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Partial to complete antagonism by putative antagonists at the wild-type α_{2C}-adrenoceptor based on kinetic analyses of agonist : antagonist interactions

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1 Activation of the recombinant human α_{2C}-adrenoceptor (α_{2C} AR) by (−)-adrenaline in CHO-K1 cells transiently co-expressing a chimeric $G_{\alpha q/i1}$ protein induced a rapid, transient Ca^{2+} response with a high-magnitude followed by a low-magnitude phase which continued throughout the recorded time period (15 min).

2 Activation of the α_{2C} AR by various α_2 AR agonists revealed the following rank order of high-magnitude Ca^{2+} response [E_{max} (%) *versus* 10 μM (−)-adrenaline]: UK 14304 (102 ± 4) = talipexole (101 ± 3) = (−)-adrenaline (100) = d-medetomidine (98 ± 1) > oxymetazoline (81 ± 4) ≃ clonidine (75 ± 5).

3 The methoxy- (RX 821002) and ethoxy-derivatives (RX 811059) of idazoxan and the dexefaroxan analogue atipamezole were fully effective as antagonists of both the high- and the low-magnitude Ca^{2+} response. However, though acting as full antagonists of the high-magnitude response, the further putative α_2 AR antagonists idazoxan (27%), SKF 86466 (29%) and dexefaroxan (59%) reversed the low-magnitude response only partially.

4 In conclusion, kinetic analyses of agonist : antagonist interactions at the α_{2C} AR demonstrate a wide spectrum of partial to complete antagonism of the low-magnitude Ca^{2+} response for structurally related α_2 AR ligands.

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Abbreviations: α_{2C} AR, α_{2C}-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

Efficacy relates to what happens to the receptor system as a result of ligand binding. This may promote a physiological response, in which case the ligand demonstrates positive efficacy and is therefore defined as an agonist. Conversely, the ligand may do nothing to the receptor but bind to it and by its presence preclude activation of the receptor by an agonist. This would make it a neutral antagonist with zero efficacy (Kenakin, 1996). More recently, certain putative antagonists have been referred to as inverse agonists that display negative efficacy (see Milligan *et al*., 1995). In the absence of constitutive receptor activity, however, neutral antagonists and inverse agonists would be indistinguishable in terms of their intrinsic effects on the receptor system.

The observed spectrum of intrinsic activities of ligands at the α_{2A}-adrenoceptor (α_{2A} AR) suggests that most common antagonists behave as either inverse agonists or partial agonists (Pauwels *et al*., 2000b). This wide spectrum of intrinsic activities becomes even more apparent by measuring activity at facilitating mutant α_{2A} ARs (Pauwels & Colpaert, 2000). Ligands may demonstrate distinct pharmacological effects, depending on which G proteins and effector pathways are involved (Berg *et al*., 1998; Yang & Lanier, 1999); for instance, a ligand that acts as a neutral antagonist at a receptor with one particular G protein may act as a partial agonist at another receptor : G protein combination. In the present study, kinetic analyses of agonist : antagonist interactions were carried out whilst using a wild-type (wt) recombinant α_{2C} AR as a model system. Receptor activation was monitored by measuring time-dependent Ca^{2+} responses using a chimeric $G_{\alpha q/i1}$ protein, as it couples the α_{2C} AR efficaciously to a Ca^{2+} response.

Methods

Construction of wild-type α_{2C} AR and chimeric $G_{\alpha q/i1}$ protein genes

The α_{2C} AR (R.C. 2.1.ADR.A2C, Genbank accession number: U72648) was prepared by PCR. The chimeric $G_{\alpha q/i1}$ protein was also constructed by PCR, by exchanging its last five amino acids (Glu^{355}-Tyr-Asn-Leu-Val) of the mouse $G_{\alpha q}$ protein (Genbank accession number: M55412) by the corresponding sequence of the rat $G_{\alpha i1}$ protein (Asp^{350}-Ile-Gly-Leu-Phe) mutated at Cys^{351} into an Ile to yield resistance to pertussis toxin (Dupuis *et al*., 1999).

