G-protein activation by putative antagonists at mutant Thr³⁷³Lys α_{2A} adrenergic receptors

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1 Replacement of a threonine by a lysine at position 373 in the C-terminal portion of the third intracellular loop of the human α_{2A} -adrenergic receptor (α_{2A} AR) has been reported to generate a constitutively active mutant receptor in analogy with similar mutations in the α_{1B} and β_2 AR (Ren *et al.*, 1993). In the present study, the mutant Thr³⁷³Lys α_{2A} AR receptor was investigated by measuring the formation of inositol phosphates in either the absence or presence of mouse $G_{\alpha 15}$ protein in Cos-7 cells.

2 Increased affinity, potency and/or efficacy for the agonists [(–)-adrenaline, UK 14304, clonidine, guanabenz and oxymetazoline] was observed, consistent with a precoupled mutant α_{2A} AR: G-protein state. The basal inositol phosphates response was similar at the wild-type (wt) and mutant α_{2A} AR, but was enhanced at the mutant α_{2A} AR upon co-expression with the mouse $G_{\alpha 15}$ protein. This enhanced response could not be attenuated in the presence of any of the tested α_2 AR antagonists (10 μ M), suggesting that inverse agonist activity did not occur at the mutant α_{2A} AR.

3 Ligands that so far have been identified as antagonists at the wt α_{2A} AR demonstrated either no intrinsic activity (MK 912, WB 4101, RS 15385, RX 811059 and RX 821002) or positive efficacy [$E_{\max}$, % vs. 1 μ M UK 14304: dexefaroxan (27 ± 7), idazoxan (34 ± 9), atipamezole (27 ± 4), BRL 44408 (59 ± 5) and SKF 86466 (54 ± 9)] at the mutant α_{2A} AR, but only in the presence of the mouse $G_{\alpha 15}$ protein. The ligand potencies corresponded with their respective pK_i values at the mutant α_{2A} AR receptor.

4 The partial agonist effect of SKF 86466 was resistant to pertussis toxin treatment (100 ng ml^{–1}) and not affected by co-expression of the rat $G_{\alpha i1}$ protein. It was virtually absent in the presence of 10 μ M RS 15385. SKF 86466 was without intrinsic activity upon co-expression of the mouse $G_{\alpha q}$ protein.

5 Some putative α_2 AR antagonists exerted a partial agonist activity that was highly dependent on the presence of specific G-protein α -subunits, suggesting that these ligands cause selective G-protein activation at the mutant α_{2A} AR.

Keywords: α_{2A} -adrenergic receptor; G-protein coupling; intrinsic ligand activity; antagonist trafficking

Abbreviations: α_{2A} AR, α_{2A} -adrenergic receptor; BRL 44408, ($\pm$)-2-[(4,5-dihydro-1H-imidazol-2-yl)methyl]-2,3-dihydro-1-methyl-1H-isoindole; CSS, controlled salt solution; DMEM, Dulbecco's modified Eagle Medium; MK 912, (2S,12bS) 1',3'-dimethylspiro (1,3,4,5',6,6',7,12b-octahydro-2H-benzo[b]furo[2,3-a]quinolizine)-2,4'-pyrimidin-2'-one; RS 15385, (+)-(8aR,12aS,13aS)-3-methoxy-12-(methylsulphonyl)-5,8,8a,9,10,11,12,12a,13,13a-decahydro-6H-isoquino[2',1-g][1,6]naphthyridine; RX 811059, 2-[2-(2-ethoxy-1,4-benzodioxan-2-yl)-1-imidazoline; [³H] RX 821002, [³H] (1,4-[6,7(n)-[³H]-benzodioxan-2-methoxy-2-yl)-2-imidazoline hydrochloride; SKF 86466, 6-chloro-2,3,4,5-tetrahydro-3-methyl-1H-3-benzazepine; UK 14304, 5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline tartrate; WB 4101, 2-(2,6-dimethoxyphenoxyethyl)aminoethyl-1,4-benzodioxane hydrochloride

Introduction

α_2 -Adrenergic receptors (α_2 AR) are implicated in the control of noradrenergic neurotransmission in the brain and modulate a number of physiological processes peripherally, including aggregation of platelets, release of insulin from the pancreas, and inhibition of lipolysis (Timmermans *et al.*, 1990; Szabadi & Bradshaw, 1996). Pharmacological results including ligand-binding data suggest that α_2 AR are not a homogeneous class of receptors. At least three distinct genes encoding α_2 AR subtypes ($\alpha_{2C10/A}$ AR, $\alpha_{2C2/B}$ AR, $\alpha_{2C4/C}$ AR) have been isolated in man and characterized in heterologous expression systems (Regan *et al.*, 1988; Fraser *et al.*, 1989; Lomasney *et al.*, 1990). The α_2 AR belong to the superfamily of G-protein-coupled receptors, mediating their effects through the activation of heterotrimeric G-proteins. The α_{2A} AR, *via* its interaction with

the pertussis toxin-sensitive G_i/G_o subclass of G-proteins, modulates multiple effector systems, including inhibition of adenylate cyclase, opening of Ca^{2+} channels and activation of K^+ channels (Lakhlani *et al.*, 1996). Eason *et al.* (1992) and Federman *et al.* (1992) have demonstrated paradoxical α_2 AR-mediated increases in cyclic AMP level *via* coupling to G_s . The degree of G_s coupling depends initially 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 (Eason *et al.*, 1994). The α_2 AR may also couple to other intracellular pathways involving Na^+/H^+ exchange, phospholipase A_2 and phospholipase C activation (Limbird, 1988; Cotecchia *et al.*, 1990).

Mutagenesis studies of several G-protein-coupled receptors indicate that the coupling to specific G-proteins is predominantly mediated by the intracellular loops (Dalman and Neubig, 1991). The second and third intracellular loops are clearly involved in α_{2A} AR:G-protein interactions (Dalman &

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Neubig, 1991; Näsman *et al.*, 1997). Replacement of a threonine by a lysine or a cysteine at position 373 in the BBXXB motif (where B represents a basic residue and X a non-basic residue) of the third intracellular loop of the human α_{2A} AR, results in a constitutively active mutant receptor in analogy with mutations affecting the same region in the α_{1B} AR and β_2 AR (Ren *et al.*, 1993). The mutant α_{2A} AR demonstrates an increased affinity for the agonist UK 14304 associated with an agonist-independent inhibition of adenylyl cyclase in HEK 293 cells. The functional consequences of this mutant receptor have also been studied in a stably transfected 3T3F442A preadipose cell line (Bétuing *et al.*, 1997). Similar (in the absence of agonist) and amplified (in the presence of agonist) cellular responses were measured at the preadipocyte stage indicating constitutive activation of the mutant receptor. However, the α_{2A} AR-dependent antilipolysis at the adipocyte stage was neither reproduced nor amplified by the mutant receptor. Conversely, down-regulation of G_{xi}-proteins and an increase in the maximal response to lipolytic agents have been observed in cell lines expressing the mutant α_{2A} AR (Bétuing *et al.*, 1997). Other than the agonist UK 14304, very few α_2 AR ligands have been evaluated at this mutant α_2 AR. Also, it has yet to be determined whether the inhibition of constitutive activity by α_2 AR inverse agonists can be reversed. Tian *et al.* (1994) demonstrated that some α_2 AR antagonists display inverse agonist activity at precoupled wild-type (wt) rat α_{2D} AR in recombinant PC12 cells; the rank order of maximal effectiveness differed from that of receptor binding affinities.

