glycine residue by the absence of a side chain.

A second major observation in our study consists in the decrease of the equilibrium dissociation constant of the agonists [^{3}H]clonidine and [^{3}H]UK 14304, but not that of the antagonist [^{3}H]RX 821002, for binding to the α_{2A}-AR in the co-presence of −3 Glu-containing mutant $G_{\alpha o}$ proteins. These data may be interpreted in view of a conformational change of the α_{2A}-AR state, dependent on the co-expressed mutant $G_{\alpha o}$ protein. Although the antagonist recognizes both the G protein-coupled and -uncoupled states of the α_{2A}-AR (Kenakin, 1995), the dissociation constants of the agonists are modulated by the coupling efficiency of the mutant $G_{\alpha o}$ protein to the α_{2A}-AR. Mutant $G_{\alpha o}$ proteins containing a Glu residue as the −3 amino acid (i.e., $G_{\alpha o}$/QYELL, $G_{\alpha o}$/GYELL, and $G_{\alpha o}$/DEIGLL proteins) yielded a 10- to 50- fold decreased dissociation constant for the radiolabeled agonists at the α_{2A}-AR. These data suggest that the interaction between these mutant $G_{\alpha o}$ proteins, and the α_{2A}-AR induces tiny modifications in the binding site for the agonists UK 14304 and clonidine, whereas the interaction with the antagonist RX 821002 is unaffected. Both α_2 AR agonists contain a common imidazoline ring, which constitutes a binding domain to the α_{2A}-AR (Salminen et al., 1999) and may therefore explain why these two ligands, apart being agonists compared with the antagonist RX 821002, are similarly affected by the mutations in the $G_{\alpha o}$ protein. Thus, the data described

TABLE 5

K_D and B_{max} values for the binding of [^{3}H]RX 821002, [^{3}H]clonidine, and [^{3}H]UK 14304 to membrane preparations of COS-7 cells expressing the α_{2A}-AR in either the absence or presence of various mutant $G_{\alpha o}$ proteins

Co-expression of α_{2A}-AR and either empty plasmid or respective mutant $G_{\alpha o}$ protein was performed as described under *Experimental Procedures*. All conditions were treated with PTX (20 ng/ml). The equilibrium dissociation constant (K_D, nM) and maximal radioligand binding capacity (B_{max}, pmol/mg of protein) were determined for each condition, as described under *Experimental Procedures*, according to a monophasic Scatchard analysis. Data represent mean values ± S.E.M. of four independent transfection experiments, each performed in duplicate. The bold amino acids correspond to those that are different between $G_{\alpha o/s}$ ($G_{\alpha o}$/QYELL) and the various mutant $G_{\alpha o}$ proteins. Statistical analysis was performed on ligand's K_D and B_{max} values between $G_{\alpha o}$/GYGLY and the other mutant $G_{\alpha o}$ proteins or empty plasmid.

G_α Protein	[^{3}H]RX 821002		[^{3}H]Clonidine		[^{3}H]UK 14304	
	K_D	B_{max}	K_D	B_{max}	K_D	B_{max}
	nM	*pmol/mg protein*	*nM*	*pmol/mg protein*	*nM*	*pmol/mg protein*
None	0.92 ± 0.09	30.70 ± 5.45	37.91 ± 2.16[a]	5.04 ± 0.33[a]	14.81 ± 2.80[a]	4.12 ± 0.37[a]
$G_{\alpha o}$/**GYGLY***	0.92 ± 0.12	32.70 ± 6.90	1.41 ± 0.20	2.39 ± 0.38	0.33 ± 0.03	2.34 ± 0.30
$G_{\alpha o}$/QYELL**	1.14 ± 0.17	44.17 ± 14.1[a]	26.10 ± 3.83[a]	5.08 ± 1.34[a]	3.07 ± 0.76[a]	1.87 ± 0.47
$G_{\alpha o}$/**G**YELL	0.91 ± 0.10	31.97 ± 8.47	42.39 ± 2.92[a]	4.68 ± 0.72[a]	9.76 ± 2.88[a]	2.06 ± 0.69
$G_{\alpha o}$/QY**G**LL	0.91 ± 0.06	29.42 ± 5.12	1.08 ± 0.12	2.83 ± 0.43	0.30 ± 0.03	2.48 ± 0.30
$G_{\alpha o}$/**GYG**LL	0.88 ± 0.09	29.76 ± 4.66	2.15 ± 0.25	2.53 ± 0.39	0.38 ± 0.02	2.03 ± 0.21

*, $G_{\alpha o}Cys^{351}Tyr$ protein; **, $G_{\alpha o/s}$ protein.
[a] $P < 0.05$.

