Biochemical Pharmacology, Vol. 59, pp. 1531–1538, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00276-8

α_{2A}-Adrenoceptor: $G_{\alpha i1}$ Protein-Mediated Pertussis Toxin-Resistant Attenuation of G_s Coupling to the Cyclic AMP Pathway

Isabelle Rauly, Marie-Christine Ailhaud, Thierry Wurch and Petrus J. Pauwels*

Department of Cellular and Molecular Biology, Centre de Recherche Pierre Fabre, 81106 Castres cédex 06, France

ABSTRACT. Fusion proteins were constructed between a recombinant human α_{2A}-adrenoceptor and either a rat wild-type $G_{\alpha i1}$ or putative pertussis toxin-resistant form of the $G_{\alpha i1}$ protein ($G_{\alpha i1}Cys^{351}Gly$). [^{3}H]2-[2-(2-Methoxy-1,4-benzodioxanyl)]imidazoline hydrochloride (RX 821002) saturation binding experiments demonstrated that both fusion proteins were expressed at a similar level as the α_{2A}-adrenoceptor co-expressed with either a wild-type $G_{\alpha i1}$ or mutant $G_{\alpha i1}Cys^{351}Gly$ protein in COS-7 cells, and displayed a ligand binding profile similar to that for the α_{2A}-adrenoceptor protein. In α_{2A}-adrenoceptor-transfected COS-7 cells, 5-bromo-6-(2-imidazolin-2-yl-amino) quinoxaline tartrate (brimonidine, 10 μM) induced stimulation (151 ± 28%) of adenosine 3′,5′-cyclic monophosphate (cAMP) formation which was prevented by cholera toxin treatment, demonstrating a direct coupling of the α_{2A}-adrenoceptor to an endogenous $G_{\alpha s}$ protein in COS-7 cells. Expression of either the wild-type $G_{\alpha i1}$ or mutant $G_{\alpha i1}Cys^{351}Gly$ protein in co-expression or fusion with the α_{2A}-adrenoceptor in COS-7 cells suppressed the brimonidine-induced stimulation of cAMP formation, both in the presence and absence of pertussis toxin pretreatment. Hence, the $G_{\alpha i1}$ protein apparently blocks the G_s-coupled α_{2A}-adrenoceptor-mediated pathway in a pertussis toxin-non-sensitive way. BIOCHEM PHARMACOL **59**;12:1531–1538, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. recombinant human α_{2A}-adrenoceptor; rat $G_{\alpha i1}$ protein; fusion protein; cAMP formation; COS-7 cells

α_2-Adrenoceptors act mainly by inhibiting neuronal firing and release of catecholamines and other neurotransmitters in the central nervous system, but they are also involved in a wide range of functions in peripheral tissues, including aggregation of platelets, release of insulin from the pancreas, and inhibition of lipolysis [1, 2]. Pharmacologically, α_2-adrenoceptors can be subdivided into three defined subtypes, α_{2A}, α_{2B}, and α_{2C}, corresponding to three distinct genes, $\alpha_{2C10/A}$, $\alpha_{2C2/B}$, and $\alpha_{2C4/C}$, that have been isolated in man and characterized in heterologous expression systems [3]. α_2-Adrenoceptors, via interaction with the pertussis toxin-sensitive G_i/G_0 subclass of G proteins, modulate multiple effector systems, including suppression of cAMP† accumulation, inhibition of voltage-activated Ca^{2+} current, and activation of K^+ channels [4]. Eason *et al.* [5] and Federman *et al.* [6] have demonstrated paradoxical α_2-adrenoceptor-mediated increases in cAMP level via coupling to a G_s protein. The degree of G_s protein coupling initially depends on the agonist's structural features, and ligands that act as full agonists for G_i coupling are not necessarily full agonists for G_s coupling [7]. α_2-Adrenoceptors may also couple to other intracellular pathways involving Na^+/H^+ exchange and phospholiphase A_2 and phospholipase C activation [8, 9].

Recently, receptor: G_α subunit fusion proteins have been developed as a new strategy to examine the intrinsic activity of ligands at receptors covalently coupled to a specific G_α protein subunit [10] in which the stoichiometry of the receptor to G_α protein is fixed at 1 to 1. Bertin *et al.* [11] linked the β_2-adrenoceptor to a $G_{\alpha S}$ subunit; the agonist-dependent activation of the adenylyl cyclase was more potent and more efficacious in transfected than in control mouse lymphoma S49 cells. Wise *et al.* [12] reported on a fusion protein between a porcine α_{2A}-adrenoceptor and a pertussis toxin-resistant rat $G_{\alpha il}Cys^{351}Gly$ protein to study agonist efficacy by measuring either high-affinity GTPase activity or [^{35}S]GTPγS binding responses. Wise *et al.* [13] also reported the capacity of the α_{2A}-adrenoceptor: $G_{\alpha i1}Cys^{351}Gly$ fusion protein to interact with a βγ complex, which increased agonist-mediated GTPase activity. In experiments performed by co-expression of the porcine α_{2A}-adrenoceptor and a pertussis toxin-experiments resistant $G_{\alpha i}Cys^{351}Gly$ mutant, Wise *et al.* [14] showed that functional interaction of the α_{2A}-adrenoceptor with the wt $G_{\alpha il}$ protein was greater than with the pertussis toxin-resistant mutant $G_{\alpha il}Cys^{351}Gly$ proteins. However, functional evidence that α_{2A}-adrenoceptor fusion proteins in-

* Corresponding author: Dr. Isabelle Rauly, Centre de Recherche Pierre Fabre, Department of Cellular and Molecular Biology, 17 avenue Jean Moulin, 81106 Castres cédex 06, France. Tel. (33) 5.63.71.43.87/42.65; FAX (33) 5.63.71.43.63; E-mail: isabelle.rauly@pierre-fabre.com

Received 14 June 1999; accepted 18 November 1999.

hibit cAMP formation has not been clearly demonstrated [15]. The situation is rendered even more complex by the fact that agonist occupation of the α_{2A}-adrenoceptor: $G_{\alpha i1}$ fusion protein results in activation of both receptor-fused and endogenous G_α proteins [15].