Our studies of constitutively active mutant α_{2A} AR provide apparently opposite results for ligands that so far have been characterized as α_2 AR antagonists. By measuring the positive coupling of the mutant receptor to the formation of inositol phosphates, partial agonist instead of either neutral antagonist or inverse agonist activity was observed for a series of α_2 AR ligands at the mutant Thr³⁷³Lys α_{2A} AR in the presence of mouse G_{z15} protein. Experiments were performed in parallel with the wt α_{2A} AR, and were compared to receptor activity as measured by inhibition of stimulated cyclic AMP formation in stably transfected C6-glia cells. We found an increased affinity of the mutant α_{2A} AR receptor for diverse agonists, whereas no functional evidence could be observed for inverse agonist activity. In contrast, a number of putative α_2 AR antagonists displayed positive efficacy and behaved as partial agonists at the mutant α_{2A} AR in the presence of mouse G_{z15} protein. The partial agonist activity is discussed with regard to the receptor binding properties, G-protein coupling and partial agonist activity of antagonists at other G-protein-coupled receptor mutants.

Methods

Cloning of human wt and mutant Thr³⁷³Lys α_{2A} -adrenergic receptors, rat G_{xi} protein, and mouse G_{z15} and G_q protein genes

Genes were cloned by PCR using primers designed at the start and stop codons of the respective nucleotide sequences (Fraser *et al.*, 1989; Jones & Reed, 1987; Strathmann & Simon, 1990; Wilkie *et al.*, 1991). The PCR mixture (50 μ l) consisted of 250 ng of reverse-transcribed poly(A⁺) RNA from either human, mouse or rat total brain, or 1 μ g of mouse genomic DNA, dNTP (350 μ M), primers (400 nM), DMSO (10% for the α_{2A} AR gene only) and 1 μ l of Expand long template DNA polymerase mix in PCR buffer [mM: (NH₄)₂SO₄, 16; MgCl₂, 1.75; Tris-HCl, 50 (pH 9.2)]. The PCR program consisted of 30

repetitive cycles with a strand separation step at 96°C for 30 s, an annealing step at 60°C for 1 min and an elongation step at 68°C for 1.5 min. The amplification cycle was preceded by a hot start step at 96°C for 5 min in which the DNA polymerase mix was omitted. PCR fragments were purified by 1% agarose gel electrophoresis, purified using a Geneclean II kit and subsequently cloned into 50 ng of a pCR3.1 vector. Sequencing was performed automatically on an ABI Prism 310 Genetic analyser using a dichloro-rhodamine terminator cycle sequencing kit. Nucleotide sequences were identical to the published sequences. Site-directed mutagenesis of the α_{2A} AR was performed using a modified overlap extension technique based on PCR (Wurch *et al.*, 1998) using appropriate complementary primers carrying the Thr (ACG codon) to Lys (AAA codon) mutation. The obtained PCR fragment contained the presence of the mutated codon: Thr³⁷³Lys.

Transient co-transfection of α_{2A} -adrenergic receptors and various G-protein genes in Cos-7 cells

Ten micrograms of plasmid containing either wt α_{2A} AR or mutant Thr³⁷³Lys α_{2A} AR and 10 μ g of the indicated G-protein plasmid were co-transfected with 5×10^6 exponentially-growing Cos-7 cells using a Bio-Rad electroporator (250 mV, 250 μ F) in DMEM supplemented with 1% DMSO. Cultures were grown in 24-well tissue culture plates with DMEM supplemented with 10% foetal calf serum, washed and treated after 24 h with 2% dialysed foetal calf serum and [2-³H](N) myo-inositol (2 μ Ci per well) for a period of 24–48 h before functional receptor analysis. For radioligand experiments, cells were grown for 48 h in DMEM supplemented with 10% foetal calf serum.

Stable transfection of wt α_{2A} -adrenergic receptor gene in C6-glia cells

C6-glia cells were transfected as described above with 10 μ g pCR3.1/human α_{2A} AR and were subcultured after 48 h in DMEM in the presence of 1.25 mg geneticin ml⁻¹. After about 10 days, individual clones were isolated and subcultured as described (Pauwels *et al.*, 1996). Intact cells were assayed for [³H] (1,4-[6,7(n)-³H]-benzodioxan-2-methoxy-2-yl)-2-imidazoline hydrochloride (RX 821002) binding and inhibition of forskolin-induced cyclic AMP formation. Results presented in this study were obtained with a clone that expresses 1.3 pmol mg⁻¹ protein of [³H] RX 821002 binding sites.

Receptor binding assay for α_{2A} -adrenergic receptors

Membrane preparations were prepared in Tris-HCl pH 7.6 (50 mM) as described (Pauwels *et al.*, 1996). Binding assays were performed with [³H] RX 821002 (2 nM). Incubation mixtures consisted of 0.4 ml of cell membranes (6 μ g protein), 0.05 ml of radioligand and 0.05 ml of displacing compound or phentolamine (10 μ M) to determine nonspecific binding. K_i values were calculated according to the equation $K_i = IC_{50}/(1 + C/K_d)$ with C representing the concentration and K_d the equilibrium dissociation constant of the [³H] ligand. pK_d values were obtained from saturation binding studies performed as previously described (Pauwels *et al.*, 1996).

α_{2A} -adrenergic receptor-mediated inhibition of forskolin-induced cyclic AMP formation

Inhibition of forskolin (100 μ M)-stimulated cyclic AMP formation in the C6-glia cell line stably expressing the wt α_{2A} AR was measured as previously described (Pauwels *et al.*,

1996). The cellular cyclic AMP content was assayed using a radioimmunoassay kit. Inhibition of forskolin-induced cyclic AMP formation was expressed as the percentage of that obtained with UK 14304 (1 μ M). EC₅₀ values (concentrations of the compounds producing 50% inhibition of their own maximal inhibition) were derived. Antagonists were added 15 min prior to agonists. pK_B values were calculated as $K_B = (B)/(A'/A) - 1$ where B is the concentration of the antagonist, and A and A' are the EC₅₀ values of agonist concentration measured in the absence and presence of antagonist, respectively.