Measurement of intracellular Ca^{2+} responses

Subconfluent CHO-K1 cells were transiently transfected by electroporation and assayed for intracellular Ca^{2+} responses

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with 2 μM Fluo-3 fluorescent calcium indicator dye between 24 and 48 h upon transfection as described (Pauwels *et al*., 2000a). Antagonists were either pre-incubated for 10 min before the agonist, co-incubated with the agonist at time zero or added 3.5 min after the addition of the agonist. Fluorescent readings were made every 2 s for the first 3.5 min and subsequently every 5 s for 10 min using a fluorometric imaging plate reader (FLIPR, Molecular Devices; Coward *et al*., 1998). Data for Ca^{2+} responses were obtained in arbitrary fluorescence units and were not translated into Ca^{2+} concentrations. [^{3}H]-1,4-[6,7(n)-[^{3}H]-benzodioxan-2-methoxy-2-yl)-2-imidazoline hydrochloride (RX 821002) binding (2.5 nM) and protein levels were determined in intact transfected CHO-K1 cells as described (Pauwels *et al*., 2000a).

Materials

Molecular biology reagents were either from Clontech (Palo Alto, U.S.A.) or In Vitrogen (San Diego, U.S.A.). CHO-K1 cells were obtained from ATCC (Rockville, U.S.A.). Fluo-3 was obtained from Molecular Probes (Oregon, U.S.A.). Clonidine, (−)-adrenaline and oxymetazoline were from Sigma (St. Louis, U.S.A.). *Bordetella pertussis* toxin was from Gibco Biocult Laboratories (Paisley, U.K.). 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. The other ligands [5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline tartrate (UK 14304), dexefaroxan, atipamezole, 2-(2-ethoxy-2,3-dihydro-benzo[1,4]-dioxin-2-yl)-4,5-dihydro-1*H*-imidazole (RX 811059) and (−)-efaroxan] were prepared *intramuros*.

Results

In contrast to its lack of effect in non-transfected cells, (−)-adrenaline produced a time- and concentration-dependent increase (pEC_{50}: 8.40±0.10) in the intracellular Ca^{2+} concentration in CHO-K1 cells transiently co-transfected with a wt α_{2C} AR and a chimeric $G_{\alpha q/i1}$ protein. A high-magnitude Ca^{2+} response occurred within 11.8±0.5 s after agonist addition, whereafter the signal decreased to 48±2% (Figure 1) of its maximal amplitude. Thereafter, the low-magnitude response was maintained for at least the 15 min period during which recordings were made. This Ca^{2+} response seems to be exclusively mediated by the pertussis

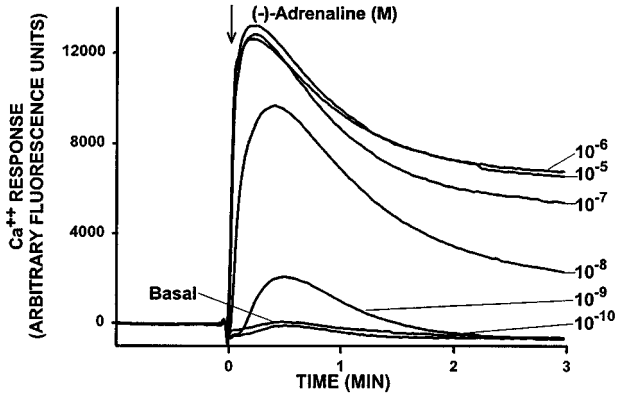

Figure 1 Ca^{2+} response as obtained with CHO-K1 cells co-transfected with a wt α_{2C} AR and chimeric $G_{\alpha q/i1}$ protein. Ca^{2+} responses were measured as described in Methods. In addition to the basal condition, indicated concentrations of (−)-adrenaline were applied at minute 0 and their effects monitored every 2 s for 3 min. Curves illustrate a representative experiment performed in quadruplicate. Transfected cells expressed 1.3±0.1 pmol mg^{-1} protein of [^{3}H]-RX 821002 (2.5 nM) binding sites.