The present study was undertaken to measure the cAMP* responses of the fusion protein made up of a recombinant human α_{2A}-adrenoceptor and a rat $G_{\alpha i1}$ protein in order to demonstrate its utility in monitoring at the effector level, as has been shown for the β_2-adrenoceptor [11, 16]. cAMP formation was compared in COS-7 cells expressing the fusion protein made by combining the α_{2A}-adrenoceptor with either the wt $G_{\alpha i1}Cys^{351}$ protein or a putative pertussis toxin-resistant rat $G_{\alpha i1}Cys^{351}Gly$ protein, and upon co-expression of the α_{2A}-adrenoceptor and each of the $G_{\alpha i1}$ proteins. The receptor expression level of the various constructs was estimated by [^{3}H]RX 821002 binding. Since α_{2A}-adrenoceptor-mediated agonist responses appear to depend on the agonist's structural features [7], three structurally different agonists were selected: the catecholamine (−)-adrenaline, the imidazoline brimonidine (UK 14304), and the azepine talipexole (BHT 920). This study demonstrates that α_{2A}-adrenoceptor: $G_{\alpha i1}$ fusion proteins are correctly expressed, although they attenuate endogenous G_s-coupled cAMP formation in a pertussis toxin-non-sensitive way.

MATERIALS AND METHODS

Materials

The ABI PRISM 310 Genetic Analyzer and the dichloro-rhodamine terminator cycle sequencing kit were from Perkin Elmer. The pCR3.1 vector was from Invitrogen. The Geneclean II kit was purchased from Bio 101 Inc. The Expand Long Template PCR kit was from Boehringer Mannheim. COS-7 cells were obtained from ATCC. All materials for cell culture were supplied by Life Technologies. The cAMP radioimmunoassay kit was purchased from Immunotech. [^{3}H]2-[2-(2-Methoxy-1,4-benzodioxanyl)] imidazoline hydrochloride ([^{3}H]RX 821002) (56.5 Ci/mmol) was from Amersham. 6-Allyl-2-amino-5, 6, 7, 8-tetrahydro-4*H*-thiazolo[4, 5-*d*]azepine dihydrochloride (BHT 920, talipexole) was a gift from Boehringer Ingelheim. 5-Bromo-6-(2-imidazolin-2-yl-amino)quinoxaline tartrate (UK 14304, brimonidine) and (+)-(8a*R*, 12a*S*, 13a*S*)-3-methoxy-12-(methylsulphonyl)-5, 8, 8a, 9, 10, 11, 12, 12a, 13, 13a-decahydro-6*H*-isoquino[2′, 1-*g*][1, 6]naphthyridine (RS 15385) were prepared intra-muros. All other chemicals were from Sigma.

* *Abbreviations:* cAMP, cyclic AMP, adenosine 3′5′-cyclic monophosphate; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; and wt, wild-type.

Cloning of α_{2A}-Adrenoceptor, $G_{\alpha i1}$ Protein, and G_{α_s} Protein Genes

The wt rat G protein α subunit i1 gene ($G_{\alpha i1}$), the wt rat G protein α subunit s gene ($G_{\alpha s}$), and the human α_{2A}-adrenoceptor (RC: 2.1.ADR.A2A) gene were cloned by PCR as previously described [17]. The mutant rat $G_{\alpha i1}Cys^{351}Gly$ protein was amplified in a similar way, except that the reverse primer carried the mutation TGT instead of GGT. The PCR mixture (50 μL) consisted of 250 ng of reverse-transcribed poly(A^+) RNA from rat 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 [16 mM $(NH_4)_2SO_4$, 1.75 mM $MgCl_2$, 50 mM Tris–HCl (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 vector. Sequencing, performed automatically on an ABI PRISM 310 Genetic Analyzer using a dichloro-rhodamine terminator cycle sequencing kit, confirmed the corresponding sequences.

Construction of α_{2A}-Adrenoceptor Fusion Proteins

The fusion proteins between the human α_{2A}-adrenoceptor and either the rat $G_{\alpha i1}$ protein or mutant $G_{\alpha i1}Cys^{351}Gly$ protein were constructed by a modified PCR-based overlap extension technique [18]. The α_{2A}-adrenoceptor gene was PCR-amplified by adding to its 3′ end a 12-base pair sequence (underlined) corresponding to the 5′ end of the $G_{\alpha i1}$ protein gene, thereby mutating its stop codon into a glycine (GGA) codon (<u>TCTGCAGCCCAT</u>TCCCAC GAT-CCGCTTCCTGTCCCC). Similarly, the wt or mutant $G_{\alpha i1}$ protein genes were PCR-amplified separately by adding to their 5′ end a 12-base pair sequence (underlined) corresponding to the 3′ end of the α_{2A}-adrenoceptor gene (<u>CGGATCGTGGGA</u>ATGGGCTGCA-CACTGAGCG CTGAG). The α_{2A}-adrenoceptor gene and the respective G protein gene were subsequently fused in a second PCR step, and the fusion product was amplified in a third PCR reaction as described [18]. Sequencing confirmed the sequence of the respective fusion proteins.

Cell Transfection

For transient transfection, 10 μg of either α_{2A}-adrenoceptor plasmid or fusion protein plasmid or the same amount of α_{2A}-adrenoceptor plasmid with either 10 μg of wt $G_{\alpha i1}$ protein or $G_{\alpha i1}Cys^{351}Gly$ protein plasmids, with or without 10 μg of wt $G_{\alpha s}$ protein, were transfected with 1×10^7 exponentially growing COS-7 cells using a Bio-Rad electroporator (250 mV, 250 μF) in 200 μL PBS supplemented with 1% DMSO. The total amount of plasmid in the transfection experiments was kept constant by adding

TABLE 1. K_d and B_{max} values for [^{3}H]RX 821002 binding to COS-7 cells transfected with α_{2A}-adrenoceptor and $G_{\alpha i1}$ proteins

	K_d (nM)	B_{max} (pmol/mg protein)
α_{2A}-Adrenoceptor	0.95 ± 0.10	7.19 ± 2.16
α_{2A}-Adrenoceptor + $G_{\alpha i1}$ protein	1.07 ± 0.20	7.52 ± 1.58
α_{2A}-Adrenoceptor + $G_{\alpha i1}Cys^{351}Gly$ protein	0.99 ± 0.16	16.54 ± 5.18
α_{2A}-Adrenoceptor: $G_{\alpha i1}$ fusion protein	0.90 ± 0.28	3.26 ± 2.20
α_{2A}-Adrenoceptor: $G_{\alpha i1}Cys^{351}Gly$ fusion protein	0.91 ± 0.38	2.73 ± 0.86

Saturation binding experiments were performed as described in Methods. α_{2A}-Adrenoceptor + $G_{\alpha i1}$ protein or α_{2A}-adrenoceptor + $G_{\alpha i1}Cys^{351}Gly$ protein corresponds to the co-transfection experiments. Values represent the means ± SD of two independent experiments, each one performed in duplicate. The corresponding Hill coefficients were between 0.940 ± 0.014 and 0.970 ± 0.014.

carrier pCR3.1 vector plasmid. The efficiency for the electroporation procedure was about 10%. Cultures were grown in 24-well tissue culture plates in 1 mL complete DMEM supplemented with 10% heat-inactivated foetal bovine serum and 1% DMSO for 72 hr. Pretreatment of cells with either pertussis toxin (100 ng/mL) or cholera toxin (100 ng/mL) was performed 16 hr before the cAMP experiments. Radioligand binding experiments were performed on membrane preparations of transfected cells grown for 72 hr in complete growth medium.

