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Disparate ligand-mediated Ca^{2+} responses by wild-type, mutant Ser^{200}Ala and Ser^{204}Ala α_{2A}-adrenoceptor : $G_{\alpha 15}$ fusion proteins: evidence for multiple ligand-activation binding sites

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1 Ligand : receptor interactions were analysed at wt, mutant Ser^{200}Ala and Ser^{204}Ala α_{2A} ARs by measuring Ca^{2+} responses in CHO-K1 cells either by co-expression with a $G_{\alpha 15}$ protein or at a receptor : $G_{\alpha 15}$ protein stoichiometry of 1.0 using fusion proteins.

2 The magnitude of the UK 14304-mediated Ca^{2+} response as elicited by a $G_{\alpha 15}$ protein was largest with both mutant Ser^{200}Ala and Ser^{204}Ala α_{2A}ARs compared to the wt α_{2A} AR in the co-expression and fusion protein experiments.

3 The activation profiles of the wt and both mutant α_{2A} ARs as analysed by a series of α_2 AR agonists differed. d-Medetomidine and clonidine appeared most efficacious at the Ser^{204}Ala α_{2A} AR, whereas oxymetazoline was also partially active at the Ser^{200}Ala α_{2A} AR. Talipexole was silent at both mutant α_{2A} ARs. The intrinsic activity of (−)-adrenaline was either absent or partial at the Ser^{204}Ala and Ser^{200}Ala α_{2A} AR, respectively. This latter observation is related to its lower binding affinity for both mutant α_{2A} ARs.

4 Ligands characterized as antagonists at wt and Ser^{200}Ala α_{2A} ARs demonstrated either no intrinsic activity (i.e., RX 811059) or positive efficacy with a different rank order of maximal response at the Ser^{204}Ala α_{2A} AR (atipamezole = SKF 86466 = idazoxan > dexefaroxan) than Asp^{79}Asn α_{2A} AR (atipamezole > idazoxan ≃ SKF 86466 > dexefaroxan) and Thr^{373}Lys α_{2A} AR (SKF 86466 > atipamezole ≃ idazoxan > dexefaroxan). These effects were only observed in the co-expression experiments at concentrations in line with their binding affinities.

5 In conclusion, these Ca^{2+} data suggest that multiple activation binding sites exist for these ligands at the α_{2A} AR, and that their activation may be affected in different ways by the mutations being investigated.

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Abbreviations: α_2 AR, α_2-adrenoceptor; RX 811059, 2-(2-ethoxy-2,3-dihydro-benzo[1,4]dioxin-2-yl)-4,5-dihydro-1*H*-imidazole; RX 821002, 2-(2-methoxy-2,3-dihydro-benzo[1,4]dioxin-2-yl)-4,5-dihydro-1*H*-imidazole; SKF 86466, 6-chloro-2,3,4,5-tetrahydro-3-methyl-1*H*-3-benzazepine; UK 14304, 5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline tartrate

Introduction

α_2-Adrenoceptors (α_2 ARs) are implicated in the control of noradrenergic and non-noradrenergic neurotransmission in the central nervous system and modulate several physiological processes peripherally (Timmermans *et al*., 1990; French, 1995; Szabadi & Bradshaw, 1996). There are now three characterized α_2 AR subtypes: α_{2A}, α_{2B} and α_{2C}; these are G protein-coupled receptors which are predominantly coupled to the $G_{i/o}$ signalling system, inhibiting and/or stimulating the activity of adenylate cyclase, inhibiting the opening of voltage-gated Ca^{2+} channels and activating K^+ channels (see Hein & Kobilka, 1997). The α_2 ARs may also couple to other intracellular pathways involving Na^+/H^+ exchange and the activation of phospholipase A_2, C and D (Limbird, 1988; Cotecchia *et al*., 1990; Fraser *et al*., 1989; Kukkonen *et al*., 1998; McNulty *et al*., 1992). The α_2 AR subtypes are distributed differentially in cells and tissues (MacDonald *et al*., 1997), endowing these receptors with different physiological functions and pharmacological activity profiles. However, most available ligands have only marginal α_2 AR subtype selectivity.

Several reports (Wang *et al*., 1991; Eason *et al*., 1994; Airriess *et al*., 1997; Rudling *et al*., 1999) have shown that both naturally occurring and synthetic agonists may induce agonist-specific α_{2A} AR-mediated responses. This specificity was based on coupling of the α_{2A} AR to different second messenger pathways. We recently analysed ligand-mediated Ca^{2+} responses between wt, Asp^{79}Asn and Thr^{373}Lys α_{2A} ARs by examining their coupling to a single signal transduction pathway *via* a $G_{\alpha 15}$ protein (Pauwels & Colpaert, 2000). The data suggest that most of the α_2 AR antagonists may actually possess partial agonist activity, which is augmented by the facilitating Asp^{79}Asn and Thr^{373}Lys α_{2A} AR mutations. A different rank order of maximal responses was observed for the agonists and putative antagonists being investigated between the wt and Asp^{79}Asn α_{2A} ARs, and between Asp^{79}Asn and Thr^{373}Lys α_{2A} ARs. Therefore, some of these ligands activate the α_{2A} AR *via* a specific binding site, which can be influenced by the Asp^{79}Asn and/or Thr^{373}Lys α_{2A} AR mutations. We

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concluded that there are probably multiple molecular mechanisms to activate a single α_{2A} AR subtype. Thus, pharmacological diversity not only occurs between various receptor subtypes, but may also occur at a single α_{2A} AR subtype.