α_2 A-adrenergic receptor-mediated phosphatidyl inositol phosphates hydrolysis

Cos-7 cells were washed with 1.0 ml CSS, and were incubated for 1 h at 37°C with 1.0 ml CSS containing LiCl (10 mM) either in the presence or absence of agonist. The reaction was stopped by the addition of 0.1 ml 1 N HClO₄ and neutralized prior to the addition of 0.25 ml of sample buffer (mM: Na₂B₄O₇, 30; EDTA, 3). The fraction of total [³H]-inositol phosphates produced after agonist stimulation was separated from free [³H]-myoinositol and [³H]-glycerophosphoinositol by chromatography on a strong anion exchange AG1-X8 resin equilibrated in water. A 1.3 ml aliquot of the cell lysate was loaded on a column as described (Pauwels *et al.*, 1990). After two washes with 10.0 ml water, the glycerophosphoinositol was washed out in 15.0 ml of [mM: Na₂B₄O₇, 5; CH₃NO₂, 60]. The total [³H]-inositol phosphates were eluted with 2.0 ml of [mM: CH₃NO₂, 2; HCOOH, 0.1]. The radioactivity was extracted in 16 ml Ultima Gold XR and counted with a Tri-Carb 2500 liquid scintillation counter. Maximal stimulation of the total production of inositol phosphates was defined in the presence of UK 14304 (1 μ M). EC₅₀ values were defined as the concentrations of compounds producing 50% of their own maximal response. Antagonist studies were performed by pre-incubating the cells with the antagonist for 10 min before the addition of the agonist. pK_B values were calculated as described above. In the case of pertussis toxin treatment (100 ng ml⁻¹), cells were pretreated for 16 h before inositol phosphates were measured.

Statistical analysis

Statistical comparison of ligand binding affinities, agonist and antagonist potencies, and basal and UK 14304-mediated responses between wt α_2 A and mutant Thr³⁷³Lys α_2 A AR were made using the two-tailed Student's *t*-test.

Materials

Cos-7 and C6-glia cells were obtained from ATCC (Rockville, U.S.A.). The ABI Prism 310 Genetic analyzer and the dichloro-rhodamine terminator cycle sequencing kit were from Perkin Elmer (Forster City, U.S.A.). The pCR3.1 vector was from Invitrogen (San Diego, U.S.A.). The GeneClean II kit was purchased from Bio 101 Inc. (La Jolla, U.S.A.). The Expand long template PCR kit was from Boehringer Mannheim (Mannheim, Germany). The AG1-X8 resin was from Bio-Rad Laboratories (Hercules, U.S.A.). [³H] RX 821002 (50.6 Ci mmol⁻¹) and [2-³H](N)-myoinositol (20 Ci mmol⁻¹) were obtained respectively from Amersham (Les Ulis, France) and New England Nuclear (Les Ulis, France). 2-(2,6-dimethoxyphenoxyethyl)aminoethyl-1,4-benzodioxane hydrochloride (WB 4101) was from RBI (Natick, U.S.A.). Clonidine, (–)-adrenaline, oxymetazoline and gua-

nabenz were from Sigma. (±)-2-[(4,5-dihydro-1H-imidazol-2-yl)methyl]-2,3-dihydro-1-methyl-1H-isoindole (BRL 44408) and 6-chloro-2,3,4,5-tetrahydro-3-methyl-1H-3-benzazepine (SKF 86466) were from Smith Kline Beecham (Herts, U.K.). (2S,12bS) 1',3'-dimethylspiro (1,3,4,5',6,6',7,12b-octahydro-2H-benzo[b]furo[2,3-a]quinolizine)-2,4'-pyrimidin-2'-one (MK 912) was from Merck (Essex, U.K.). 5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline tartrate (UK 14304), dexefaroxan, (–)-efaroxan, atipamezole, (+)-(8aR,12aS,13aS)-3-methoxy-12-(methylsulphonyl)-5,8,8a,9,10,11,12,12a, 13,13a-decahydro-6H-isoquino [2',1-g][1,6]naphthyridine (RS 15385) and 2-[2-(2-ethoxy-1,4-benzodioxan-2-yl)-1-imidazoline (RX 811059) were prepared intra-muros. Idazoxan and (1,4-benzodioxan-2-methoxy-2-yl)-2-imidazoline hydrochloride (RX 821002) were from Reckitt and Colman (Kingston-upon-Hull, U.K.).

Results

Binding properties of α_2 -adrenergic ligands at wt α_2 A and mutant Thr³⁷³Lys α_2 A-adrenergic receptors

Saturation binding experiments with [³H] RX 821002 in membrane preparations of Cos-7 cells transfected with either a wt α_2 A or mutant Thr³⁷³Lys α_2 A AR gene indicated the presence of a single high affinity binding site with similar affinity for [³H] RX 821002 (pK_d: 8.98 ± 0.05 and 8.84 ± 0.02 for wt α_2 A and mutant α_2 A AR receptors, respectively). The maximal binding capacity for [³H] RX 821002 in these transfected cells was similar (4.0 ± 0.4 and 4.5 ± 1.0 pmol mg⁻¹ protein, for the wt and mutant α_2 A AR, respectively). A series of eleven α_2 -adrenergic antagonists and five agonists were characterized for inhibition of [³H] RX 821002 binding to wt α_2 A and mutant α_2 A AR (Table 1). The mutation significantly decreased (1.8–2.9 fold) the affinity of RS 15385, RX 811059 and WB 4101, and left unchanged that of the other antagonists. In contrast, each of the agonists appeared to be significantly more potent at the mutant α_2 A AR; the following rank order of increased affinity was obtained (fold-increase): (–)-adrenaline (20) > UK 14304 (8.7) > clonidine (3.5) = guanabenz (3.2) > oxymetazoline (1.9) (Table 1). Co-expression of either the wt α_2 A or mutant Thr³⁷³Lys α_2 A AR with the mouse G_{z15} protein enhanced the maximal binding capacity for [³H] RX 821002 without affecting the affinity constant for [³H] RX 821002. Binding affinities of the ligands tested were not affected upon co-expression with mouse G_{z15} protein or co-incubation with GTP (100 μ M) (not shown).