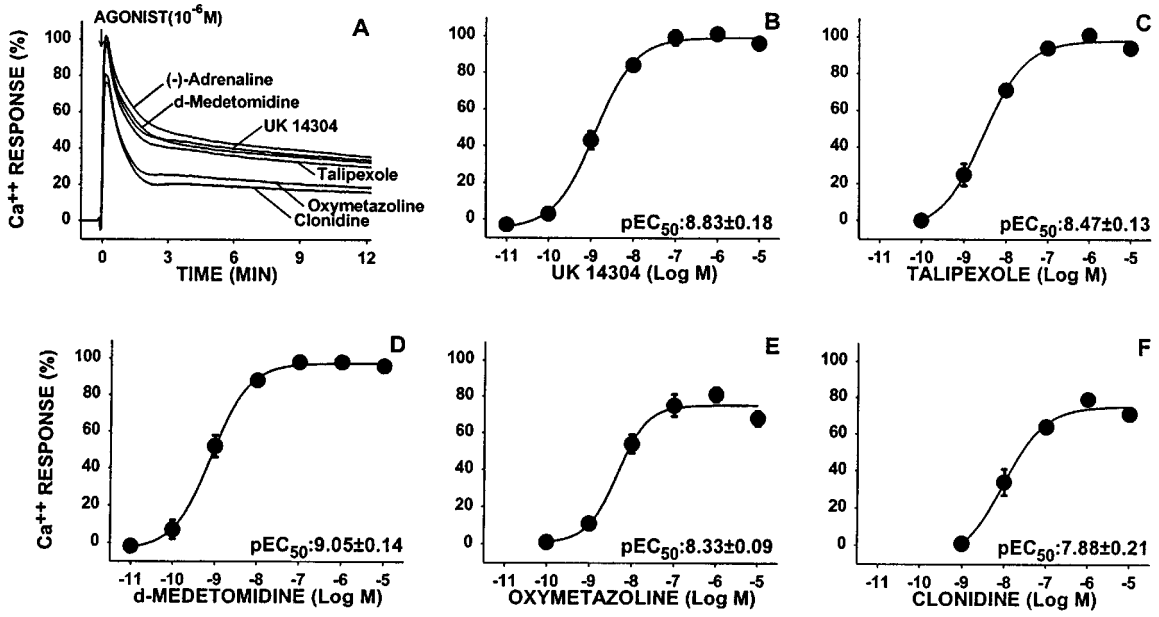

Figure 2 Comparison between time- and dose-dependent α_2 AR agonist-induced Ca^{2+} responses at wt α_{2C} AR co-expressed with $G_{\alpha q/i1}$ protein in CHO-K1 cells. Ca^{2+} responses were measured as described in Methods. (A) Time-course of indicated agonist-induced Ca^{2+} responses. Curves were normalized to the respective peak Ca^{2+} response by 10 μM (−)-adrenaline and illustrate a representative experiment performed in quadruplicate. (B–F) Dose-dependent high-magnitude Ca^{2+} responses by indicated agonists. Data are expressed as a percentage of the respective peak Ca^{2+} response induced by 10 μM (−)-adrenaline. Curves were constructed using mean values±s.e.mean obtained in 3–4 independent transfection experiments, each one performed in quadruplicate.