Radioligand Binding Assay to α_{2A}-Adrenoceptor

Membrane preparations of COS-7 cells were done in 50 mM Tris–HCl pH 7.6 as described [19]. Binding assays were performed with 2 nM [^{3}H]RX 821002. Incubation mixtures consisted of 0.4 mL cell membranes (5 μg protein), 0.05 mL radioligand, and 0.05 mL compound for inhibition or 10 μM phentolamine to determine nonspecific binding. The reactions were stopped after a 30-min incubation at 25° by adding 3 mL ice-cold 50 mM Tris–HCl pH 7.6 followed by rapid filtration over Whatman GF/B glass fiber filters using a Brandel harvester, after which the filters were washed and counted as described [19]. Data were analyzed graphically with inhibition curves, and IC_{50} values (concentrations of the compounds producing 50% inhibition of specific binding) were derived and converted to pK_i values (pK_i = −log K_i; K_i = IC_{50}/[1 + (concentration/K_d)]. Saturation binding experiments were performed with 0.15 to 20 nM of [^{3}H]RX 821002 as described [19].

Western Blotting

Membrane preparations of COS-7 cells containing the α_{2A}-adrenoceptor co-expressed with either the rat $G_{\alpha i1}$ protein or rat $G_{\alpha i1}Cys^{351}Gly$ protein were done as described above. Total proteins were separated by SDS/PAGE (12.5% (w/v) gel), as described [20]. Thereafter, the proteins were blotted on a nylon membrane by semi-dry electrotransfer (23 V, 45 min) in 190 mM glycine/20% (v/v) methanol/25 mM Tris–HCl buffer (pH 8.3). Proteins were probed with a polyclonal antibody raised against a peptide corresponding to residues 159–168 of the rat $G_{\alpha i1}$ protein. The incubation was performed in PBS containing 0.1% (w/v) Tween 20, 5% (w/v) BSA, and the antibody at a dilution of 1:2000. Proteins were detected with an anti-rabbit immunoglobulin G antibody coupled to alkaline phosphatase by using a colorimetric reaction [0.12 mM 5-bromo-4-chloroindol-3-yl phosphate *p*-toluidine salt/5 mM $MgCl_2$ in 100 mM diethanolamine (pH 9.6)].

[^{35}S] GTPγS Binding Responses

Basal and agonist-dependent [^{35}S]GTPγS binding [21] to the above-mentioned membrane preparation was performed in 20 mM HEPES (pH 7.4) supplemented with 30

TABLE 2. pK_i and Hill coefficient values of α_{2A}-adrenergic agonists for COS-7 cells transfected with α_{2A}-adrenoceptor and $G_{\alpha i1}$ proteins

	(−)-Adrenaline		Talipexole		Brimonidine	
	pK_i	nHill	pK_i	nHill	pK_i	nHIll
α_{2A}-Adrenoceptor	6.01 ± 0.00	0.950 ± 0.014	6.76 ± 0.24	0.990 ± 0.000	7.15 ± 0.02	0.955 ± 0.007
α_{2A}-Adrenoceptor + $G_{\alpha i1}$ protein	5.98 ± 0.06	0.910 ± 0.071	6.70 ± 0.22	0.960 ± 0.028	7.04 ± 0.14	0.980 ± 0.014
α_{2A}-Adrenoceptor + $G_{\alpha i1}Cys^{351}Gly$ protein	6.05 ± 0.00	0.910 ± 0.085	6.71 ± 0.02	0.990 ± 0.000	7.05 ± 0.00	0.985 ± 0.021
α_{2A}-Adrenoceptor: $G_{\alpha i1}$ fusion protein	6.24 ± 0.18	0.975 ± 0.021	6.90 ± 0.20	0.970 ± 0.014	7.53 ± 0.20	0.975 ± 0.007
α_{2A}-Adrenoceptor: $G_{\alpha i1}Cys^{351}Gly$ fusion protein	6.45 ± 0.06	0.860 ± 0.127	6.92 ± 0.04	0.945 ± 0.007	7.51 ± 0.24	0.955 ± 0.007

Inhibition of [^{3}H]RX 821002 binding was performed as described in Methods. α_{2A}-Adrenoceptor + $G_{\alpha i1}$ protein or α_{2A}-Adrenoceptor + $G_{\alpha i1}Cys^{351}Gly$ protein corresponds to the co-transfection experiments. Values represent the means ± SD of two independent experiments, each one performed in duplicate.

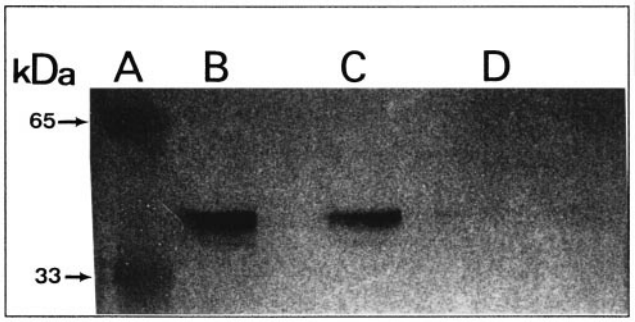

FIG. 1. Western blot of $G_{\alpha i1}$ protein expression in COS-7 cell expressing α_{2A}-adrenoceptor with either wt $G_{\alpha i1}$ or $G_{\alpha i1}Cys^{351}Gly$ protein. Total membrane proteins (80 μg) of COS-7 cells transfected with either 10 μg of α_{2A}-adrenoceptor with 10 μg of $G_{\alpha i1}Cys^{351}Gly$ protein (B), 10 μg of α_{2A}-adrenoceptor with 10 μg of wt $G_{\alpha i1}$ protein (C), or 10 μg of α_{2A}-adrenoceptor with 10 μg pCR3.1 plasmid vector (D) were analyzed by SDS/PAGE. Immunodetection was performed as described in Methods with an antibody raised against a peptide corresponding to residues 159–168 of the rat $G_{\alpha i1}$ protein. Protein molecular-mass markers are indicated in the left margin (A).