Intrinsic activity of α_2 -adrenergic ligands at wt α_2 A and mutant Thr³⁷³Lys α_2 A-adrenergic receptors

In contrast to nontransfected Cos-7 cells, UK 14304 produced a concentration-dependent stimulation of inositol phosphates formation in cells transfected with either wt α_2 A or mutant Thr³⁷³Lys α_2 A AR. The basal activity as well as the maximal response to UK 14304 were the highest with the mutant Thr³⁷³Lys α_2 A AR in the presence of mouse G_{z15} protein (Table 2). Otherwise, for the wt α_2 A AR, the basal and maximal responses to UK 14304 were similar in the presence and absence of mouse G_{z15} protein. The UK 14304-mediated responses are likely to occur *via* different G-protein subclasses as they could be differentially blocked by pretreatment of the cells with pertussis toxin. The responses at wt α_2 A and mutant Thr³⁷³Lys α_2 A AR were respectively blocked by 84 and 67% in the presence of pertussis toxin but in the absence of mouse G_{z15} protein. In contrast, the UK 14304 responses were less affected

upon pertussis toxin treatment in the presence of mouse $G_{\alpha_{15}}$ protein, although this effect was variable between experiments (Table 2). Further studies were conducted unless otherwise specified in the presence of mouse $G_{\alpha_{15}}$ protein. UK 14304 and clonidine stimulated the production of inositol phosphates to the same magnitude as (–)-adrenaline at the wt α_{2A} AR, whereas guanabenz and oxymetazoline were less active (73 and 51%, respectively; Figure 1). The corresponding potencies and $E_{\max}$ values are summarized in Table 3, and are compared with the respective agonist potencies as measured by inhibition of 100 μ M forskolin-stimulated cyclic AMP formation in C6-glia cells stably transfected with the wt α_{2A} AR, and stimulation of the production of inositol phosphates at mutant Thr³⁷³Lys α_{2A} AR in Cos-7 cells. Agonist potencies were systematically lower in the cyclic AMP assay, in particular for (–)-adrenaline (21 fold) in the presence of propranolol (1 μ M) (to block β AR). This may be due to the lower levels of receptor expression in C6-glia cells (1.3 pmol mg^{–1} protein) versus Cos-7 cells (4.0–

4.5 pmol mg^{–1} protein). The cyclic AMP assay with the C6-glia cell line appeared to be also less sensitive in differentiating between the maximal responses of the agonists. Nonetheless, the $E_{\max}$ values of guanabenz and oxymetazoline were 24 and 20% inferior to that of UK 14304. Potencies for each of the agonists in the inositol phosphates assay for the mutant Thr³⁷³Lys α_{2A} were significantly greater than at wt α_{2A} AR, and their maximal responses were similar to that of UK 14304 (Figure 1 and Table 3).

Table 4 summarizes the intrinsic activities of the α_2 -adrenergic antagonists as measured at wt α_{2A} and mutant Thr³⁷³Lys α_{2A} AR. Two distinct subgroups were revealed within the antagonists by measuring their stimulation of inositol phosphates at the mutant Thr³⁷³Lys α_{2A} AR. One group consisted of RS 15385, RX 811059, RX 821002 and WB 4101 which displayed neither positive nor negative efficacy at mutant and wt α_{2A} AR. The other group consisted of atipamezole, dexefaroxan, SKF 86466, idazoxan and BRL 44408 which demonstrated between +27 to +59% stimulation of inositol phosphates (versus 1 μ M of UK 14304) at the mutant α_{2A} AR. These ligands were apparently free of intrinsic activity (–1.3 to +13.0% versus 1 μ M of UK 14304) at the wt α_{2A} AR receptor. The observed potencies for the partial agonist activities of these ligands corresponded with their respective pK_i values at the mutant α_{2A} AR (Tables 1 and 4). The partial intrinsic activity of these ligands was attenuated or absent in the presence of RS 15385 (10 μ M, Figure 2). The partial agonist effect was also resistant to pertussis toxin treatment and virtually absent without expression of the mouse $G_{\alpha_{15}}$ protein. This is shown for SKF 86466 when compared to RS 15385 in Table 5.

In order to demonstrate that the partial agonist effect of SKF 86466 is selective for the mouse $G_{\alpha_{15}}$ protein, further experiments were performed by co-expression of the mutant α_{2A} AR with either mouse $G_{\alpha_{15}}$ protein or mouse $G_{\alpha_{15}}$ plus rat $G_{\alpha_{11}}$ protein. The data summarized in Table 6 illustrate that SKF 86466 (1 μ M) was without intrinsic activity upon co-expression of the mouse $G_{\alpha_{15}}$ protein. The partial agonist effect of SKF 86466 was not affected by co-expression of the rat $G_{\alpha_{11}}$ and the mouse $G_{\alpha_{15}}$ proteins. SKF 86466 and RS 15385 did not display intrinsic activity at the wt α_{2A} AR by co-expression of mouse $G_{\alpha_{15}}$ or mouse $G_{\alpha_{15}}$ + rat $G_{\alpha_{11}}$ proteins (not shown).

Blockade of UK 14304-mediated responses by α_2 -adrenergic antagonists at wt α_{2A} and mutant Thr³⁷³Lys α_{2A} -adrenergic receptors

UK 14304 was selected as a highly efficacious agonist to characterize the potencies of the antagonists at either wt α_{2A} and mutant Thr³⁷³Lys α_{2A} AR as measured both by stimulation

Table 1 pK_i values of α_2 -adrenergic ligands at human wt α_{2A} and mutant Thr³⁷³Lys α_{2A} AR in transfected Cos-7 cells

Ligand	Mean pK_i values $\pm$ s.e.mean	
	wt α_{2A} AR	Mutant Thr ³⁷³ Lys α_{2A} AR
<i>Putative antagonist:</i>		
RS 15385	9.19 $\pm$ 0.04	8.94 $\pm$ 0.04*
RX 811059	8.95 $\pm$ 0.07	8.49 $\pm$ 0.06**
MK 912	8.89 $\pm$ 0.08	8.76 $\pm$ 0.07
RX 821002	8.87 $\pm$ 0.07	8.67 $\pm$ 0.05
Atipamezole	8.60 $\pm$ 0.06	8.47 $\pm$ 0.07
Dexefaroxan	8.28 $\pm$ 0.02	8.29 $\pm$ 0.07
SKF 86466	8.00 $\pm$ 0.23	7.88 $\pm$ 0.08
Idazoxan	7.86 $\pm$ 0.06	7.79 $\pm$ 0.09
WB 4101	7.86 $\pm$ 0.08	7.42 $\pm$ 0.07*
BRL 44408	7.80 $\pm$ 0.03	7.64 $\pm$ 0.06
(–)-efaroxan	<5	<5
<i>Agonist:</i>		
Oxymetazoline	7.73 $\pm$ 0.05	8.00 $\pm$ 0.09*
Guanabenz	7.53 $\pm$ 0.03	8.04 $\pm$ 0.03***
Clonidine	7.21 $\pm$ 0.07	7.76 $\pm$ 0.16*
UK 14304	7.15 $\pm$ 0.11	8.09 $\pm$ 0.09***
(–)-Adrenaline	5.91 $\pm$ 0.05	7.21 $\pm$ 0.09***

[³H]RX 821002 binding was performed with membrane preparations of Cos-7 cells as described in Methods. Total and nonspecific binding represented 1639 $\pm$ 223 and 204 $\pm$ 57 d.p.m. for wt α_{2A} AR, and 2409 $\pm$ 136 and 167 $\pm$ 41 d.p.m. for mutant Thr³⁷³Lys α_{2A} AR, respectively. Values were obtained from 3–4 independent experiments, performed in duplicate. Statistical analysis was performed using Student's *t*-test: * P < 0.05; ** P < 0.01; *** P < 0.001.