toxin resistant $G_{\alpha q/i1}$ protein; overnight treatment of cells with pertussis toxin (20 ng ml^{-1}) did not modify both phases of the (−)-adrenaline-mediated Ca^{2+} response. Activation of the α_{2C} AR by various α_2 AR agonists displayed for each of these ligands a high-magnitude Ca^{2+} response followed by a low-magnitude phase (Figure 2A). These ligands revealed the following rank order of high-magnitude Ca^{2+} response [E_{max} (%) *versus* 10 μM (−)-adrenaline]: UK 14304 (102±4) = talipexole (101±3) = (−)-adrenaline (100) = d-medetomidine (98±1) > oxymetazoline (81±4) ≃ clonidine (75±5). This rank order of agonists is similar to that obtained earlier by measuring [^{35}S]-GTPγS binding, Ca^{2+} and GTPase responses in CHO and HEK 293 cells stably transfected with a wt α_{2C} AR (Jasper *et al*., 1998; Kukkonen *et al*., 1998; Jansson *et al*., 1999). Co-exposure of cells to (−)-adrenaline with the putative antagonist RX 811059 displayed competitive antagonism of the (−)-adrenaline-mediated high-magnitude Ca^{2+} response with a pA_2 value of 7.57±0.14 (Figure 3). Whereas RX 811059 (1 μM) was silent (E_{max}: +1±0%, n=7), some intrinsic activity (E_{max}: +19±3%, n=9) was observed with idazoxan (1 μM). However, idazoxan did antagonize the high-magnitude Ca^{2+} response to a same extent as RX 811059 and with a similar potency (Figure 3 and Table 1). The antagonist potency of RX 811059 and idazoxan was, respectively, 2 fold enhanced and 3 fold attenuated by pre-incubation of cells for 10 min to the antagonist prior to (−)-adrenaline exposure. Figure 4 illustrates the effects of the two putative antagonists for the low-magnitude Ca^{2+} response by exposure of cells for 3.5 min to increasing concentrations of (−)adrenaline prior to antagonist. At each of the (−)-adrenaline concentrations, RX 811059 (1 μM) reversed rapidly the low-magnitude Ca^{2+} response to the basal Ca^{2+} level. In contrast, the capacity of idazoxan (1 μM) to reverse the low-magnitude Ca^{2+} signal was greatly determined by the (−)-adrenaline concentration. Whereas at 10 nM of (−)-adrenaline, idazoxan attained a similar, maximal reversal to that obtained with RX 811059; the reversal which it produced at 1 μM of (−)-adrenaline was particularly partial and short. Comparison of the reversal effect by idazoxan and RX 811059 at 10 μM *versus* the low-magnitude Ca^{2+} response mediated by (−)-adrenaline (1 μM) displayed a greater reversal capacity for RX 811059 than idazoxan (Figure 5A). The weaker reversal capacity by idazoxan *versus* RX 811059 was also observed by measuring the low-magnitude Ca^{2+} response as mediated by 0.1 μM UK 14304 (Figure 5B). This suggests that the different reversal capacity between these antagonists for the late-phase Ca^{2+} response is not agonist-specific.

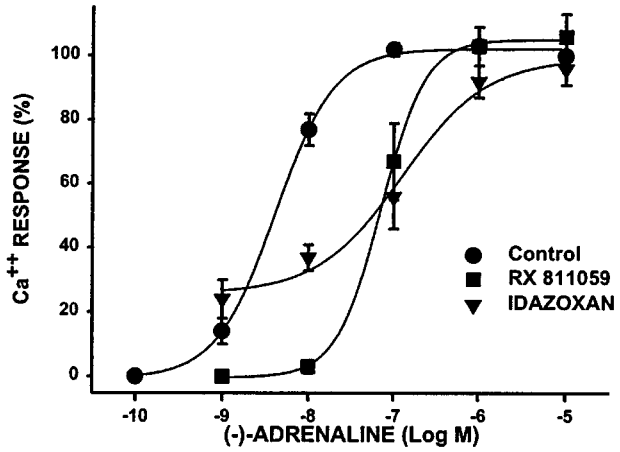

Figure 3 Concentration-response curves of (−)-adrenaline in producing the high-magnitude Ca^{2+} response in either the absence or the presence of 1 μM of idazoxan and RX 811059. (−)-Adrenaline and antagonist were co-incubated at time zero. Data were obtained as shown in Figure 1, and peak values of Ca^{2+} responses were corrected for basal Ca^{2+} response and plotted in percentage *versus* the response of (−)-adrenaline (10 μM). Curves were constructed using mean values±s.e.mean obtained in three independent transfection experiments, each one performed in quadruplicate. pA_2 values are summarized in Table 1.