In the present study, analyses of ligand:receptor interactions were made at Ser^{200}Ala and Ser^{204}Ala α_{2A} ARs. Previous studies (Strader *et al*., 1989; Wang *et al*., 1991; Hwa & Perez, 1996) have emphasized the importance of conserved serine residues in transmembrane domain V of adrenoceptors in possible hydrogen bond interactions with the *para*- and *meta*-hydroxyl groups of the phenyl ring of catecholamines in activation processes. Studies on the α_{2A} AR suggest that only Ser^{204}, which is thought to interact with the *para*-hydroxyl of the catecholamine ring, appears to contribute partially to agonist-binding and receptor activation. Ser^{200} does not appear to be directly involved in receptor activation in contrast to the corresponding serine residue (Ser^{204}) in the β_2 AR, which has been postulated to interact with the *meta*-hydroxyl group of catecholamines. Recently, Ser^{204} has been shown to prevent *meta*-octopamine from generating a receptor-agonist conformation that can increase cyclic AMP levels, illustrating the importance of this residue in the agonist-specific coupling of the α_{2A} AR to different second messenger systems (Rudling *et al*., 1999). The present report considers the agonist properties of several imidazoline derivatives, the azepine derivative talipexole and putative α_2 AR antagonists by comparing wt and both mutant Ser^{200}Ala and Ser^{204}Ala α_{2A} ARs under conditions of co-expression and fusion with a $G_{\alpha 15}$ protein. The fusion protein approach was chosen to exclude differences in receptor coupling efficacy to the $G_{\alpha 15}$ protein and to quantify intrinsic activities of ligands under controlled experimental conditions of a fixed receptor : $G_{\alpha 15}$ protein ratio of 1.0 (Pauwels *et al*., 2000a). Experiments were also performed in parallel by co-expression of the mutant α_{2A} ARs with a $G_{\alpha 15}$ protein. We previously observed that the amplitude of the intrinsic activity of a partial agonist at wt α_{2A} AR was greater in co-expression than fusion protein experiments (Pauwels *et al*., 2000). The data support disparate ligand-mediated Ca^{2+} responses by wt and mutant α_{2A} ARs. These data are compared with the Ca^{2+} data as obtained by fusion of the Asp^{79}Asn α_{2A} AR and Thr^{373}Lys α_{2A} AR to a $G_{\alpha 15}$ protein. Multiple ligand-activation binding sites exist at the α_{2A} AR; their activation is affected in different ways by the mutations being investigated.

Method

Construction of mutant α_{2A} ARs and their fusion to a $G_{\alpha 15}$ protein

The mutant Ser^{200}Ala α_{2A} AR cDNA (T^{600}CG to GCG codon) was generated starting from the wt human α_{2A} AR (R.C: 2.1.ADR.A2A, Genbank accession number: M23533) cloned in a pCR3.1 expression plasmid as previously described (Wurch *et al*., 1999) by using a Quick Change site-directed mutagenesis kit according to the supplier's instructions. The fusion of the mutant Ser^{200}Ala α_{2A} AR to a $G_{\alpha 15}$ protein (Genbank accession number: M80632) was realized in two steps. First, the stop codon of the Ser^{200}Ala α_{2A} AR cDNA was exchanged by PCR for an alanine as part of a *Not*I restriction site in frame with its coding sequence; a *Not*I site was also inserted at the 5′ end of a $G_{\alpha 15}$ protein cDNA according to its reading frame. Second, the fusion of the mutant Ser^{200}Ala α_{2A} AR to the $G_{\alpha 15}$ protein was made after *Not*I restriction of both plasmids and subsequent ligation, as previously described (Pauwels *et al*., 2000b). The resulting fusion protein consists of the mutant Ser^{200}Ala α_{2A} AR in which the stop codon has been replaced by an alanine, followed by two additional alanine residues generated by the *Not*I restriction site and by the entire $G_{\alpha 15}$ protein coding sequence. The mutant Ser^{200}Ala α_{2A} AR and the Ser^{200}Ala α_{2A} AR : $G_{\alpha 15}$ fusion protein cDNA were cloned into a pCR3.1 plasmid and fully sequenced on an ABI 310 Genetic Analyser, confirming the respective nucleotide sequences. The mutant Ser^{204}Ala α_{2A} AR cDNA (T^{612}CC to GCC codon) was constructed and fused to a $G_{\alpha 15}$ protein cDNA in a similar way. Fusion of the mutant $Asp^{79}Asn_{\alpha 2A}$ AR and Thr^{373}Lys α_{2A} AR (Pauwels and Colpaert, 2000) to a $G_{\alpha 15}$ protein was realized after exchange of the α_{2A} AR stop codons for a *Not*I restriction site and subsequent ligation as described above.

Measurement of intracellular Ca^{2+} responses

Subconfluent CHO-K1 cells were transiently transfected by electroporation (Bio-Rad electroporator: 250 mV, 250 μF) with the indicated receptor and/or $G_{\alpha 15}$ protein construct (10 μg of each plasmid) and plated into 96-well plates with 0.2 ml nutrient mixture Ham's F12 plus 10% heat-inactivated foetal calf serum and 1% DMSO at about 60,000 cells per well. Cells were assayed for intracellular Ca^{2+} responses between 24–48 h upon transfection as previously described (Pauwels *et al*., 2000b). The culture medium was removed by aspiration and replaced by 0.1 ml of complete growth medium containing 20 mM HEPES, 2.5 mM probenicid acid and 2 μM Fluo 3 fluorescent calcium indicator dye for 1 h. Cells were washed and fluorescent readings were made for 3 min every 2 s using a fluorometric imaging plate reader (FLIPR, Molecular Devices). Maximal fluorescent counts were used to determine agonist activity. The instrument software normalizes the fluorescence reading to give initial readings for each well at time zero. Ca^{2+} responses were systematically measured in either the absence (basal) or presence of either 10 μM (−)-adrenaline or 1 μM UK 14304, and the difference with the basal condition was defined as the maximal Ca^{2+} response. E_{max} values of ligand responses were referred to the maximal response as mediated by 10 μM (−)-adrenaline for wt, mutant Asp^{79}Asn and Thr^{373}Lys α_{2A} ARs. In case of Ser^{200}Ala and Ser^{204}Ala α_{2A} ARs, responses were referred to the maximal response as mediated by 1 μM UK 14304. pEC_{50} values were defined as the ligand concentration at which 50% of its own maximal stimulation of Ca^{2+} response was obtained. In antagonist experiments, (+)-RX 811059 (1 μM) was co-incubated with the agonist. 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 EC_{50} values of the agonist measured in either the absence or presence of antagonist, respectively. Statistical analysis was performed by one-way analysis of variance followed by all pairwise multiple comparison procedures (Tukey-test).