TABLE 6

K_D and B_{max} values for the binding of various radioligands to membrane preparations of COS-7 cells expressing the α_{2A}-AR and mutant $G_{\alpha o}$/DEINLL and $G_{\alpha o}$/DEIGLL proteins

Co-expression of α_{2A}-AR and mutant $G_{\alpha o}$ protein was performed as described under *Experimental Procedures*. All conditions were treated with PTX (20 ng/ml). Saturation [^{35}S]GTPγS binding responses mediated by the α_{2A}-AR were performed as described. Membranes were incubated with 0.5 nM [^{35}S]GTPγS, 30 μM GDP, and either without or with 0.1 to 300 nM unlabeled GTPγS. K_D (nM) and B_{max} (pmol/mg of protein) values were deduced from saturation analysis for specific (−)-epinephrine (10 μM) and/or clonidine (10 μM)-stimulated [^{35}S]GTPγS binding. The equilibrium dissociation constant (K_D, nM) and maximal radioligand binding capacity (B_{max}, pmol/mg of protein) were determined for each condition as described under *Experimental Procedures* according to a monophasic Scatchard analysis. Data represent mean values ± S.E.M. of four independent transfection experiments, each performed in duplicate. Statistical analysis was performed on ligand's K_D and B_{max} values between $G_{\alpha o}$/DEINLL and $G_{\alpha o}$/DEIGLL proteins.

G_α protein	[^{35}S]GTPγS Binding			
	(−)-Epinephrine		Clonidine	
	K_D	B_{max}	K_D	B_{max}
	nM	*pmol/mg protein*	*nM*	*pmol/mg protein*
$G_{\alpha o}$/DEINLL*	11.68 ± 1.56	5.80 ± 0.25	weak stimulation (not determined)	
$G_{\alpha o}$/DEIGLL	4.49 ± 1.02[a]	5.11 ± 0.79	6.59 ± 1.28	6.24 ± 1.04

	[^{3}H]RX 821002		[^{3}H]Clonidine		[^{3}H]UK 14304	
	K_D	B_{max}	K_D	B_{max}	K_D	B_{max}
	nM	*pmol/mg protein*	*nM*	*pmol/mg protein*	*nM*	*pmol/mg protein*
$G_{\alpha o}$/DEINLL*	0.85 ± 0.03	34.41 ± 6.71	64.26 ± 7.12	4.87 ± 1.10	11.24 ± 0.53	2.63 ± 0.47
$G_{\alpha o}$/DEIGLL	0.83 ± 0.05	32.77 ± 5.35	1.25 ± 0.06[a]	2.24 ± 0.04[a]	0.24 ± 0.04[a]	1.96 ± 0.30

* $G_{\alpha o/15}$ protein.
[a] $P < 0.05$.

here indicate that a single mutation in the $G_{\alpha o/s}$ protein carboxy-terminal portion increased the affinity of the chimeric G_{α} protein for the α_{2A}-AR. Several mutations within the receptor sequence have been described that are able to increase the basal G protein activation level because the tri-dimensional structure of the receptor was probably modified by the amino acid exchange; these mutant receptors display constitutive activity (Lefkowitz et al., 1993; Pauwels and Wurch, 1998). In the present study, a mutation in a G_{α} protein exhibits a retrograde modulatory effect on the ligand binding properties of α_2 AR agonists. Recently, Grishina and Berlot (2000) showed that a chimeric $G_{\alpha s/i2}$ protein switching their α_3/β_5 domains yielded an increased population of co-expressed β_2 AR in a high-affinity state (34%) compared with a wt $G_{\alpha s}$ protein (19%) and a concomitant increase in the isoproterenol high- and low-affinity dissociation constants. These results also suggest, for another G_{α} protein domain, a modulatory effect on GPCR/G_{α} protein interactions. In contrast, the affinity constant of the antagonist yohimbine or the agonists clonidine and (−)-epinephrine were either not decreased or maximally 2-fold decreased between the α_{2A}-AR:$G_{\alpha i1}Cys^{351}Gly$ and α_{2A}-AR:$G_{\alpha i1}Cys^{351}Ile$ fusion proteins (Jackson et al., 1999). The apparent absence of effect on agonist binding for the Cys to Gly and Ile mutations of the −4 C-terminal residue in the $G_{\alpha i1}$ portion of the fusion proteins may be due to the use of a labeled antagonist as a radioligand instead of an agonist. Other explanations may be: 1) the constrained interaction between the α_{2A}-AR and the mutant $G_{\alpha i1}Cys^{351}Gly/Ile$ proteins due to the fusion process, which may restrict the flexibility of the $G_{\alpha i1}$ protein partner, thereby masking effects that are uncovered by the co-expression experiments presented here, and 2) a weaker influence of the −4 C-terminal position of the $G_{\alpha i1}$ protein compared with the −3 Glu residue detailed here toward α_{2A}-AR states.