μM GDP, 100 mM NaCl, 3 mM $MgCl_2$, and 0.2 mM ascorbic acid. Maximal stimulation of [^{35}S]GTPγS binding was defined in the presence of 10 μM brimonidine and calculated versus basal [^{35}S]GTPγS binding.

cAMP Formation

Cellular cAMP formation was measured as described [19]. Cultures were incubated for 5 min at 37° with 1 mL of controlled salt solution (CSS: 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 5 mM glucose, 25 mM Tris–HCl pH 7.4) containing 1 mM isobutylmethylxanthine, with or without 10 μM forskolin and with 10 μM of agonist and/or antagonist. The reaction was stopped by the addition of 0.1 mL $HC1O_4$ to a final concentration of 0.04 M and afterwards neutralized. The cellular cAMP content was assayed using a radioimmunoassay kit. Results were expressed in percentage relative to basal cAMP values obtained in the absence of agonist.

Statistical Analysis

Statistical differences between the amount of cAMP formation were determined using a Student's *t*-test.

RESULTS

Binding Properties of α_{2A}-Adrenoceptors in the Presence of $G_{\alpha i1}$ Proteins

In a first series of experiments, the expression level of the α_{2A}-adrenoceptor: $G_{\alpha i1}$ and α_{2A}-adrenoceptor: $G_{\alpha i1}Cys^{351}$ Gly fusion proteins was compared to that of the α_{2A}-adrenoceptor protein co-transfected with either control plasmid, $G_{\alpha i1}$ protein, or $G_{\alpha i1}Cys^{351}Gly$ protein. Saturation binding experiments with [^{3}H]RX 821002 on membrane preparations were compatible with the presence of a single nanomolar affinity binding site for [^{3}H]RX 821002 (Table 1). The maximal binding capacity for [^{3}H]RX 821002 was similar for the α_{2A}-adrenoceptor protein and the α_{2A}-adrenoceptor co-transfected with the $G_{\alpha i1}$ protein. Two-fold variations in binding site density were apparent with the fusion proteins. Binding affinities for the agonists brimonidine, talipexole, and (−)-adrenaline were not modified in any of the constructions compared to the values obtained with the α_{2A}-adrenoceptor protein (Table 2).

Expression Level of Endogenous and Recombinant $G_{\alpha i1}$ Proteins and Their Coupling to α_{2A}-Adrenoceptors

The expression of endogenous and co-expressed $G_{\alpha i1}$ proteins was analyzed by Western blotting. No $G_{\alpha i1}$ protein could be detected upon expression of the α_{2A}-adrenoceptor in COS-7 cells (Fig. 1). On the other hand, a strong and equivalent expression of $G_{\alpha i1}$ protein was detected upon co-transfection of the α_{2A}-adrenoceptor with the $G_{\alpha i1}$ or the $G_{\alpha i1}Cys^{351}Gly$ protein (Fig. 1). [^{35}S]GTPγS binding experiments were performed to estimate α_{2A}-adrenoceptor coupling to the endogenous and co-expressed $G_{\alpha i1}$ proteins. A stimulation of [^{35}S]GTPγS binding of about 47 ± 12% was observed with brimonidine (10 μM) in the absence of recombinant $G_{\alpha i1}$ protein (Table 3). This effect is likely the result of α_{2A}-adrenoceptor coupling to endogenous G_i/G_0 proteins. An enhanced [^{35}S]GTPγS binding was observed upon co-transfection of the $G_{\alpha i1}$ or $G_{\alpha i1}Cys^{351}Gly$ protein (113 ± 22% and 66 ± 18%, respectively). In the case of the expression of $G_{\alpha i1}Cys^{351}Gly$ protein, the cells were treated with pertussis toxin. This result suggests that both $G_{\alpha i1}$ proteins couple to the α_{2A}-adrenoceptor, but that the wt $G_{\alpha i1}$ protein is more efficacious in its coupling than the mutant $G_{\alpha i1}Cys^{351}Gly$ protein.

TABLE 3. [^{35}S]GTPγS binding responses by α_{2A}-adrenoceptor co-expressed with either wt $G_{\alpha i1}$ protein or $G_{\alpha i1}Cys^{351}Gly$ protein in COS-7 cells

	[^{35}S]GTPγS binding response (E_{max}, %)		
	α_{2A}-Adrenoceptor	α_{2A}-Adrenoceptor + $G_{\alpha i1}$ protein	α_{2A}-Adrenoceptor + $G_{\alpha i1}Cys^{351}Gly$ protein + PTX (100 ng/mL)
Brimonidine (1 μM)	47 ± 11.7	113 ± 21.8	65.9 ± 18.3

Cells were transfected and [^{35}S]GTPγS binding to membrane preparation was performed as described in Methods. Values represent the means ± SEM of four independent experiments, each one performed in duplicate.

cAMP Formation by α_{2A}-Adrenoceptors in the Presence of $G_{\alpha i1}$ Proteins