Table 2 Comparison between inositol phosphates formation in Cos-7 cells transfected with either wt α_{2A} or mutant Thr³⁷³Lys α_2 AR in the absence or presence of mouse $G_{\alpha_{15}}$ protein and blockade by pertussis toxin

Mouse $G_{\alpha_{15}}$	Inositol phosphates formation			
	wt α_{2A} AR	Mutant Thr ³⁷³ Lys α_{2A} AR	wt α_{2A} AR	Mutant Thr ³⁷³ Lys α_{2A} AR
	–	+	–	+
Basal (d.p.m.)	455 $\pm$ 104	378 $\pm$ 135	401 $\pm$ 28	1310 $\pm$ 244*
1 μ M UK 14304 (d.p.m.)	2021 $\pm$ 471	2248 $\pm$ 664	1932 $\pm$ 598	5124 $\pm$ 1083**
1 μ M UK 14304 + pertussis toxin treatment (%)	15.6 $\pm$ 2.8	81.6 $\pm$ 13.0	32.8 $\pm$ 9.9	81.0 $\pm$ 18.0

Transfection of Cos-7 cells was performed as described in Methods. Pertussis toxin treatment (100 ng ml^{–1}) was performed 16 h before stimulation of inositol phosphates in Cos-7 cells and was measured as described in Methods. Basal and UK 14304 (1 μ M)-mediated inositol phosphates formation are expressed as d.p.m. $\pm$ s.e.mean obtained from 3–4 independent experiments, each one performed in quadruplicate. UK 14304 (1 μ M) + pertussis toxin treatment data, expressed versus the corresponding maximal response obtained with UK 14304 (1 μ M) in nontreated cells, are shown as mean $\pm$ s.e.mean obtained from 5–8 independent experiments, performed in quadruplicate. * P < 0.05 versus the other basal responses; ** P < 0.05 versus UK 14304 response at mutant α_{2A} AR without mouse $G_{\alpha_{15}}$ protein expression (Student's *t*-test).

Figure 1 Stimulation of inositol phosphates formation and inhibition of forskolin-induced cyclic AMP formation by α_2 AR agonists in transfected cells expressing either wt α_2 A AR or mutant Thr³⁷³Lys α_2 A AR. Stimulation of agonist-stimulated inositol phosphates formation in Cos-7 cells expressing either wt α_2 A AR or mutant Thr³⁷³Lys α_2 A AR upon co-expression of mouse $G_{\alpha 15}$, and agonist-mediated inhibition of 100 μ M forskolin-induced cyclic AMP formation in C6-glia cells expressing wt α_2 A AR were measured as described in Methods. Representative dose-response curves are shown from 3–4 independent experiments. pEC₅₀ and E_{max} values are summarized in Table 3.

Table 3 pEC₅₀ and E_{max} values of α -adrenergic agonists for stimulating inositol phosphates formation in the presence of mouse $G_{\alpha 15}$ protein, or for inhibiting forskolin-induced cyclic AMP formation in transfected cells expressing either wt α_2 A or mutant Thr³⁷³Lys α_2 A AR

Ligand	wt α_2 A AR Stimulation of inositol phosphates formation in Cos-7 cells		wt α_2 A AR Inhibition of forskolin-induced cyclic AMP formation in C6-glia cells		Mutant Thr ³⁷³ Lys α_2 A AR Stimulation of inositol phosphates formation in Cos-7 cells	
	pEC ₅₀	E _{max} (%)	pEC ₅₀	E _{max} (%)	pEC ₅₀	E _{max} (%)
UK 14304	8.69 ± 0.10	100	8.23 ± 0.13	100	8.99 ± 0.07*	100
Clonidine	8.11 ± 0.10	91.0 ± 14.0	7.36 ± 0.18	89.0 ± 7.0	8.95 ± 0.06***	96.4 ± 2.2
(-)-Adrenaline	8.51 ± 0.04	90.0 ± 5.0	7.18 ± 0.18	94.0 ± 12.0	8.85 ± 0.05**	89.2 ± 6.3
Guanabenz	8.13 ± 0.11	73.0 ± 11.5	7.34 ± 0.21	76.0 ± 10.0	8.91 ± 0.22*	89.0 ± 3.6
Oxymetazoline	8.26 ± 0.07	51.0 ± 3.5	7.34 ± 0.21	80.0 ± 13.0	8.89 ± 0.06***	96.8 ± 5.5

Stimulation of inositol phosphates in Cos-7 cells in the presence of mouse $G_{\alpha 15}$ protein, and inhibition of 100 μ M forskolin-induced cyclic AMP formation in C6-glia cells were measured as described in Methods. E_{max} values are expressed *versus* the response obtained with UK 14304 (1 μ M). pEC₅₀ values are defined as the concentration at which 50% of the maximal response was obtained for each individual compound. Basal and forskolin-stimulated cyclic AMP levels in C6-glia cells were 5.07 ± 0.79 and 85.5 ± 6.26 pmol ml⁻¹ respectively, for wt α_2 A AR. Maximal inhibition with UK 14304 represented 81 ± 2% of forskolin-stimulated cyclic AMP formation. Results are expressed as means ± s.e.mean obtained from 3–4 independent experiments, performed in triplicate. Statistical analysis was performed using Student's *t*-test: **P* < 0.05; ***P* < 0.01; ****P* < 0.001 *versus* pEC₅₀ values for stimulation of inositol phosphates at wt α_2 A AR in Cos-7 cells.

of inositol phosphates in the presence of mouse $G_{\alpha 15}$ protein (Figure 3), and by inhibition of stimulated cyclic AMP formation at wt α_2 A AR (Table 7). In both assay systems, antagonists (1 μ M) inhibited the UK 14304-mediated response at the wt α_2 A AR. (-)-Efaroxan (1 μ M) did not affect the UK 14304-mediated response. The calculated pK_B values (Table 7) for antagonism of the UK 14304-mediated stimulation of inositol phosphates were similar to their corresponding pK_i values. The pK_B values obtained in the cyclic AMP assay were inferior compared to their pK_i values and the pK_B values in the inositol phosphates assay. Partial blockade of the UK 14304-mediated response at the mutant Thr³⁷³Lys α_2 A AR was obtained with atipamezole, dexefaroxan, SKF 86466, idazoxan and BRL 44408 (Figure 3). In contrast, RS 15385, RX 811059,

RX 821002 and WB 4101 antagonized the UK 14304-mediated inositol phosphates response at the mutant α_2 A AR in a manner that appeared competitive (Figure 3) and consistent with their lack of intrinsic activity. The antagonist potencies were inferior (2–7 fold) to their respective pK_i values, and the pK_B values (5–13 fold) at the wt α_2 A AR (Table 7).