Ligand binding assay to α_{2A}-adrenoceptors

Membrane preparations of the transfected CHO-K1 cells were prepared in 50 m Tris-HCl pH 7.6 as previously described (Wurch *et al*., 1999). Binding assays were performed with 0.3–10 or 100 (Ser^{204}Ala α_{2A} AR) nM [^{3}H]-(1,4-[6,7(n)-[^{3}H]-benzodioxan-2-methoxy-2-yl)-2-imidazoline hydrochloride (RX 821002). Incubation mixtures consisted of 0.4 ml cell membranes (30 μg protein), 0.05 ml radioligand and 0.05 ml of compound or phentolamine (10 μM) to determine non-specific binding. The reactions were stopped after a 30 min incubation at 25°C by adding 3.0 ml ice-cold 50 mM Tris-HCl pH 7.6 and rapid filtration over Whatman GF/B glass fibre

filters using a Brandel harvester, washed and counted as described (Wurch *et al*., 1999). pK_D and B_{max} values were obtained from saturation binding studies performed as previously described (Wurch *et al*., 1999). pIC_{50} values for ligands as obtained from dose-response curves performed at six concentrations were converted into pK_i values as described (Wurch *et al*., 1999).

Protein content

Membrane protein levels were estimated with a dye-binding assay using a Bio-Rad kit, bovine serum albumin was used as a standard (Bradford, 1976).

Materials

The pCR3.1 vector was from In Vitrogen (San Diego, U.S.A.). The ABI Prism 310 Genetic Analyser and the dichlororhodamine terminator cycle sequencing kit were purchased from Perkin Elmer (Foster City, U.S.A.). The Quick change site-directed mutagenesis kit was from Stratagene (La Jolla, U.S.A.). CHO-K1 cells were obtained from ATCC (Rockville, U.S.A.). [^{3}H]-RX 821002 (50 Ci mmol^{-1}) was obtained from Amersham (Les Ulis, France). Fluo-3 was obtained from Molecular Probes (Oregon, U.S.A.). Probenicid acid, clonidine, (−)-adrenaline and oxymetazoline were from Sigma (St. Louis, U.S.A.). 5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline tartrate (UK 14304), dexefaroxan, atipamezole and (+)-2-(2-ethoxy-2,3-dihydro-benzo[1,4]dioxin-2-yl)-4,5-dihydro-1*H*-imidazole (RX 811059) were prepared intramuros. 6-Chloro-2,3,4,5-tetrahydro-3-methyl-1*H*-3-benzazepine (SKF 86466) was from Smith Kline Beecham (Herts, U.K.). Idazoxan and RX 821002 were from Reckitt and Colman (Kingston-upon-Hill, U.K.). Talipexole was a gift from Boehringer Ingelheim (Biberach an der Riss, Germany). d-Medetomidine was purchased from Smith Kline Beecham. Stock solutions of ligands were prepared at 10^{-3} M. Serial dilutions were made in the respective incubation buffer.

Results

Binding properties of Ser^{200}Ala α_{2A} AR and Ser^{204}Ala α_{2A} AR compared to wt α_{2A} AR

[^{3}H]-RX 821002 saturation binding curves performed to membrane preparations of CHO-K1 cells transfected with either a wt α_{2A} AR, mutant Ser^{200}Ala α_{2A} AR or mutant Ser^{204}Ala α_{2A} AR in the co-presence of $G_{\alpha 15}$ protein indicated for each receptor a single class of high affinity binding sites for [^{3}H]-RX 821002. The pK_D value of [^{3}H]-RX 821002 was twice enhanced at the mutant Ser^{200}Ala α_{2A} AR, whereas it was 9 fold attenuated at the Ser^{204}Ala α_{2A} AR (Table 1). The binding capacity of [^{3}H]-RX 821002 was also reduced by 78% at the Ser^{204}Ala α_{2A} AR. Analysis of the binding profile for each of these α_{2A} ARs with a diverse series of α_2 AR ligands indicated a loss in affinity for the native agonist (−)-adrenaline at both mutant α_{2A} ARs. The binding affinity of the other ligands being investigated were either not affected or maximally 2.6 fold enhanced at the mutant Ser^{200}Ala α_{2A} AR. The putative antagonists (+)-RX 811059, RX 821002, atipamezole, SKF 86466 and idazoxan showed a decreased affinity (6–15 fold) at the mutant Ser^{204}Ala α_{2A} AR. In contrast, the binding affinities of the imidazoline derivatives oxymetazoline, d-medetomidine, clonidine and UK 14304, and the azepine derivative talipexole were either not affected or 2–3 fold higher at the mutant Ser^{204}Ala α_{2A} AR (Table 2).

Ligand-mediated Ca^{2+} responses by Ser^{200}Ala α_{2A} AR compared to wt α_{2A} AR

UK 14304 (1 μM) produced a time-dependent increase in the intracellular Ca^{2+} concentration in CHO-K1 cells transiently co-transfected with a Ser^{200}Ala α_{2A} AR and a $G_{\alpha 15}$ protein (Figure 1); its magnitude was about 2 fold greater compared to the UK 14304-mediated response by the wt α_{2A} AR. The kinetic properties of the UK 14304-mediated Ca^{2+} response

Table 1 pK_D and B_{max} values of [^{3}H]-RX 821002 binding to wt and mutant α_{2A} AR

	wt α_{2A} AR	*Ser^{200}Ala α_{2A} AR*	*Ser^{204}Ala α_{2A} AR*
pK_D	8.54±0.02	8.90±0.03	7.58±0.04
B_{max} (pmol mg^{-1} protein)	29.58±4.10	32.92±4.08	6.63±0.49

[^{3}H]-RX 821002 saturation binding and Scatchard analysis to membrane preparations of CHO-K1 cells co-transfected with 10 μg of either wt α_{2A} AR, Ser^{200}Ala α_{2A} AR or Ser^{204}Ala α_{2A} AR and 10 μg of $G_{\alpha 15}$ protein was performed as described in Methods. Data are presented as mean values±s.e.mean of three independent transfection experiments.