Following transient expression of the α_{2A}-adrenoceptor in COS-7 cells, the imidazoline derivative brimonidine (10 μM) and the native agonist (−)-adrenaline (10 μM) stimulated cAMP formation by 151 ± 28 and 633 ± 35%, respectively versus the basal cAMP level (Fig. 2A). In contrast, the azepine derivative talipexole (10 μM) neither stimulated nor inhibited basal cAMP formation. No stimulation by brimonidine could be observed on forskolin (10 μM)-stimulated cAMP formation (data not shown). The cAMP-mediated stimulation by brimonidine was dose-dependent (EC_{50}: 300 ± 50 nM) with a maximal effect at 10 μM (Fig. 2B). This effect was specific to the recombinant α_{2A}-adrenoceptor: it was absent in non-transfected cells and could be fully blocked by the α_{2A}-adrenoceptor antagonist RS 15385 (10 μM). RS 15385 (10 μM) did not modify the basal cAMP level. The (−)-adrenaline-mediated stimulation of cAMP formation could not be blocked by RS 15385 (10 μM). This effect is likely mediated by an endogenous β-adrenoceptor subtype in COS-7 cells. The β-adrenoceptor antagonist propranolol (10 μM) blocked the cAMP effect of (−)-adrenaline, while it was without effect on basal cAMP formation and brimonidine-stimulated cAMP accumulation. Pertussis toxin pretreatment (100 ng/mL) did not significantly modify either brimonidine or (−)-adrenaline-mediated cAMP formation compared to untreated COS-7 cells. To control the efficacy of pertussis toxin, C6 glial cells permanently expressing α_{2A}-adrenoceptors were treated with this toxin and assayed for cAMP formation [17]. In non-treated cells, brimonidine inhibited (−73 ± 2%) forskolin (100 μM)-induced stimulation of cAMP formation. When these cells were pretreated overnight with 100 ng/mL of pertussis toxin, an activation (+ 52.3 ± 4.2% above forskolin stimulation) of cAMP formation by brimonidine was observed. Therefore, we conclude that the batch of pertussis toxin used in this study was effective. Cholera toxin (100 ng/mL) pretreatment decreased both brimonidine-and (−)-adrenaline-mediated cAMP formation in COS-7 cells by 79 ± 7 and 84 ± 1%, respectively versus the basal cAMP level (Fig. 2A).

Expression of the α_{2A}-adrenoceptor: $G_{\alpha i1}$ and α_{2A}-adrenoceptor: $G_{\alpha i1}Cys^{351}Gly$ fusion proteins in COS-7 cells induced a significant decrease (−92 ± 12 and − 115 ± 4%, respectively, Fig. 3A) in brimonidine-mediated cAMP formation compared to COS-7 cells expressing the α_{2A}-adrenoceptor protein. Pretreatment with either pertussis toxin or cholera toxin did not modify this effect. A comparison between the cAMP responses mediated by the α_{2A}-adrenoceptor protein co-expressed with either the rat $G_{\alpha i1}$ or rat $G_{\alpha i1}Cys^{351}Gly$ protein is shown in Fig. 3B. A significant decrease in brimonidine-mediated cAMP formation (−69 ± 9 and − 78 ± 11%, respectively) was observed in this case compared to COS-7 cells expressing the α_{2A}-adrenoceptor protein. The attenuation was not as great as that observed in the expression of the corresponding fusion proteins. No modification in cAMP formation by brimonidine was observed by either pertussis toxin or cholera toxin pretreatment. Expression of the rat $G_{\alpha s}$ protein together with the α_{2A}-adrenoceptor co-expressed or fused with either the rat $G_{\alpha i1}$ or rat $G_{\alpha i1}Cys^{351}Gly$ protein also did not modify any of the cAMP responses.

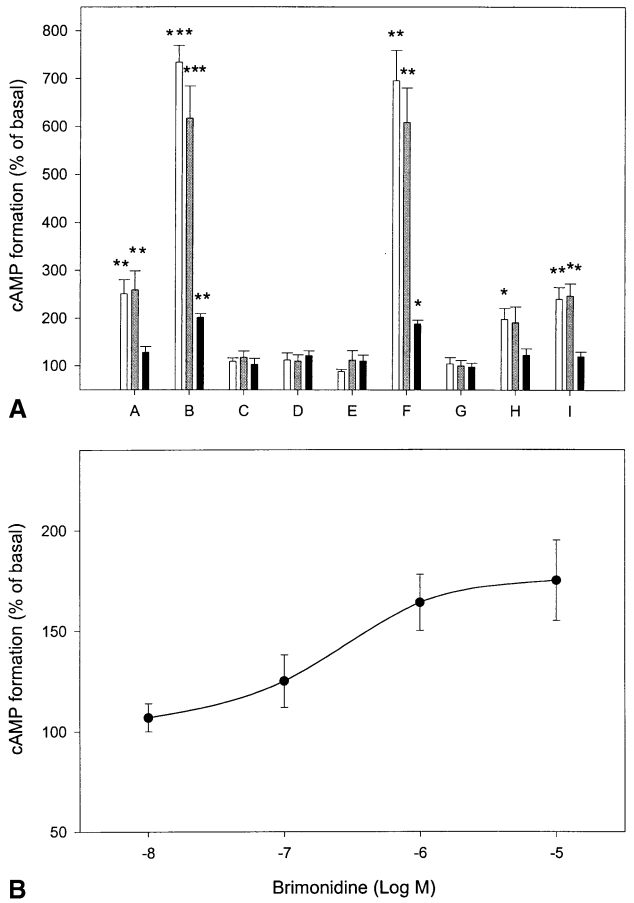

FIG. 2. (A) cAMP formation by α_{2A}-adrenoceptor ligands in COS-7 cells transiently transfected with the α_{2A}-adrenoceptor gene. Transfection and cAMP formation were assessed as described in Methods. Results are expressed in percentage relative to basal cAMP values obtained in the absence of agonist. Data are means ± SEM of five individual experiments, each one performed in triplicate. The ligands are 10 μM brimonidine (A), 10 μM (−)-adrenaline (B), 10 μM talepixole (C), 10 μM RS 15385 (D), 10 μM brimonidine + 10 μM RS 15385 (E), 10 μM (−)-adrenaline + 10 μM RS 15385 (F), 10 μM propranolol (G), 10 μM (−)-adrenaline + 10 μM propranolol (H), and 10 μM brimonidine + 10 μM propranolol (I). Cells were either not treated (empty bars) or pretreated with 100 ng/mL of pertussis toxin (▨) or with 100 ng/mL of cholera toxin (■). Statistical analysis of the basal cAMP level and ligand cAMP responses was performed using a Student's *t*-test. *P < 0.05, **P < 0.01, ***P < 0.001. (B) Concentration–response curve of brimonidine for stimulation of cAMP formation in COS-7 cells transiently transfected with the α_{2A}-adrenoceptor gene. cAMP formation was measured as described in Methods. Results are expressed in percentages relative to cAMP values obtained in the absence of brimonidine. The curve was constructed with mean values ± SEM of three independent experiments, each one performed in triplicate.