In conclusion, the present data highlight a critical role for the C-terminal portion of the $G_{\alpha o}$ protein in the modulation of α_{2A}-AR states and the particular involvement of the third amino acid away from the G_{α} protein C-terminal extremity to determine the transition from a partial to efficacious agonist or antagonist at the α_{2A}-AR. A retrograde modulatory effect of the $G_{\alpha o}$ protein on the α_{2A}-AR agonist binding site(s) is hypothesized, which probably involves transmission of the mutation-induced conformational change from the $G_{\alpha o}$ protein to the ligand-bound α_{2A}-AR.

Acknowledgments

We sincerely thank S. Tardif and C. Cathala for expert technical assistance and S. Brignatz for skillful secretarial work.

References

Airriess CN, Rudling JE, Midgley JM and Evans PD (1997) Selective inhibition of adenylyl cyclase by octopamine via a human cloned α_{2A}-adrenoceptor. *Br J Pharmacol* **122:**191–198.

Bockaert J and Pin J-P (1999) Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO (Eur Mol Biol Organ) J* **18:**1723–1729.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.

Brink CB, Wade SM and Neubig RR (2000) Agonist-directed trafficking of porcine α_{2A} adrenergic receptor signaling in Chinese hamster ovary cells: *l*-isoproterenol selectively activates Gs. *J Pharmacol Exp Ther* **294:**539–547.

Conklin BR, Farfel Z, Lustig KD, Julius D and Bourne HR (1993) Substitution of three amino acids switches receptor specificity of $G_{q\alpha}$ to that of $G_{i\alpha}$. *Nature (Lond)* **363:**274–276.

Conklin BR, Herzmark P, Ishida S, Voyno-Yasenetskaya TA, Sun Y, Farfel Z and Bourne HR (1996) Carboxyl-terminal mutations of $G_{q\alpha}$ and $G_{s\alpha}$ that alter the fidelity of receptor activation. *Mol Pharmacol* **50:**885–890.

Cotecchia S, Kobilka BK, Daniel KW, Nolan RD, Lapetina EY, Caron MG, Lefkowitz RJ and Regan JW (1990) Multiple second messenger pathways of α-adrenergic receptor subtypes expressed in eukaryotic cells. *J Biol Chem* **265:**63–69.

Dorn GW, Oswald KJ, Mc Cluskey TS, Kuhel DG and Liggett SB (1997) α_{2A}-adrenergic receptor stimulated calcium release is transduced by G_i-associated $G_{\beta\gamma}$-mediated activation of phospholipase C. *Biochemistry* **36:**6415–6423.

Eason MG, Jacinto MT and Liggett SB (1994) Contribution of ligand structure to activation of α_2-adrenergic receptor subtype coupling to G_s. *Mol Pharmacol* **45:** 696–702.

Eason MG, Kurose H, Holt BD, Raymond JR and Liggett SB (1992) Simultaneous coupling of α_2-adrenergic receptors to two G-proteins with opposing effects. *J Biol Chem* **267:**15795–15801.

Eason MG and Liggett SB (1996) Chimeric mutagenesis of putative G-protein coupling domains of the α_{2A}-adrenergic receptor. *J Biol Chem* **271:**12826–12832.

Fraser CM, Arakawa S, McCombie WR and Venter JC (1989) Cloning, sequence analysis, and permanent expression of a human α_2-adrenergic receptor in Chinese hamster ovary cells. Evidence for independent pathways of receptor coupling to adenylate cyclase attenuation and activation. *J Biol Chem* **264:** 11754–11761.

Grishina G and Berlot CH (2000) A surface-exposed region of $G_{s\alpha}$ in which substitutions decrease receptor-mediated activation and increase receptor affinity. *Mol Pharmacol* **57:**1081–1092.

Gudermann T, Schöneberg T and Schultz G (1997) Functional and structural complexity of signal transduction via G-protein-coupled receptors. *Annu Rev Neurosci* **20:**399–427.