Discussion

The present study compares the intrinsic activities of α_2 AR ligands at the wt α_2 A and mutant Thr³⁷³Lys α_2 A AR. This receptor mutant has previously been reported to be constitutively active in HEK 293 cells as well as in a 3T3F442A

preadipose cell line (Ren *et al.*, 1993; Bétuing *et al.*, 1997). We also found evidence for constitutive activity at the mutated α_{2A} AR. Enhanced affinity, potency and/or efficacy for the agonists was observed in line with a precoupled mutant α_{2A} AR:G-protein state. The basal inositol phosphates response was also enhanced but only upon co-expression of the mutant α_{2A} AR

Table 4 Intrinsic activity of antagonists at wt α_{2A} and mutant Thr³⁷³Lys α_{2A} AR in the presence of mouse G_{z15} protein in Cos-7 cells

Ligand	Inositol phosphates formation		
	wt α_{2A} AR <i>E</i> _{max} (%)	Mutant Thr ³⁷³ Lys α_{2A} AR <i>E</i> _{max} (%)	<i>pEC</i> ₅₀
RS 15385	-5.1 ± 6.5	3.2 ± 2.6	
RX 811059	1.2 ± 1.0	-0.4 ± 2.5	
MK 912	0.8 ± 2.1	13.0 ± 1.1	
RX 821002	-0.3 ± 0.7	-1.3 ± 2.2	
Atipamezole	13.0 ± 4.2	27.2 ± 3.5	8.59
Dexefaroxan	3.5 ± 1.3	27.4 ± 6.9	7.52
SKF 86466	3.1 ± 1.4	54.4 ± 9.0	8.00
Idazoxan	3.5 ± 0.5	33.6 ± 9.1	7.52
WB 4101	2.7 ± 2.9	7.2 ± 1.4	
BRL 44408	-1.3 ± 2.0	59.2 ± 4.5	7.62
(-)-Efaroxan		2	

Stimulation of inositol phosphates in Cos-7 cells in the presence of mouse G_{z15} protein was measured as described in Methods. Ligands were tested at 10 μ M, whereas dose-response curves were performed for atipamezole, dexefaroxan, SKF 86466, idazoxan and BRL 44408 at the mutant Thr³⁷³Lys α_{2A} AR. Mean *E*_{max} values $\pm$ s.e.mean are expressed *versus* the maximal response obtained with UK 14304 (1 μ M). *pEC*₅₀ values were derived from the mean dose-response curves and were defined as the concentrations at which 50% of the maximal response was obtained for each individual ligand. Results were obtained from 2–5 independent experiments for wt α_{2A} and mutant Thr³⁷³Lys α_{2A} AR, performed in triplicate.

with the mouse G_{z15} protein. However, this response could not be attenuated in the presence of any of the tested antagonists. Indeed, ligands previously characterized as antagonists at the

Table 5 Effects of pertussis toxin treatment and mouse G_{z15} protein on the intrinsic activity of SKF 86466 and RS 15385 at mutant Thr³⁷³Lys α_{2A} AR in Cos-7 cells

	Inositol phosphates formation Mutant Thr ³⁷³ Lys α_{2A} AR (<i>E</i> _{max} , %)		
	Mouse G _{z15}	+	+
Pertussis toxin	-	-	+
SKF 86466	6.8 ± 3.1	43.8 ± 8.2	54.0 ± 8.8
RS 15385	6.8 ± 7.2	6.8 ± 6.9	-1.3 ± 4.8

Pertussis toxin treatment (100 ng ml⁻¹) was performed 16 h before stimulation of inositol phosphates in Cos-7 cells. Ligands were tested at 1 μ M. Mean *E*_{max} values $\pm$ s.e.mean, expressed *versus* the maximal response of UK 14304 (1 μ M), were obtained from 4 independent experiments, performed in triplicate.

Table 6 Effects of various G-protein subunits on the intrinsic activity of SKF 86466 and RS 15385 at mutant Thr³⁷³Lys α_{2A} AR in Cos-7 cells

G protein subtype	Inositol phosphates formation Mutant Thr ³⁷³ Lys α_{2A} AR (<i>E</i> _{max} , %)	
	SKF 86466	RS 15385
Mouse G _{z15}	48.2 ± 6.1	6.2 ± 2.5
Mouse G _{zq}	4.3 ± 4.1	-0.7 ± 2.2
Mouse G _{z15} + rat G _{z11}	41.3 ± 4.7	-0.7 ± 2.1

Stimulation of inositol phosphates in Cos-7 cells was measured as described in Methods. Ligands were tested at 1 μ M. Mean *E*_{max} values $\pm$ s.e.mean, expressed *versus* the maximal response of UK 14304 (1 μ M), were obtained from 2–6 independent experiments, performed in triplicate.

Figure 2 Intrinsic activity of putative α_{2A} AR antagonists at mutant Thr³⁷³Lys α_{2A} AR upon co-expression of mouse G_{z15} protein. Stimulation of inositol phosphates formation by putative α_{2A} AR antagonists in Cos-7 cells expressing mutant Thr³⁷³Lys α_{2A} AR and mouse G_{z15} protein, in either the absence or presence of RS 15385 (10 μ M), was measured as described in Methods. Dose-response curves were constructed using mean $\pm$ s.e.mean of five independent experiments. *pEC*₅₀ values are summarized in Table 4.

wt α_{2A} AR demonstrated either no intrinsic activity or positive efficacy at the mutant α_{2A} AR upon co-expression with the mouse $G_{\alpha_{15}}$ protein. This observation suggests that some of these ligands may actually possess a partial agonist activity at the wt α_{2A} AR receptor, which is augmented by the facilitating Thr³⁷³Lys mutation. Importantly, this partial agonist effect is dependent on the presence of specific G-protein α -subunits. In particular, SKF 86466 and BRL 84408 displayed in the presence of mouse $G_{\alpha_{15}}$ protein, an intrinsic activity with maximal responses similar to that of oxymetazoline at the wt α_{2A} AR. The partial agonist effect was not attenuated by the

co-expression of the rat $G_{\alpha_{11}}$ protein, and was not observed upon co-expression of the mouse G_{α_q} protein.