Table 2 pK_i values of α_2 AR ligands for inhibition of [^{3}H]-RX 821002 binding to wt, Ser^{200}Ala and Ser^{204}Ala α_{2A} ARs in CHO-K1 cellular membranes

	Ser^{200}Ala α_{2A} AR		*Ser^{204}Ala α_{2A} AR*		*wt α_{2A} AR*	
	pK_i	*s.e.mean*	*pK_i*	*s.e.mean*	*pK_i*	*s.e.mean*
(+)-RX 811059	8.84	0.03	7.81	0.11	8.86	0.12
RX 821002	8.66	0.01	7.57	0.14	8.57	0.08
Atipamezole	8.60	0.04	7.63	0.07	8.39	0.07
Dexefaroxan	8.20	0.02	7.10	0.06	8.18	0.07
Oxymetazoline	8.11	0.01	8.22	0.08	7.69	0.06
d-Medetomidine	7.95	0.02	7.60	0.07	7.58	0.08
SKF 86466	7.77	0.03	6.77	0.15	7.68	0.10
Idazoxan	7.73	0.03	6.46	0.09	7.63	0.08
Clonidine	6.84	0.01	6.54	0.04	6.91	0.09
UK 14304	6.58	0.02	7.31	0.01	6.82	0.13
Talipexole	6.37	0.02	6.23	0.06	6.12	0.05
(−)-Adrenaline	<5.00		<5.00		5.60	0.13

Membrane preparations were prepared from CHO-K1 cells co-transfected with either wt or mutant α_{2A} AR and $G_{\alpha 15}$ protein as described in Table 1. Radioligand binding was performed at respective pK_D values (see Table 1) as described in Methods. Data are presented as mean values±s.e.mean of three independent transfection experiments.

were similar for both the wt and mutant $Ser^{200}Ala$ α_{2A} AR. A comparison between the Ca^{2+} responses of various α_2 AR agonists at the mutant $Ser^{200}Ala$ α_{2A} AR in the co-presence of a $G_{\alpha15}$ protein is illustrated in Figure 2. The corresponding pEC_{50} and E_{max} values are compared in Table 3 to the values as obtained under similar experimental conditions with the wt α_{2A} AR. (−)-Adrenaline showed still the capacity to maximally activate the $Ser^{200}Ala$ α_{2A} AR though with a 45 fold decrease in potency as compared to the wt α_{2A} AR. The Ca^{2+} response as mediated by d-medetomidine was similar to that of UK 14304 at both the wt and mutant $Ser^{200}Ala$ α_{2A} AR. Oxymetazoline and clonidine behaved more efficaciously at the $Ser^{200}Ala$ α_{2A} AR; their maximal response was enhanced by +29 and +23% respectively. Talipexole displayed a weak partial agonist response at the $Ser^{200}Ala$ α_{2A} AR; its potency (71 fold) and maximal response (−59%) were attenuated. Each of these agonist-mediated Ca^{2+} responses was antagonized in a competitive manner by 1 μM of the silent antagonist (+)-RX 811059; the pK_B values for the antagonism of the UK 14304 response by (+)-RX 811059 were similar for both the wt and mutant $Ser^{200}Ala$ α_{2A} AR (Table 3).

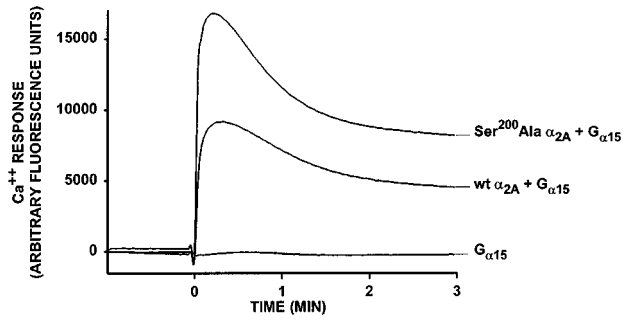

Figure 1 Comparison between kinetics of Ca^{2+} responses as mediated by UK 14304 in CHO-K1 cells transfected with either wt α_{2A} AR or $Ser^{200}Ala$ α_{2A} AR in combination with a $G_{\alpha15}$ protein. Ca^{2+} responses were measured as described in Methods. Tracings were expressed in arbitrary fluorescence units and illustrate a representative experiment. The magnitude of Ca^{2+} response was 8033±914 (n=51) and 15 498±1431 (n=10) arbitrary fluorescence units for respectively the wt α_{2A} AR and $Ser^{200}Ala$ α_{2A} AR. The time of onset for maximal activation (15.4±1.2 s, n=10) and the residual activity upon 1 min of maximal activation (69.6±2.1%, n=10) for the $Ser^{200}Ala$ α_{2A} AR were not different from the properties at the wt α_{2A} AR.

Ligand-mediated Ca^{2+} responses by $Ser^{204}Ala$ α_{2A} AR compared to wt α_{2A} AR

UK 14304 (1 μM) also induced a Ca^{2+} response at the $Ser^{204}Ala$ α_{2A} AR with kinetics similar to the response mediated by the wt α_{2A} AR (Figure 3). Figure 4 shows agonist-dependent Ca^{2+} responses at the $Ser^{204}Ala$ α_{2A} AR. Ca^{2+} responses as mediated by UK 14304, d-medetomidine

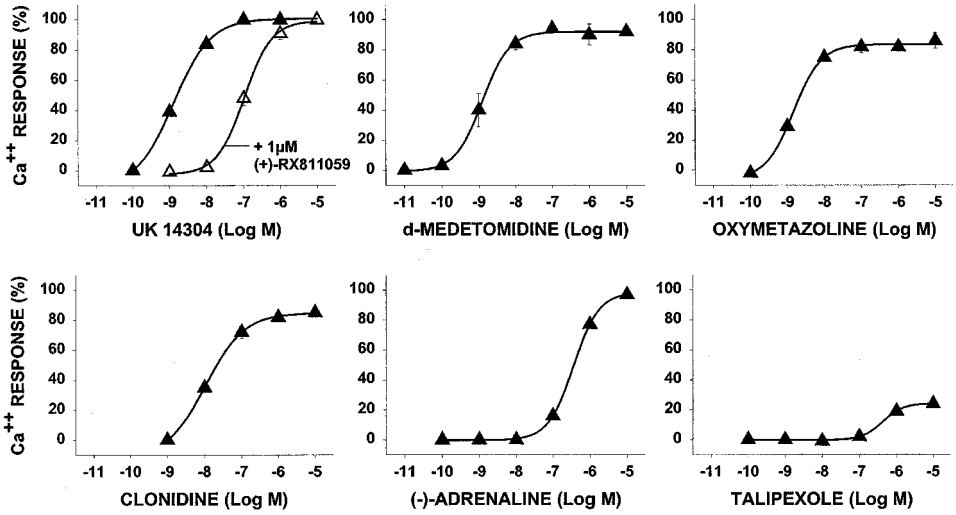

Figure 2 Comparison between α_2 AR agonist-induced Ca^{2+} responses as obtained in CHO-K1 cells co-transfected with a $Ser^{200}Ala$ α_{2A} AR and $G_{\alpha15}$ protein. Ca^{2+} responses were measured as described in Methods and expressed as a percentage of the maximal Ca^{2+} response induced by UK 14304 (1 μM). Curves were constructed using mean values±s.e.mean obtained in 2–3 independent transfection experiments. pEC_{50}, pK_B and E_{max} values are summarized in Table 3.