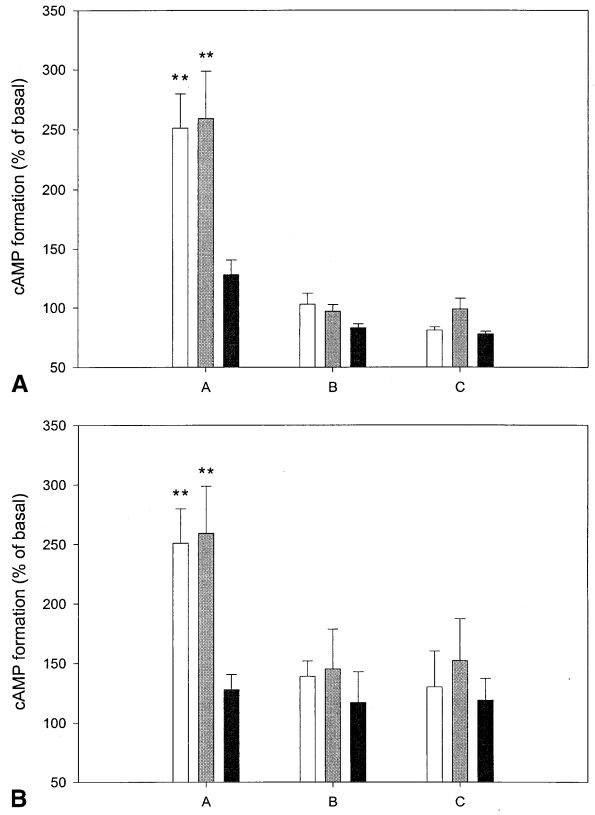

FIG. 3. (I) The effect of brimonidine on cAMP formation mediated by α_{2A}-adrenoceptor: $G_{\alpha i1}$ fusion proteins. Brimonidine (10 μM) was tested in COS-7 cells expressing α_{2A}-adrenoceptor (A), α_{2A}-adrenoceptor: $G_{\alpha i1}$ fusion protein (B), or α_{2A}-adrenoceptor: $G_{\alpha i1}Cys^{351}Gly$ fusion protein (C). cAMP formation was measured as described in Methods. Values for (A) were taken from Fig. 1, values for (B) and (C) represent means ± SEM of four independent transfection experiments, each one performed in triplicate. Cells were either not treated (empty bars) or pretreated with 100 ng/mL of pertussis toxin (▨) or with 100 ng/mL of cholera toxin (■). Statistical analysis of the basal cAMP level and ligand cAMP responses was performed using a Student's *t*- test. **$P < 0.01$. Absolute basal levels (nmol/well): α_{2A}-adrenoceptor basal 8.6 ± 1.9; pertussis toxin 7.2 ± 1.4; cholera toxin 112.6 ± 9.6. α_{2A}-adrenoceptor: $G_{\alpha i1}$ fusion protein basal 12.4 ± 1.4; pertussis toxin 12.7 ± 1.5; cholera toxin 102.5 ± 6.4. α_{2A}-Adrenoceptor: $G_{\alpha i1}Cys^{351}Gly$ fusion protein basal 14.7 ± 0.9; pertussis toxin 12.5 ± 1.1; cholera toxin 115.4 ± 10.2 (II) The effect of brimonidine on cAMP formation mediated by α_{2A}-adrenoceptor co-expressed with $G_{\alpha i1}$ proteins. Brimonidine (10 μM) was tested in COS-7 cells expressing the α_{2A}-adrenoceptor (A) or co-expressed with $G_{\alpha i1}$ protein (B) or $G_{\alpha i1}Cys^{351}Gly$ protein (C). cAMP formation was measured as described in Methods. Values for (A) were taken from Fig. 1, values for (B) and (C) represent means ± SEM of four independent transfection experiments, each one performed in triplicate. Cells were either not treated (empty bars) or pretreated with 100 ng/mL of pertussis toxin (▨) or with 100 ng/mL of cholera toxin (■). Statistical analysis of the basal cAMP level and the different cAMP responses were performed using a Student's *t*-test. **$P < 0.01$. Absolute basal levels (nmol/well): α_{2A}-adrenoceptor basal 8.6 ± 1.9; pertussis toxin 7.2 ± 1.4; cholera toxin 112.6 ± 9.6. α_{2A}-Adrenoceptor co-expressed with $G_{\alpha i1}$ protein basal 13.1 ± 0.5; pertussis toxin 14.1 ± 1.4; cholera toxin 124.4 ± 13.6. α_{2A}-Adrenoceptor co-expressed with $G_{\alpha i1}Cys^{351}Gly$ protein basal 13.0 ± 1.4; pertussis toxin 13.0 ± 3.3; cholera toxin 101.9 ± 11.9.

DISCUSSION

The present study reports on measuring the functional expression of α_{2A}-adrenoceptor: $G_{\alpha i1}$ fusion proteins as followed by cAMP responses. A mutant $G_{\alpha i1}Cys^{351}Gly$ protein was used to obtain putative resistance to pertussis toxin [12] so as to be able to differentiate between α_{2A}-adrenoceptor responses mediated by endogenous $G_{\alpha i}/G_{\alpha 0}$ proteins. Therefore, the wt and mutant $G_{\alpha i1}Cys^{351}Gly$ protein's were systematically compared to each other. The α_{2A}-adrenoceptor fusion proteins were expressed at a similar level as the wt α_{2A}-adrenoceptor protein in COS-7 cells, indicating that the fusion of the α_{2A}-adrenoceptor with $G_{\alpha i1}$ protein does not interfere with correct folding and membrane targeting of the fusion protein. Similarly, the binding affinities of the investigated α_2 agonists were unchanged by the fusion protein approach.