The partial agonists did not display modifications in their binding affinities for the mutant α_{2A} AR. The ligands characterized as antagonists at both the wt α_{2A} and mutant α_{2A} AR showed less affinity at the mutant α_{2A} AR, in line with what would be expected for inverse agonists (Westphal & Sanders-Bush, 1994). However, no direct functional evidence was obtained for the occurrence of such inverse agonist activity. The α_2 AR antagonists appear under the present experimental conditions to behave as either neutral antagonists

Figure 3 Antagonism of UK 14304-mediated stimulation of inositol phosphates formation by α_{2A} AR antagonists in transfected Cos-7 cells expressing either wt α_{2A} AR or mutant Thr³⁷³Lys α_{2A} AR and mouse $G_{\alpha_{15}}$ protein. Stimulation of UK 14304-mediated inositol phosphates formation in Cos-7 cells upon co-expression of mouse $G_{\alpha_{15}}$ was measured in either the absence and presence of 1 μ M antagonist as described in Methods. Representative curves are shown from 3–5 independent experiments. pK_B values are summarized in Table 6. In each panel, the dotted lines represent the UK 14304-mediated dose-responses for stimulation of inositol phosphates at wt α_{2A} AR and mutant Thr³⁷³Lys α_{2A} AR, as shown in the upper left-hand panel for RS 15385.

Table 7 Antagonist potencies of α_2 -adrenergic ligands against UK 14304-mediated stimulation of inositol phosphates formation in the presence of mouse $G_{\alpha 15}$ protein or inhibition of forskolin-induced cyclic AMP formation in transfected cells expressing either wt α_{2A} or mutant Thr³⁷³Lys α_{2A} AR

Ligand	wt α_{2A} AR Antagonism of UK 14304-mediated stimulation of inositol phosphates in Cos-7 cells pK_B	wt α_{2A} AR Antagonism of UK 14304-mediated inhibition of forskolin-induced cyclic AMP formation in C6-glia cells pK_B	Mutant Thr ³⁷³ Lys α_{2A} AR Antagonism of UK 14304-mediated stimulation of inositol phosphates in Cos-7 cells pK_B
RS 15385	9.32 ± 0.14	8.29 ± 0.18	8.47 ± 0.13*
RX 811059	8.74 ± 0.08	8.07 ± 0.15	8.05 ± 0.04**
MK 912		8.10 ± 0.26	
RX 821002	8.75 ± 0.07	8.07 ± 0.22	7.83 ± 0.09**
Atipamezole	8.76 ± 0.06	8.10 ± 0.26	
Dexefaroxan	8.27 ± 0.10	7.66 ± 0.32	
SKF 86466	7.69 ± 0.00	7.28 ± 0.20	
Idazoxan	7.70 ± 0.08	7.18 ± 0.24	
WB 4101	8.14 ± 0.05	7.08 ± 0.20	7.04 ± 0.11**
BRL 44408	8.23 ± 0.16	6.96 ± 0.22	

Antagonism of UK 14304-mediated stimulation of inositol phosphates in Cos-7 cells in the presence of mouse $G_{\alpha 15}$ protein and inhibition of 100 μ M forskolin-induced cyclic AMP formation in C6-glia cells were measured as described in Methods. Mean pK_B values $\pm$ s.e.mean were obtained from 3–5 independent experiments, performed in triplicate. Statistical analysis was performed using Student's *t*-test: * $P < 0.01$, ** $P < 0.001$ versus pK_B values for antagonism of UK 14304-mediated stimulation of inositol phosphates at wt α_{2A} AR in Cos-7 cells.

or partial agonists at the mutant α_{2A} AR, depending on the co-expressed G-protein α -subunit.

Specificity of the α_{2A} AR-mediated inositol phosphates responses in Cos-7 cells

The following findings suggest that the observed inositol phosphates responses in transfected Cos-7 cells are mediated by stimulation of a human α_{2A} AR: (i) the absence of a UK 14304-mediated inositol phosphates response in the non-transfected cell type together with a lack of specific [³H] RX 821002 binding; (ii) the observed agonist stimulation of inositol phosphates at wt α_{2A} AR which is consistent with the agonist data on inhibition of forskolin-stimulated cyclic AMP formation in C6-glia cells; (iii) the competitive blockade of UK 14304-mediated responses by the various antagonists at the wt α_{2A} AR, and in particular the rank order of potency of dexefaroxan and its inactive enantiomer (–)-efaroxan, at concentrations similar to their binding affinities; and (iv) the correlation of the binding affinities for UK 14304 at the wt α_{2A} and mutant Thr³⁷³Lys α_{2A} AR in membrane preparations of Cos-7 cells with those measured in transfected HEK 293 cells (Ren *et al.*, 1993). This further extends the observation that α_{2A} AR may mediate multiple second messenger pathways, including the stimulation of polyphosphoinositide hydrolysis (Cotecchia *et al.*, 1990). The ligand binding characteristics at the wt α_{2A} AR in either the absence or the presence of mouse $G_{\alpha 15}$ protein were similar, thus excluding a spontaneous coupling of the wt α_{2A} receptor to this particular G-protein. Inositol phosphates responses at wt α_{2A} AR could be more readily measured in the presence of mouse $G_{\alpha 15}$; the intrinsic activity of the various ligands was apparently not affected by this G-protein. However, the partial agonist effect for some of the antagonists at the mutant α_{2A} AR was only observed upon co-expression of mouse $G_{\alpha 15}$. Therefore, inositol phosphates responses mediated by the coupling of mutant α_{2A} AR to pertussis-toxin insensitive mouse $G_{\alpha 15}$ protein (Milligan *et al.*, 1996; Table 2) are distinct from the responses evoked by mutant α_{2A} AR- pertussis-toxin sensitive G protein coupling in Cos-7 cells. Specific coupling to multiple second messenger pathways by synthetic agonists has previously been observed for α_{2A} , α_{2B} and α_{2C} AR (Eason *et al.*, 1994). Differential G-

protein activation by agonists has been suggested for a splice variant of the pituitary adenylate cyclase activating polypeptide (PACAP) receptor (Spengler *et al.*, 1993) and the *Drosophila* octopamine-tyramine receptor (Robb *et al.*, 1994). In the present study, $G_{\alpha 15}$ protein activation by putative α_2 AR antagonists was observed. The concept that G-protein-coupled receptors can assume different conformations that can selectively and differentially permit their coupling to specific second messenger pathways also receives support from mutagenesis studies. Conversion of Asp79 to asparagine in the second transmembrane domain of the α_{2A} AR results in a selective uncoupling of the receptor from K^+ currents while retaining the receptor's ability to inhibit cyclic AMP production and open voltage-sensitive Ca^{2+} channels (Lakhilani *et al.*, 1996). Ligands may differentially affect multiple active receptor states. As a consequence, multiple receptor:G-protein interactions, each of them specific for a particular G-protein/effector pathway, can be induced and lead to different, maybe distinct, receptor-mediated responses. Therefore, the possibility exists for the development of new ligands for these receptors which could selectively activate a desired second messenger pathway and reduce collateral side effects mediated via other undesired pathways.