Table 3 pEC_{50}, E_{max} and pK_B values of ligands' Ca^{2+} responses mediated by wt and mutant α_{2A} ARs in the co-presence of $G_{\alpha15}$ protein in CHO-K1 cells

	$Ser^{200}Ala$		*$Ser^{204}Ala$*		*wt†*	
α_{2A} AR:	*pEC_{50}*	*E_{max} (%)*	*pEC_{50}*	*E_{max} (%)*	*pEC_{50}*	*E_{max} (%)*
UK 14304	8.81±0.02	100	9.28±0.08	100	8.89	96
UK 14304 plus 1 μM (+)-RX 811059	7.94±0.10*		7.55±0.20*		8.05*	
(−)-Adrenaline	6.41±0.11	97±1	5.66±0.06	33±5#	8.06	100
d-Medetomidine	8.83±0.24	94±2	9.30±0.14	97±6	8.92	88
Oxymetazoline	8.70±0.21	86±5	8.29±0.10	52±5#	8.57	57
Clonidine	7.81±0.06	85±0	8.43±0.18	95±1	8.24	62
Talipexole	6.24±0.14	24±3#	6.09±0.13	15±2#	8.09	83

Ca^{2+} responses at $Ser^{200}Ala$ and $Ser^{204}Ala$ α_{2A} ARs were measured as described in Methods. Ligand-mediated responses are expressed as a percentage of the respective maximal response induced by UK 14304 (1 μM) and values are given as mean values±s.e.mean*. pK_B value of (+)-RX 811059; †values are taken from Pauwels and Colpaert (2000); # $P<0.05$ *versus* UK 14304 value.

and oxymetazoline were similar at both the wt and mutant Ser^{204}Ala α_{2A} ARs (Table 3). The response as mediated by clonidine was highly efficacious; its maximal response was enhanced by +33% compared to the wt α_{2A} AR. Otherwise, micromolar concentrations of (−)-adrenaline and talipexole only demonstrated a small Ca^{2+} effect at the Ser^{204}Ala α_{2A} AR. Besides the agonists, most of the putative α_2 AR antagonists displayed intrinsic activity at nanomolar concentrations at the Ser^{204}Ala α_{2A} AR (Figure 5). RX 811059 appeared to be the only antagonist with no observable intrinsic activity at this mutant α_{2A} AR. Its positive enantiomer antagonized in a potent manner (pK_B: 7.55±0.20) the UK 14304-mediated response (Figure 4) in line with its binding affinity for the Ser^{204}Ala α_{2A} AR (Table 2).

Ligand-mediated Ca^{2+} responses as controlled by mutant α_{2A} AR : $G_{\alpha 15}$ fusion proteins

Another set of Ca^{2+} experiments was performed with fusion proteins between either the Ser^{200}Ala or Ser^{204}Ala α_{2A} AR and a $G_{\alpha 15}$ protein in order to determine the ligand-mediated Ca^{2+} responses under controlled expression conditions at a receptor : G_α protein stoichiometry of 1.0. The kinetics of the UK 14304-mediated responses for the fusion proteins and the corresponding co-expression experiments are shown in Figure 6; both fusion proteins displayed a slower onset time ($P<0.05$) and a maximal Ca^{2+} response with a lower magnitude ($P<0.05$). The attenuation of the Ca^{2+} response upon maximal activation was only significantly ($P<0.05$) different between the mutant Ser^{204}Ala α_{2A} AR co-expressed or fused with a $G_{\alpha 15}$ protein. Table 4 compares the agonist-mediated maximal Ca^{2+} responses as obtained with mutant Ser^{200}Ala, Ser^{204}Ala and wt α_{2A} ARs as fused with a $G_{\alpha 15}$ protein together with those obtained with the Asp^{79}Asn and Thr^{373}Lys α_{2A} ARs. The degree of maximal activation by d-medetomidine and clonidine *versus* that of UK 14304 was enhanced at the Ser^{204}Ala α_{2A} AR : $G_{\alpha 15}$, Asp^{79}Asn α_{2A} AR : $G_{\alpha 15}$ and Thr^{373}Lys

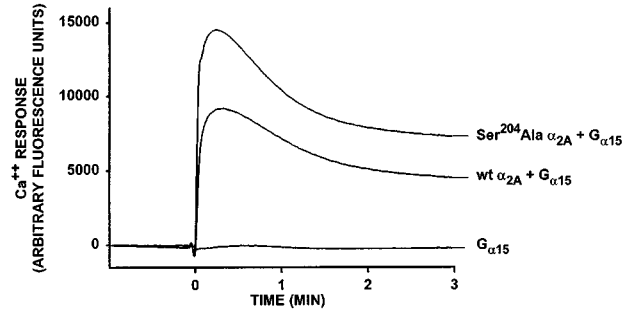

Figure 3 Comparison between kinetics of Ca^{2+} responses as mediated by UK 14304 in CHO-K1 cells transfected with either wt α_{2A} AR or Ser^{204}Ala α_{2A} AR in combination with a $G_{\alpha 15}$ protein. Ca^{2+} responses were measured as described in Methods. Tracings were expressed in arbitrary fluorescence units and illustrate a representative experiment. The magnitude of Ca^{2+} response was 8033±914 ($n=51$) and 14 180±1232 ($n=13$) arbitrary fluorescence units for respectively the wt α_{2A} AR and Ser^{204}Ala α_{2A} AR. The time of onset for maximal activation (16.5±1.0 s, $n=13$) and the residual activity upon 1 min of maximal activation (71.9±1.4%, $n=13$) for the Ser^{204}Ala α_{2A} AR were not different from the properties at the wt α_{2A} AR.