Hamm HE, Deretic D, Arendt A, Hargrave PA, Koenig B and Hofmann KP (1988) Site of G protein binding to rhodopsin mapped with synthetic peptides from the alpha subunit. *Science (Wash DC)* **241:**832–835.

Jackson NV, Bahia DS and Milligan G (1999) Modulation of relative intrinsic activity of agonists at the alpha-2A adrenoceptor by mutation of residue 351 of G protein $G_{i1\alpha}$. *Mol Pharmacol* **55:**195–201.

Jasper JR, Lesnick JD, Chang LK, Yamanishi SS, Chang TK, Hsu SAO, Daunt DA, Bonhaus DW and Eglen RM (1998) Ligand efficacy and potency at recombinant α_2 adrenergic receptor. *Biochem Pharmacol* **55:**1035–1043.

Kenakin T (1995) Agonist-receptor efficacy II: agonist trafficking of receptor signals. *Trends Pharmacol Sci* **16:**232–238.

Kisselev OG, Kao J, Ponder JW, Fann YC, Gautam N and Marshall GR (1998) Light-activated rhodopsin induces structural binding motif in G protein α subunit. *Proc Natl Acad Sci USA* **95:**4270–4275.

Kostenis E, Gomeza J, Lerche C and Wess J (1997) Genetic analysis of receptor-$G_{\alpha q}$ coupling selectivity. *J Biol Chem* **272:**23675–23681.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond)* **227:**680–685.

Lefkowitz RJ, Cotecchia S, Samama P and Costa T (1993) Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol Sci* **14:**303–307.

Liu J, Conklin BR, Blin N, Yun J and Wess J (1995) Identification of a receptor/G-protein contact site critical for signaling specificity and G-protein activation. *Proc Natl Acad Sci USA* **92:**11642–11646.

Mazzoni MR, Malinski JA and Hamm HE (1991) Structural analysis of rod GTP-binding protein, G_t. Limited proteolytic digestion pattern of Gt with four proteases defines monoclonal antibody epitope. *J Biol Chem* **266:**14072–14081.

Milligan G and Rees S (1999) Chimaeric G_{α} proteins: their potential use in drug discovery. *Trends Pharmacol Sci* **20:**118–124.

Pauwels PJ, Palmier C, Wurch T and Colpaert FC (1996) Pharmacology of cloned human 5-HT_{1D} receptor-mediated functional responses in stably transfected rat C6-glial cell lines: further evidence of differentiating human 5-HT_{1D} and 5-HT_{1B} receptors. *Naunyn-Schmiedeberg's Arch Pharmacol* **353:**144–156.

Pauwels PJ, Tardif S, Colpaert FC and Wurch T (2001) Modulation of ligand responses by coupling of α_{2A}-adrenoceptors to diverse G_{α} proteins. *Biochem Pharmacol* **61:**1079–1092.

Pauwels PJ and Wurch T (1998) Amino acid domains involved in constitutive activation of G-protein-coupled receptors. *Mol Neurobiol* **17:**109–136.

Rovati GE, Rodbard D and Munson PJ (1989) Optimization of experimental design for ligand binding studies: improved estimation of affinity and binding capacity. *Pharmacol Res* **21:**71–72.

Salminen T, Varis M, Nyrönen T, Pihlavisto M, Hoffren A-M, Lönnberg T, Marjamäki A, Frang H, Savola J-M, Scheinin M and Johnson MS (1999) Three-dimensional models of α_{2A}-adrenergic receptor complexes provide a structural explanation for ligand binding. *J Biol Chem* **274:**23405–23413.

Wilkie TM, Scherle PA, Strathmann MP, Slepak VZ and Simon MI (1991) Characterization of G-protein alpha subunits in the G_q class: expression in murine tissues and in stromal and hematopoietic cell lines. *Proc Natl Acad Sci USA* **88:**10049–10053.

Wurch T, Colpaert FC and Pauwels PJ (1999) G-Protein activation by putative antagonists at mutant $Thr^{373}Lys$ α_{2A} adrenergic receptors. *Br J Pharmacol* **126:** 939–948.

Yang C-S, Skiba NP, Mazzoni MR and Hamm HE (1999) Conformational changes at the carboxyl terminus of G_{α} occur during G-protein activation. *J Biol Chem* **274:**2379–2385.

Address correspondence to: Dr. Petrus J. Pauwels, Department of Cellular and Molecular Biology, Centre de Recherché Pierre Fabre, 17 Avenue Jean Moulin, 81106 Castres Cedex, France. E-mail: peter.pauwels@pierre-fabre.com