A comparison of the reversal of the low-magnitude Ca^{2+} response as induced by (−)-adrenaline by a series of related α_{2A} AR ligands, together with their intrinsic activity and their potency to block the (−)-adrenaline-mediated high-magnitude Ca^{2+} response, is summarized in Table 1. Idazoxan, atipamezole, SKF 86466 and also (−)-efaroxan produced some intrinsic effect in a way that was blocked by RX 811059 (not shown). The other ligands were virtually free of intrinsic activity. With the exception of (−)-efaroxan, each of these ligands displayed antagonism of the high-magnitude Ca^{2+} response in a manner that appeared competitive, and with potencies between 7.09 and 7.77. These ligands displayed the following rank order for reversal of the low-magnitude Ca^{2+} response: idazoxan = SKF 86466 < dexefaroxan < atipamezole = RX 821002 ≃ RX 811059. A similar rank order for reversal of the low-magnitude Ca^{2+} response was obtained upon treatment with pertussis toxin (20 ng ml^{-1}). Control experiments further indicated that not any of these

Table 1 Intrinsic activity and antagonist properties of high- and low-magnitude Ca^{2+} response by α_2 AR ligands at the recombinant α_{2C} AR in CHO-K1 cells

	Intrinsic activity (%)	*Antagonist potency (pA_2) of high-magnitude Ca^{2+} response**	*Reversal capacity (%) of low-magnitude Ca^{2+} response†*
(−)-Efaroxan	10±1	–	1±0
Idazoxan	19±3	7.31±0.19	27±5
SKF 86466	7±1	7.09±0.11	29±3
Dexefaroxan	2±0	7.77±0.19	59±3
Atipamezole	18±2	7.34±0.17	89±6
RX 821002	0±1	7.34±0.01	90±4
RX 811059	1±0	7.57±0.14	100

Intrinsic activities were determined at 1 μM and expressed in percentage *versus* high magnitude Ca^{2+} response of 10 μM (−)-adrenaline. pA_2 values were calculated for the high-magnitude Ca^{2+} response as performed in the legend to Figure 3. They were with exception of RX 821002 (pA_2: 7.64±0.25) and RX 811059 (pA_2: 7.86±0.25) not enhanced by incubation of antagonist 10 min prior to agonist. Reversal capacity of low-magnitude Ca^{2+} response was defined as the property of the ligand (1 μM, added at 3.5 min upon agonist addition) to reverse the (−)-adrenaline (1 μM) response as performed in the legend to Figure 4. This was calculated as the surface area between the (−)-adrenaline and ligand condition for a period of 10 min upon addition of the ligand. The surface areas are expressed in percentage *versus* 1 μM RX 811059. Values represent mean values±s.e.mean of three* and 7–8† independent transfection experiments, each performed in quadruplicate.