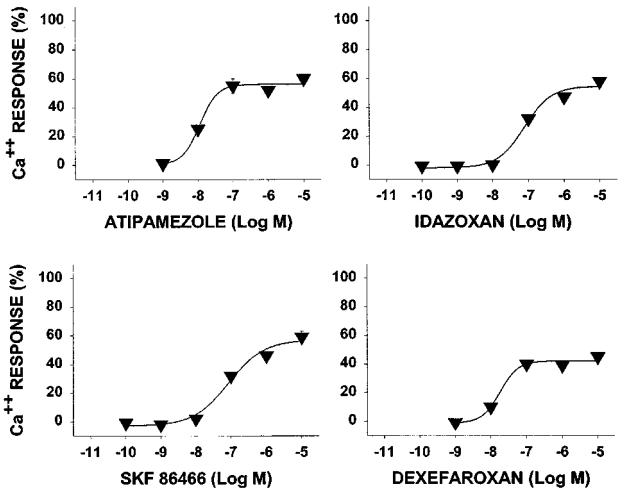

Figure 5 Comparison between Ca^{2+} responses of various putative α_2 AR antagonists in CHO-K1 cells co-transfected with a Ser^{204}Ala α_{2A} AR and $G_{\alpha 15}$ protein. Ca^{2+} responses were measured as described in Methods and expressed as a percentage of the maximal Ca^{2+} response induced by UK 14304 (1 μM). Curves were constructed using mean values±s.e.mean obtained in two independent transfection experiments. pEC_{50} and E_{max} values are summarized in Table 5.

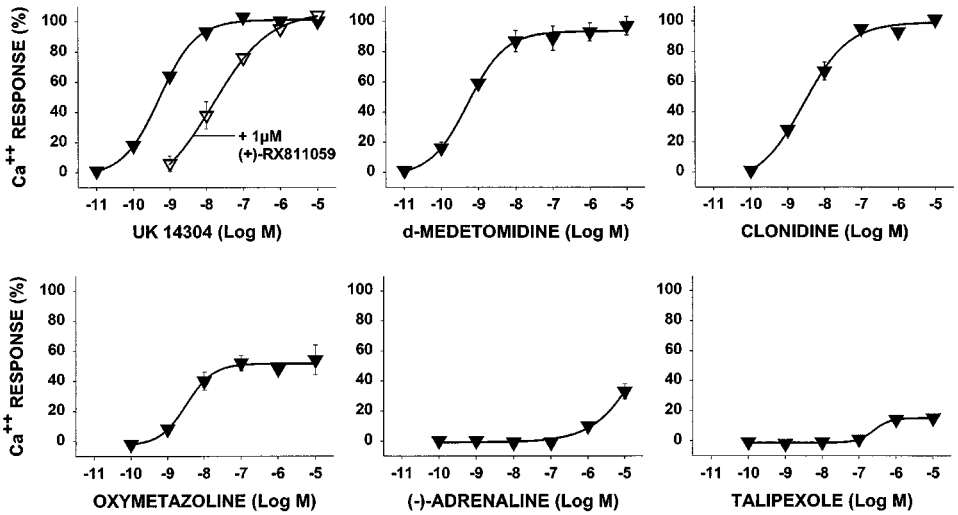

Figure 4 Comparison between α_{2A} AR agonist-induced Ca^{2+} responses as obtained in CHO-K1 cells co-transfected with a Ser^{204}Ala α_{2A} AR and $G_{\alpha 15}$ protein. Ca^{2+} responses were measured as described in Methods and expressed as a percentage of the maximal Ca^{2+} response induced by UK 14304 (1 μM). Curves were constructed using mean values±s.e.mean obtained in 2–4 independent transfection experiments. pEC_{50} pK_B and E_{max} values are summarized in Table 3.

α_{2A} AR : $G_{\alpha15}$ fusion proteins as compared to the wt α_{2A} AR : $G_{\alpha15}$ fusion protein. Oxymetazoline that appeared silent at the Ser^{204}Ala α_{2A} AR : $G_{\alpha15}$, Asp^{79}Asn α_{2A} AR : $G_{\alpha15}$ and wt α_{2A} AR : $G_{\alpha15}$ fusion proteins showed a partial agonist response at the Ser^{200}Ala α_{2A} AR : $G_{\alpha15}$ and Thr^{373}Lys α_{2A} AR : $G_{\alpha15}$ fusion proteins. The partial agonist response of talipexole at the wt α_{2A} AR : $G_{\alpha15}$ fusion protein was either fully abolished at the Ser^{200}Ala α_{2A} AR : $G_{\alpha15}$ and Ser^{204}Ala α_{2A} AR : $G_{\alpha15}$ fusion proteins or highly efficacious at the Asp^{79}Asn α_{2A} AR : $G_{\alpha15}$ and Thr^{373}Lys α_{2A} AR : $G_{\alpha15}$ fusion proteins. The response as mediated by (−)-adrenaline was respectively partial and absent at the Ser^{200}Ala α_{2A} AR : $G_{\alpha15}$ and Ser^{204}Ala α_{2A} AR : $G_{\alpha15}$ fusion proteins. None of the investigated α_2 AR antagonists revealed intrinsic activity at these fusion proteins (not shown).