Stimulation of α_{2A}-adrenoceptors by brimonidine increased the formation of cAMP in COS-7 cells, likely due to an interaction of these adrenoceptors with endogenous G_s proteins. Eason and co-workers [5, 22] have identified a sequence of 11 amino acids of the amino-terminal region of the third intracellular loop (positions 218 to 228) of the α_{2A}-adrenoceptor capable of activating G_s. α_{2A}-Adrenoceptor regulation of adenylyl cyclase in cells may be either stimulatory or inhibitory depending on the relative levels of expression of G_s versus G_i. The present study supports a direct coupling of α_{2A}-adrenoceptors to G_s, because the stimulatory effect was not decreased upon a pertussis toxin pretreatment. This observation excludes a possible stimulation of type II or type IV adenylyl cyclases by βγ subunits released from activated G_i proteins [6, 23]. Pertussis toxin pretreatment did not enhance brimonidine- and adrenaline-stimulated cAMP formation. As adenylyl cyclase is expressed at relatively low levels in many cell systems [24], we hypothesize that the adenylyl cyclase expressed in COS-7 cells is already maximally activated in the absence of pertussis toxin. The stimulatory cAMP effect by brimonidine was sensitive to cholera toxin, which ADP-ribosylates $G_{\alpha s}$ proteins and consequently blocks the modulation of the G_s-coupled pathway [25]. Stimulation by the agonist (−)-adrenaline induced a strong increase in cAMP formation, but this effect was principally mediated by an endogenous β-adrenoceptor in COS-7 cells, as suggested by the blockage with the β-adrenoceptor antagonist propranolol and not by the α_{2A}-adrenoceptor antagonist RS 15385. The α_{2A}-adrenoceptor agonist talipexole induced no effect on cAMP formation. This observation is in agreement with the study of Eason and co-workers [7], who reported that the degree of α_{2A}-adrenoceptor-mediated G_s coupling is highly dependent on the chemical structure of the α_2-adrenoceptor agonist being investigated. The coupling of α_{2A}-adrenoceptor with the G_s pathway is not necessarily due to the high level of expression of the α_{2A}-adrenoceptor. Fraser *et al.* [26] have demonstrated, for the α_{2A}-adrenoceptor stably transfected in Chinese hamster ovary cells, an increase in basal cAMP formation by adrenaline at various

receptor densities (50 to 1200 fmol of receptor/mg membrane protein). Contrary to the results of Sautel and Milligan [27], we did not observe a biphasic response for cAMP formation by brimonidine. This apparent discrepancy may be due to the experiments performed in different cell lines (Rat-1 fibroblasts versus COS-7 cells) and the porcine α_{2A}-adrenoceptor versus the human α_{2A}-adrenoceptor.

In contrast to expression of the α_{2A}-adrenoceptor protein in the absence of recombinant protein, brimonidine did not affect the cAMP formation with the fusion protein between the α_{2A}-adrenoceptor and either the wt $G_{\alpha i1}$ or $G_{\alpha i1}Cys^{351}Gly$ protein. This effect was not modified by either pertussis toxin or cholera toxin pretreatment. Moreover, similar results were obtained with both the wt and mutant $G_{\alpha i1}$ proteins. These data are inconsistent with the ability of the agonist-occupied α_{2A}-adrenoceptor: $G_{\alpha i1}$ protein or α_{2A}-adrenoceptor: $G_{\alpha i1}Cys^{351}Gly$ protein to interact effectively with endogenous G_s proteins [15]. Whereas Sautel and Milligan [27] observed an inhibition of cAMP formation by brimonidine for the fusion proteins, we did not see such an effect. This may be due to the different host cell type. Sautel and Milligan [27] suggested the combined action of endogenous G_i proteins with the fused $G_{\alpha il}$ proteins.

To confirm the hypothesis that the agonist-occupied fusion proteins could not interact with endogenous G_s protein, COS-7 cells were co-transfected with the α_{2A}-adrenoceptor and either the wt $G_{\alpha i1}$ or $G_{\alpha i1}Cys^{351}Gly$ protein. Brimonidine again did not increase the cAMP formation. Small differences in cAMP levels were apparent when compared to the fusion proteins and may be due to a likely different receptor: G protein stoichiometry between the co-transfection and fusion protein experiments. The presence of a putative pertussis toxin-sensitive wt $G_{\alpha i1}$ protein prevents stimulation of cAMP formation in response to occupancy of α_{2A}-adrenoceptors with brimonidine, even after pertussis toxin pretreatment. In this case, the lack of effect of pertussis toxin pretreatment cannot be explained by the $Cys^{351}Gly$ mutation on the $G_{\alpha il}$ protein, which has been suggested by Wise *et al.* [14] to block the interaction between the effector and the $G_{\alpha i}$ protein. Two hypotheses could explain the lack of pertussis toxin effect. The first is that the increased activity of adenylyl cyclase via $G_{\alpha s}$ is blocked by over expression of $G_{\alpha i1}$ proteins, as the cellular level of adenylyl cyclase may be a quantitatively limiting element in the effector cascade from the receptor via G_s to adenylyl cyclase [24]. In the case of the $G_{\alpha i1}$ protein, the binding site is close to the catalytic site, and the binding of the α subunit may disturb the optimal alignment and consequently the adenylyl cyclase activity [28]. A second hypothesis may involve the pertussis toxin insensitivity of the wt $G_{\alpha i1}$ protein. The preferred substrates of pertussis toxin are G_i/G_0 α subunits associated with $\beta\gamma$ complexes. Monomeric G_α subunits are known to be very poor substrates for pertussis toxin [29]. The co-expression of the $G_{\alpha i1}$ protein may not be sufficient, since $\beta\gamma$ units are not at an optimal concentration. It is known that $\beta\gamma$ units are only effective at two orders of magnitude higher than the α subunit [30].

In conclusion, the $G_{\alpha i1}$ protein can modulate the G_s-mediated cAMP response by co-expression or fusion with the α_{2A}-adrenoceptor. However, pertussis toxin treatment did not alter the cAMP response. Therefore, it appears that pertussis toxin sensitivity of α_{2A}-adrenoceptor: $G_{\alpha i1}$ fusion proteins has to be considered with caution.

We thank Frédérick Finana, Claudie Cathala, and Fabrice Lestienne for excellent technical assistance and Stéphanie Cecco for secretarial assistance.