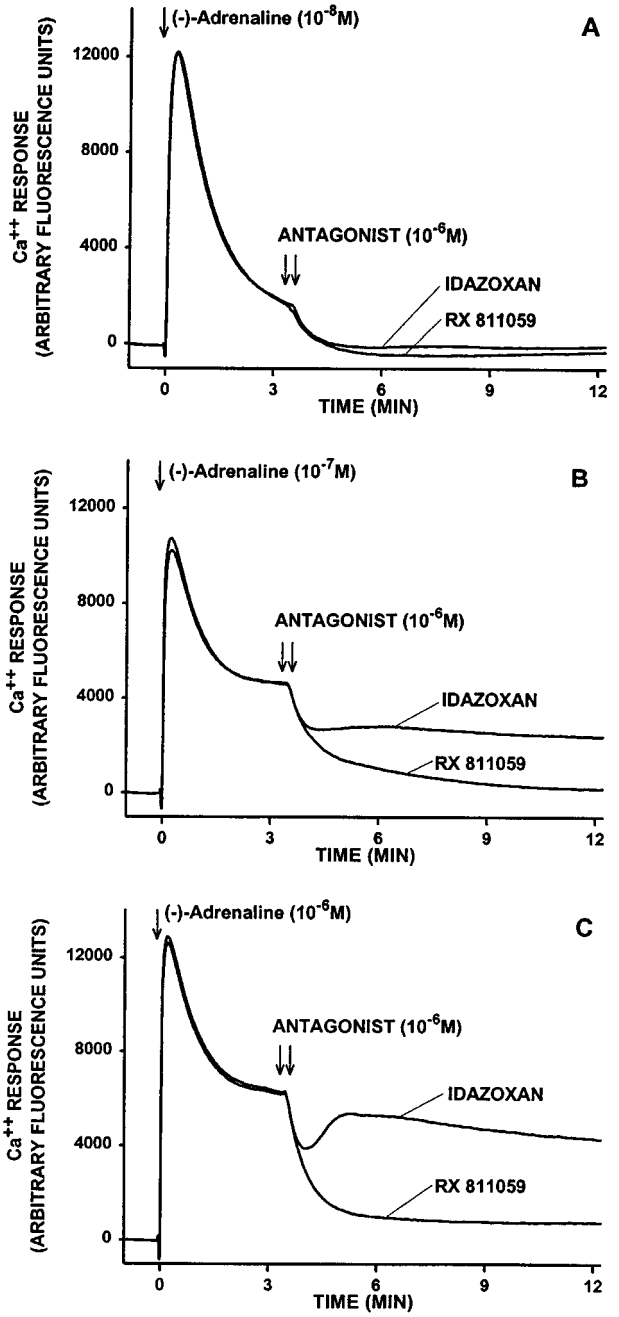

Figure 4 Reversal of low-magnitude Ca^{2+} response by RX 811059 and idazoxan in CHO-K1 cells pre-exposed to (−)-adrenaline. Ca^{2+} responses were measured as described in Methods. The indicated concentrations of (−)-adrenaline were applied at time zero and 3.5 min later either 1 μM of RX 811059 or idazoxan was added and Ca^{2+} responses were followed every 5 s for 10 min. Curves illustrate a representative experiment, performed in quadruplicate, out of 3–8 independent transfection experiments. Reversal capacity (%) of low-magnitude Ca^{2+} response mediated by 1 μM (−)-adrenaline is summarized in Table 1.