Partial agonist activity of antagonists at mutant G-protein-coupled receptors

Partial agonist activity of antagonists has been reported (Cho *et al.*, 1996; Groblewski *et al.*, 1997) at constitutively active mutant human dopamine D₁ and rat AT_{1A} angiotensin II receptors. R(+)SCH 23390, a classical antagonist at the wt D₁ receptor, behaves as a partial agonist at the mutant Leu²⁸⁶Ala D₁ receptor, with an efficacy reaching 40% of the maximal stimulation attained with the full D₁ agonist ($\pm$)SKF 82958. However, like ($\pm$)SKF 82958, R(+) SCH 23390 failed to demonstrate increased affinity at the mutated D₁ receptor, similar to our observations for the partial agonists at the mutant α_{2A} AR. In a similar way, the (Sar¹, Ile⁸)AII and (Sar¹, Ala⁸)AII derivatives, which are peptidic antagonists of the wt rat AT_{1A} angiotensin II receptor, display maximal activities of 52 and 27%, respectively, with unchanged affinities for the mutant Asn¹¹¹Ala AT_{1A} angiotensin II receptor. Otherwise,

induction of partial agonist properties of the AT₂-specific peptide ligand CGP 42112A [27% of the response to the agonist (Sar¹)AII] is associated with an increased affinity for the mutant AT_{1A} angiotensin II receptor (Grobowski *et al.*, 1997). Thus, with the exception of CGP 42112A, partial agonist activity was not accompanied by an increased binding affinity at this latter receptor. However, Noda *et al.* (1996) described a large increase in affinity with the antagonist (Sar¹, Ile⁸) A II for the mutant AT₁ angiotensin receptor. The apparent absence of increased binding affinity may be related to a lack of sensitivity in the models being investigated.

According to the extended ternary complex model of Lefkowitz and colleagues (Lefkowitz *et al.*, 1993; Samama *et al.*, 1993) and refined by Kenakin (1995), the receptor is capable of either spontaneous or ligand-facilitated isomerization to an activated state, which is then capable of coupling to G-proteins. It is hypothesized that the adoption of this activated conformation is favoured by certain mutations that display not only increases in basal activity but also increases in agonist activity, proportional to the intrinsic activity of the agonist. The model described by Lefkowitz and colleagues may hold for constitutively activating mutations that structurally mimic the ligand-activated state of the receptor throughout its entire tertiary structure. Different mutational substrates may very well result in different local effects on binding and G-protein-coupling geometries. It is conceivable that structural alterations near the ligand-binding pocket may modify the nature of ligand/receptor interactions altogether, such that some antagonists are capable of stabilizing the active conformation at the new binding interface generated by the mutation. The Leu²⁸⁶Ala mutation in transmembrane domain VI is within the ligand-binding transmembrane core of the D₁ receptor. The mutation Thr³⁷³Lys, located in the carboxyl-terminal region of the third intracellular loop of the α_2 A AR, seems to favour the coupling of the mutated receptor to the mouse G_{z15} protein. This receptor:G-protein interaction may modify the tertiary structure of the mutated receptor in such a way that some antagonists are capable of stabilizing the active receptor conformation, thereby generating positive efficacy. The Asn¹¹¹Ala mutation in transmembrane domain III of the AT_{1A} angiotensin II receptor is more difficult to interpret since peptidic ligands bind the receptor *via* both transmembrane residues and extracellular loops (Hjorth *et al.*, 1994; Noda *et al.*, 1995).

Partial agonist activity for certain antagonists has also been measured at mutated G-protein-coupled receptors which do not display constitutive activity. The inverse agonist 11-cis-retinal behaves as a partial agonist at the Gly¹²¹Leu mutation in transmembrane domain III of rhodopsin (Han *et al.*, 1997). The single amino acid substitution Asp¹¹³Glu in the third transmembrane domain of the hamster β_2 AR promotes partial agonist activity for pindolol, oxyprenolol and alprenolol, ligands that act as antagonists at the wt β_2 AR (Strader *et al.*, 1989). The other β AR antagonists being investigated did not demonstrate intrinsic activity at the mutant β_2 AR. However, the affinity of the agonist isoproterenol was also reduced 100 fold, suggesting that this mutant receptor has partially lost both affinity and specificity for activation by ligands. This contrasts with our binding data at the α_2 A AR where both ligand affinity and specificity are conserved. Strader *et al.* (1989) explained their findings by suggesting the existence of overlapping binding sites for agonists and antagonists. Claude *et al.* (1996) hypothesize that antagonists with high structural homology to agonists are capable of agonist activity if the receptor is permissive. This was based on their observation that the mutation of conserved serine residues (Ser¹⁷⁷Leu or Ser¹⁹⁶Leu) in transmembrane domain IV of μ -opioid receptors confers full agonistic properties to peptide (TIPP ϕ) and alkaloid antagonists (naloxone, naltrexone and naltriben). A similar explanation may also account for atipemazole and BRL 44408 in our study since their structures resemble the α_2 AR agonist medetomidine.

In conclusion, the results provided in this study imply that the pharmacology of constitutively active receptors is more complex than is commonly assumed. The mutant Thr³⁷³Lys α_2 A AR facilitates detection of partial agonist activity for a subclass of putative antagonists. This further supports the hypothesis that pure neutral antagonists may be relatively uncommon. Targeting of these mutant receptors may open new possibilities to manipulate the α_2 A AR receptor.

We acknowledge the cooperation of Mrs C. Palmier and Dr I. Rauly. We sincerely thank the excellent technical assistance of M.-C. Ailhaud, J.-C. Blanchet, C. Cathala, F. Finana and F. Lestienne, and fruitful secretarial assistance of S. Cecco.

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(Received July 23, 1998

Revised October 30, 1998

Accepted November 20, 1998)