References

1. Timmermans PB, Chiu AT and Thoolen MJC, α-Adrenoceptors. In: *Comprehensive Medicinal Chemistry*, (Eds. Hansch C, Sammes PG and Taylor JB), pp. 135–185, Pergamon, Oxford, 1990.
2. Szabadi E and Bradshaw CM, Autonomic pharmacology of α_{2A}-adrenoceptors. *J Psychopharmacol* **3:** 6–18, 1996.
3. Bylund DB, Eikenberg DC, Hieble JP, Langer SZ, Lefkowitz RJ, Minneman KP, Molinoff PB, Ruffolo RR Jr and Trendelenburg U, IV International Union of Pharmacology nomenclature of adrenoceptors. *Pharmacol Rev* **46:** 121–136, 1994.
4. Lakhlani PP, Lovinger DM and Limbird LE, Genetic evidence for involvement of multiple effector systems in α_{2A}-adrenergic receptor inhibition of stimulus–secretion coupling. *Mol Pharmacol* **50:** 96–103, 1996.
5. Eason MG, Kurose H, Holt BD, Raymond JR and Liggett SB, Simultaneous coupling of α_{2A}-adrenergic receptors to two G-proteins with opposing effects. Subtype-selective coupling of α_2C10, α_2C4, and α_2C2-adrenergic receptors to G_i and G_s. *J Biol Chem* **267:** 15795–15801, 1992.
6. Federman AD, Conklin BR, Schrader KA, Reed RR and Bourne HR, Hormonal stimulation of adenylyl cyclase through G_i-protein $\beta\gamma$ subunits. *Nature* **356:** 159–161, 1992.
7. Eason MG, Jacinto MT and Liggett SB, Contribution of ligand structure to activation of α_2-adrenergic receptor subtype coupling to G_s. *Mol Pharmacol* **45:** 696–702, 1994.
8. Limbird LE and Sweatt JD, α_2-Adrenoceptors: Apparent interaction with multiple effector systems. In: *The Receptors* (Ed. Conn PM), pp. 281–305. Academic Press, Orlando, 1985.
9. Cotecchia S, Kobilka BK, Daniel KW, Nolan RD, Lapetina EY, Caron MG, Lefkowitz RJ and Regan JW, Multiple second messenger pathways of α-adrenergic receptor subtypes expressed in eukaryotic cells. *J Biol Chem* **265:** 63–69, 1990.
10. Milligan G, New aspects of G-protein-coupled receptor signalling and regulation. *Trends Endocrinol Metab* **9:** 13–19, 1998.
11. Bertin B, Freissmuth M, Jockers R, Strosberg AD and Marullo S, Cellular signaling by an agonist-activated receptor/$G_{s\alpha}$ fusion protein. *Proc Natl Acad Sci USA* **91:** 8827–8831, 1994.
12. Wise A, Carr IC, Groarke DA and Milligan G, Measurement of agonist efficacy using an α_{2A}-adrenoceptor–$G_{i1}\alpha$ fusion protein. *FEBS Lett* **419:** 141–146, 1997.
13. Wise A, Carr IC and Milligan G, Measurement of agonist-induced guanine nucleotide turnover by the G-protein $G_{i1}\alpha$ when constrained within an α_{2A}-adrenoceptor–$G_{i1}\alpha$ fusion protein. *Biochem J* **325:** 17–21, 1997.
14. Wise A, Watson-Koken MA, Rees S, Lee M and Milligan G, Interactions of the α_{2A}-adrenoceptor with multiple G_i-family

G-proteins: Studies with pertussis toxin- resistant G-protein mutants. *Biochem J* **321:** 721–728, 1997.
15. Burt AR, Sautel M, Wilson MA, Rees S, Wise A and Milligan G, Agonist occupation of an α_{2A}-adrenoreceptor–$G_{i1\alpha}$ fusion protein results in activation of both receptor-linked and endogenous G_i. Comparisons of their contributions to GTPase activity and signal transduction and analysis of receptor–G protein activation stoichiometry. *J Biol Chem* **273:** 10367–10375, 1998.
16. Seifert R, Lee TW, Lam VT and Kobilka BK, Reconstitution of β_2-adrenoceptor–GTP—high coupling efficiency in a beta 2-adrenoceptor-G (S alpha) fusion protein, binding-protein interaction in Sf9 cells. *Eur J Biochem* **255:** 369–382, 1998.
17. Wurch T, Colpaert FC and Pauwels PJ, G-protein activation by putative antagonists at mutant $Thr^{373}Lys$ α_{2A}-adrenoceptors. *Br J Pharmacol* **126:** 939–948, 1999.
18. Wurch T, Lestienne F and Pauwels PJ, A modified overlap extension PCR method to create chimeric genes in the absence of restriction enzymes. *Biotechnol Tech* **12:** 653–657, 1998.
19. Pauwels PJ, Palmier C, Wurch T and Colpaert FC, Pharmacology of cloned human 5-HT_{1D} receptor-mediated functional responses in stably transfected C6-glial cell lines: Further evidence differentiating human 5-HT_{1D} and 5-HT_{1B} receptors. *Naunyn Schmiedebergs Arch Pharmacol* **353:** 144–156, 1996.
20. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacterophage T4. *Nature* **227:** 680–685, 1970.
21. Pauwels PJ, Tardif S, Palmier C, Wurch T and Colpaert FC, How efficacious are 5-$HT_{1B/D}$ receptor ligands: An answer from GTPγS binding studies with stably transfected C6-glial cell lines. *Neuropharmacology* **36:** 499–512, 1997.
22. Eason MG and Liggett SB, Identification of a G_s coupling domain in the amino terminus of the third intracellular loop of the α_{2A}-adrenergic receptor. *J Biol Chem* **270:** 24753–24760, 1995.
23. Taussig R and Gilman AG, Mammalian membrane-bound adenylyl cyclases. *J Biol Chem* **270:** 1–4, 1995.
24. MacEwan DJ, Kim GD and Milligan G, Agonist regulation of adenylate cyclase activity in neuroblastoma x glioma hybrid NG108–15 cells transfected to co-express adenylate cyclase type II and the β_2-adrenoceptor. Evidence that adenylate cyclase is the limiting component for receptor-mediated stimulation of adenylate cyclase activity. *Biochem J* **318:** 1033–1039, 1996.
25. Cassel D and Selinger Z, Mechanism of adenylate cyclase activation by cholera toxin: Inhibition of GTP hydrolysis at the regulatory site. *Proc Natl Acad Sci USA* **74:** 3307–3311, 1977.
26. Fraser CM, Arakawa S, McCombie WR and Venter JC, 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 attentuation and activation. *J Biol Chem* **264:** 11754–11761, 1989.
27. Sautel M and Milligan G, Loss of activation of G_s but not G_i following expression of an α_{2A}-adrenoceptor–$G_{i1}\alpha$ fusion protein. *FEBS Lett* **436:** 46–50, 1998.
28. Tang WJ and Hurley JH, Catalytic mechanism and regulation of mammalian adenylyl cyclases. *Mol Pharmacol* **54:** 231–240, 1998.
29. Valenti G, Procino G, Liebenhoff U, Frigeri A, Benedetti PA, Ahnert-Hilger G, Nürnberg B, Svelto M and Rosenthal W, A heterotrimeric G protein of the G_i family is required for cAMP-triggered trafficking of aquaporin 2 in kidney epithelial cells. *J Biol Chem* **273:** 22627–22634, 1998.
30. Iniguez-Lluhi J, Kleuss C and Gilman AG, The importance of G-protein βγ subunits. *Trends Cell Biol* **3:** 230–236, 1993.