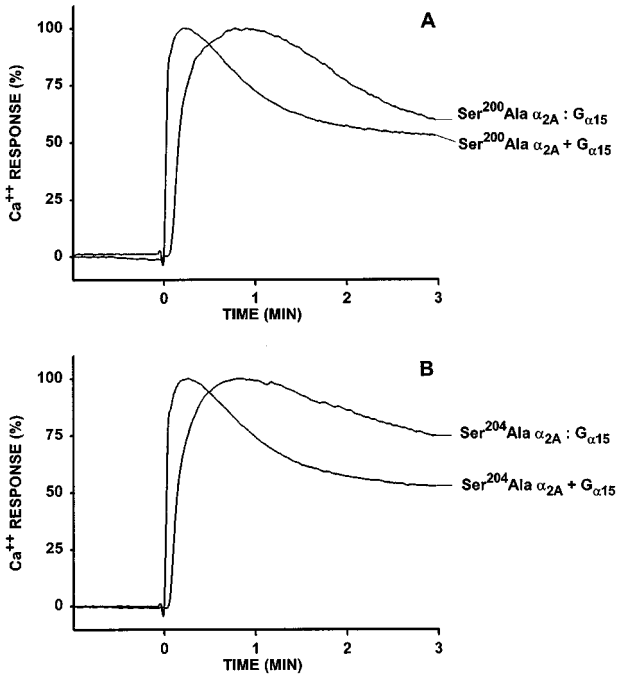

Figure 6 Comparison between kinetics of Ca^{2+} responses as mediated by UK 14304 in CHO-K1 cells transfected with either Ser^{200}Ala or Ser^{204}Ala α_{2A} AR fused or co-expressed with a $G_{\alpha15}$ protein. Ten μg of either α_{2A} AR and $G_{\alpha15}$ protein or fusion protein was transfected. Ca^{2+} responses with 1 μM UK 14304 were measured as described in Methods. Tracings were expressed in percentage of the respective maximal activation by UK 14304 (1 μM). The magnitude of Ca^{2+} response was respectively 7568 ± 2269 (n = 5) and 10 861 ± 980 (n = 5) for the Ser^{200}Ala α_{2A} AR : $G_{\alpha15}$ (A) and Ser^{204}Ala α_{2A} AR : $G_{\alpha15}$ fusion proteins (B). The time of onset for maximal activation [35.5 ± 4.0 s (n = 4) and 42.4 ± 6.2 s (n = 5) for Ser^{200}Ala α_{2A} AR : $G_{\alpha15}$ and $Ser^{204}Ala_{\alpha2A}$ AR : $G_{\alpha15}$ fusion proteins] was significantly (P < 0.05) slower than for the corresponding co-expression experiments. The residual activity upon 1 min of maximal activation was significantly different (P < 0.05) between Ser^{204}Ala α_{2A} AR : $G_{\alpha15}$ fusion protein (84.0 ± 2.1%, n = 5) *versus* co-expression condition (71.9 ± 1.4%, n = 13). No difference was observed for the Ser^{200}Ala α_{2A} AR fused (79.0 ± 3.5%, n = 4) or co-expressed (69.6 ± 2.1%, n = 10) with a $G_{\alpha15}$ protein.

Discussion

The present study reports on intrinsic activity for a diverse series of α_2 AR ligands by wt α_{2A} AR, mutant Ser^{200}Ala and Ser^{204}Ala α_{2A} AR as obtained by measuring Ca^{2+} responses *via* a $G_{\alpha15}$ protein. The magnitude of the UK 14304-mediated Ca^{2+} response as elicited by a $G_{\alpha15}$ protein was largest with both mutant Ser^{200}Ala and Ser^{204}Ala α_{2A} ARs compared to the wt α_{2A} AR in both the co-expression and fusion protein experiments. Both wt and mutant α_{2A} ARs displayed similar kinetic data for their UK 14304-induced response in the co-presence of a $G_{\alpha15}$ protein. A slower onset of the Ca^{2+} response was apparent with each of the fusion proteins assembled by either a wt or a mutant α_{2A} AR and a $G_{\alpha15}$ protein, though the reversal phase of the Ca^{2+} response followed almost similar Ca^{2+} kinetics compared to the co-expression of an α_{2A} AR with a $G_{\alpha15}$ protein. Otherwise, the activation profiles of the wt and both mutant α_{2A} ARs as analysed by a series of α_2 AR ligands were clearly different. The agonists d-medetomidine and clonidine appeared most efficacious at the Ser^{204}Ala α_{2A} AR, whereas oxymetazoline was also partially efficacious at the Ser^{200}Ala α_{2A} AR. Talipexole was silent at both mutant α_{2A} ARs. (−)-Adrenaline was inactive or partially active at the Ser^{204}Ala and Ser^{200}Ala α_{2A} AR, respectively. (−)-Adrenaline has also been reported to be less efficacious on both inhibition and potentiation of forskolin-stimulated cyclic AMP formation at Ser^{200}Ala and Ser^{204}Ala α_{2A} ARs (Wang *et al*., 1991). These observations with (−)-adrenaline are related to its lower binding affinity for both mutant α_{2A} AR (Table 2; Wang *et al*., 1991).

The other agonists being investigated did not display attenuated ligand binding properties. A different rank order of maximal Ca^{2+} responses to agonists with unmodified ligand binding properties was observed at the Ser^{200}Ala α_{2A} AR (UK 14304 > d-medetomidine > > oxymetazoline ≃ clonidine > > talipexole), compared to the Ser^{204}Ala α_{2A} AR (UK 14304 ≃ d-medetomidine > clonidine > > oxymetazoline ≃ talipexole) and wt α_{2A} AR (UK 14304 > d-medetomidine > talipexole > clonidine > > oxymetazoline). These data extend the hypothesis that multiple ligand-activation binding sites exist for these

Table 4 E_{max} values of ligands' Ca^{2+} responses mediated by wt and mutant α_{2A} ARs fused with a $G_{\alpha15}$ protein in CHO-K1 cells

	E_{max} values (%)				
α_{2A} AR:	Ser^{200}Ala	Ser^{204}Ala	Asp^{79}Asn	Thr^{373}Lys	wt*
UK 14304	100	100	67 ± 5	95 ± 2†	97
d-Medetomidine	71 ± 4	93 ± 3†	91 ± 9†	101 ± 7†	76
(−)-Adrenaline	41 ± 5	1 ± 1	100	100	100
Oxymetazoline	34 ± 9	4 ± 2	15 ± 5	69 ± 5	10
Clonidine	28 ± 8	66 ± 4	60 ± 5	86 ± 6†	39
Talipexole	2 ± 0	0 ± 0	93 ± 4†	92 ± 1†	53
RX 811059	2 ± 1	1 ± 0	1 ± 1	2 ± 1	2

Ca^{2+} responses at Ser^{200}Ala α_{2A} AR : $G_{\alpha15}$ and Ser^{204}Ala α_{2A} AR : $G_{\alpha15}$ fusion proteins were expressed *versus* the maximal activation induced by UK 14304 (1 μM). Those mediated by Asp^{79}Asn α_{2A} AR : $G_{\alpha15}$ and Thr^{373}Lys α_{2A} AR : $G_{\alpha15}$ fusion proteins were expressed *versus* the maximal activation induced by (−)-adrenaline (10 μM). Data are presented as mean values ± s.e.mean of 4–16 independent transfection experiments. *values are taken from Pauwels *et al.* (2000a); †P > 0.05 *versus* UK 14304 or (−)-adrenaline value.

Table 5 pEC_{50} and/or E_{max} values of Ca^{2+} responses as mediated by putative α_2 AR antagonists by mutant α_{2A} ARs co-expressed with a $G_{\alpha 15}$ protein

	$Ser^{200}Ala$	*$Ser^{204}Ala$*		*$Asp^{79}Asn$**	*$Thr^{373}Lys$**	*wt**
α_{2A} AR:	*E_{max} (%)*	*pEC_{50}*	*E_{max} (%)*	*E_{max} (%)*	*E_{max} (%)*	*E_{max} (%)*
Atipamezole	6±3	7.85±0.11	60±2	64	31	8
SKF 86466	3±2	7.09±0.06	59±4	29	48	9
Idazoxan	4±3	7.05±0.05	58±0	32	26	3
Dexefaroxan	3±2	7.54±0.02	45±0	21	14	2
RX 811059	1±0	–	3±1	3	2	2

Ca^{2+} responses were measured as described in Table 3 and expressed either *versus* the maximal response as obtained by 1 μM UK 14304 or 10 μM (−)-adrenaline. Data are presented as mean values±s.e.mean of 8–11 independent transfection experiments. *values are taken form Pauwels and Colpaert (2000).

agonists at the α_{2A} AR, and that their activation may be affected in different ways by the mutations being investigated (Pauwels & Colpaert, 2000). We previously observed in co-expression experiments with a $G_{\alpha 15}$ protein (Pauwels and Colpaert, 2000) that the maximal amplitude of activation by d-medetomidine and clonidine *versus* that of (−)-adrenaline was not affected by the $Asp^{79}Asn$ mutation in the second transmembrane domain of the α_{2A} AR. The activation was significantly lower for both UK 14304 and oxymetazoline in contrast to their high-efficacy responses at the $Thr^{373}Lys$ mutation in the third intracellular loop of the α_{2A} AR. Heterogeneity of Ca^{2+} responses as produced by different α_2 AR agonists is also observed by comparing the fusion proteins of the various mutant α_{2A} ARs (see Table 4). UK 14304 consistently acted as an efficacious agonist, whereas it is a partial agonist at the $Asp^{79}Asn$ α_{2A} AR fusion protein. The structurally related imidazoline derivatives d-medetomidine and in particular clonidine display either fully efficacious or enhanced Ca^{2+} responses at the $Asp^{79}Asn$, $Ser^{204}Ala$ and/or $Thr^{373}Lys$ α_{2A} AR fusion proteins. The imidazoline derivative oxymetazoline showed a partial agonist response at $Ser^{200}Ala$ and $Thr^{373}Lys$ α_{2A} AR fusion proteins. The azepine derivative talipexole yielded a highly efficacious response at the $Asp^{79}Asn$ and $Thr^{373}Lys$ α_{2A} AR in contrast to its lack of agonist effect at the $Ser^{200}Ala$ and $Ser^{204}Ala$ α_{2A} AR fusion proteins. The response by the native agonist (−)-adrenaline was not affected by the $Asp^{79}Asn$ and $Thr^{373}Lys$ α_{2A} AR fusion proteins. The effect of these mutations on α_{2A} AR function is presumably related to specific changes in the receptor conformation. The modified receptor conformation apparently affects differentially the activation of these α_2 AR agonists. It is clear that the mutations described herein differentially modulate E_{max} values of agonists belonging to the same chemical series, such as the imidazoline derivatives. It has been suggested that each agonist may induce a different receptor conformation or set of conformations, but the available evidence to this effect is sparse (see Colquhoun, 1998). Recently, Marjamäki *et al.* (1999) have combined targeted mutagenesis experiments with structural modelling to show that two molecules that covalently bind to α_{2A} AR, chloroethylclonidine and 2-aminoethyl methanethiosulphonate hydrobromide, recognize two different receptor conformations.

Ligands previously characterized as antagonists at wt α_{2A} AR (Wurch *et al.*, 1999) also demonstrated no intrinsic activity at the $Ser^{200}Ala$ α_{2A} AR. In contrast, positive efficacy with a different rank order of maximal responses was observed (see Table 5 and Pauwels & Colpaert, 2000) for the $Ser^{204}Ala$ α_{2A} AR (atipamezole = SKF 86466 = idazoxan > dexefaroxan), $Asp^{79}Asn$ α_{2A} AR (atipamezole > idazoxan ≃ SKF 86466 > dexefaroxan) and $Thr^{373}Lys$ α_{2A} AR (SKF 86466 > atipamezole ≃ idazoxan > dexefaroxan). These effects were only measured in the co-expression experiments with a $G_{\alpha 15}$ protein and not by the fusion proteins. This suggests the requirement of an enhanced $G_{\alpha 15}$ protein : α_{2A} AR density ratio to observe this positive efficacy (Pauwels *et al.*, 2000a). The binding affinities of these ligands were unmodified at the $Asp^{79}Asn$ and $Thr^{373}Lys$ α_{2A} AR in contrast to their attenuated affinity for the $Ser^{204}Ala$ α_{2A} AR. However, the positive efficacy for each of these ligands was measured at concentrations that corresponded with their binding affinity for the $Ser^{204}Ala$ α_{2A} AR. RX 811059, free of intrinsic activity, also showed a decreased binding affinity for the $Ser^{204}Ala$ α_{2A} AR. The $Ser^{204}Ala$ α_{2A} AR mutant demonstrates in a strong way the apparent positive efficacy; this activity could attain ~60% of that as measured with the efficacious agonist UK 14304. Dexefaroxan yielded the lowest level of positive efficacy for each of the mutants α_{2A} ARs. This ligand has been previously defined as an α_2 AR antagonist with low intrinsic agonist activity both *in vitro* and *in vivo* (Chopin *et al.*, 1999). However, the dexefaroxan analogue, atipamezole, was efficacious at the $Ser^{204}Ala$ and $Asp^{79}Asn$ α_{2A} ARs. Similar disparate results were obtained between the positive efficacy of idazoxan at each of the mutant α_{2A} ARs and the silent properties of its ethoxy-derivative RX 811059. Thus, even closely related compounds may act differently at the α_{2A} AR. It is conceivable that conformational alterations near the ligand-binding pocket may modify the nature of ligand : receptor interactions, such that some antagonists are capable of stabilizing an active conformation at the new binding interface generated by the mutation.

In conclusion, this study supports that most of the α_2 AR antagonists may actually possess partial agonist properties, which is augmented by the $Asp^{79}Asn$, $Ser^{204}Ala$ and $Thr^{373}Lys$ α_{2A} AR mutations. The data further suggest that several ligands activate the α_{2A} AR *via* a specific binding site, which can be unravelled by the mutations described in this study. The outcome that multiple molecular mechanisms may activate the α_{2A} AR opens perspectives for diverse signalling *via* a single α_{2A} AR subtype.

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