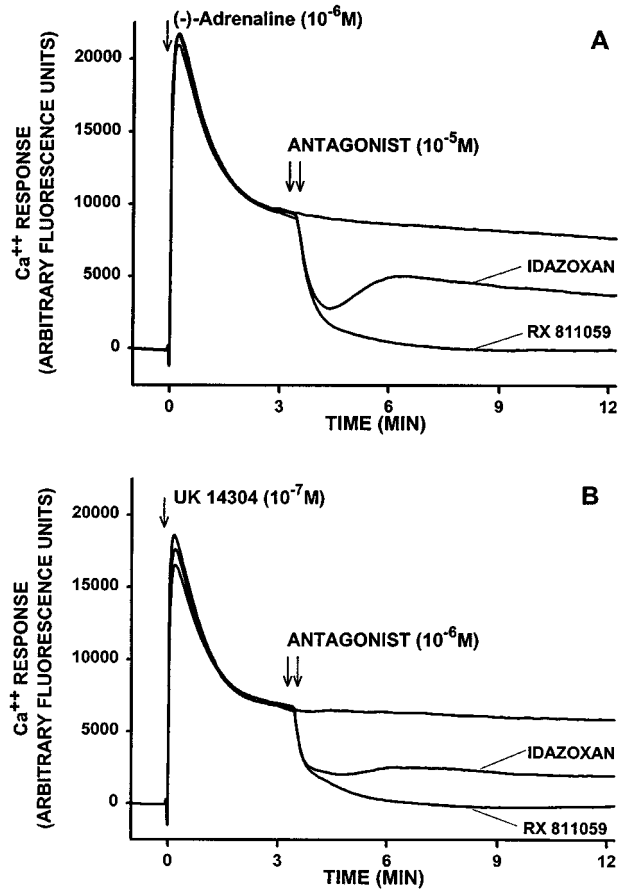

Figure 5 Reversal of low-magnitude Ca^{2+} response by RX 811059 and idazoxan in CHO-K1 cells pre-exposed to either (−)-adrenaline or UK 14304. Ca^{2+} responses were measured as described in Methods. (A) (−)-Adrenaline (1 μM) was applied at time zero and 3.5 min later either 10 μM RX 811059 or idazoxan was added and Ca^{2+} responses were followed every 5 s for 10 min. Curves illustrate a representative experiment, performed in quadruplicate, out of two independent transfection experiments. Reversal capacity of low-magnitude Ca^{2+} response mediated by 1 μM (−)-adrenaline was for idazoxan 46% ($n=2$) of that obtained by RX 811059. (B) UK 14304 (0.1 μM) was applied instead of (−)-adrenaline at time zero and 3.5 min later either 1 μM RX 811059 or idazoxan was added and Ca^{2+} responses were followed as in (A). Reversal capacity of low-magnitude Ca^{2+} response mediated by 0.1 μM UK 14304 was for idazoxan 68±6% ($n=4$) of that obtained by RX 811059.

ligands displayed a Ca^{2+} response in non-transfected CHO-K1 cells.

Discussion

The present study demonstrates a spectrum of partial to complete antagonism by a series of putative α_2 AR antagonists at the wt α_{2C} AR. This spectrum was based on kinetic analyses of agonist : antagonist interactions by measuring time-dependent Ca^{2+} responses using a pertussis toxin resistant chimeric $G_{\alpha q/i1}$ protein. It has previously been reported that the $G_{\alpha q/i1}$ protein can convert the coupling of $G_{i/o}$ protein-coupled receptors to the phospholipase C pathway (Conklin *et al.*, 1996; Liu *et al.*, 1995; Kostenis *et al.*, 1997); it is therefore suitable to monitor receptor-mediated Ca^{2+} responses. Ca^{2+} mobilization by α_{2C} ARs has recently been demonstrated in CHO-K1 cells stably expressing the receptor; this response was sensitive to the phospholipase C inhibitor U-73122 as well as the $G_{i/o}$ protein-inactivating agent pertussis toxin (Kukkonen *et al.*, 1998). Hence, this Ca^{2+} response is likely to be mediated by $\beta\gamma$ subunits of $G_{i/o}$ proteins. In the present study, a robust (−)-adrenaline-mediated Ca^{2+} response was observed with a $G_{\alpha q/i1}$ protein; it consisted of a rapid, transient response with a high-magnitude followed by a low-magnitude phase which continued for the recorded time period (15 min). Both phases could be fully antagonized by RX 811059, indicating both phases of this Ca^{2+} process to be mediated by α_{2C} ARs. The lack of Ca^{2+} response in non-transfected CHO-K1 cells

together with an activation profile as observed with several α_2 AR agonists similar to that reported by Kukkonen *et al.* (1998), further indicate that the Ca^{2+} responses described here are mediated by α_{2C} ARs. Besides the investigated α_2 AR agonists, idazoxan and atipamezole displayed weak positive intrinsic activity at the wt α_{2C} AR in contrast to the wt α_{2A} AR (Pauwels & Colpaert, 2000). Nonetheless, both ligands could antagonize the high-magnitude Ca^{2+} response to the same extent as the observed silent α_2 AR ligands, on the condition that the cells were simultaneously exposed to agonist and antagonist. The antagonist potencies for each of the ligands being investigated were in the same range. Antagonist potencies were almost not improved by incubating the cells with antagonist prior to agonist; therefore, they appear to bind rapidly to the α_{2C} AR. Otherwise, the inhibition of α_{2A} AR-mediated Ca^{2+} elevation by α_2 AR antagonists in human erythroleukaemia cells (Kukkonen *et al.*, 1997), suggested two classes: those that displayed surmountable inhibition (i.e., idazoxan) and those that displayed different degrees of insurmountable inhibition (i.e., RX 821002). These data were obtained by preincubation (5 min) of antagonist on UK 14304-induced high-magnitude Ca^{2+} response.

Remarkably, the capacity of some of the antagonists to diminish the low-magnitude phase of the (−)-adrenaline-mediated Ca^{2+} response was much attenuated (idazoxan = SKF 86466 > dexefaroxan) by incubating the cells with (−)-adrenaline prior to the antagonist. This was in stark contrast to their ability to fully antagonize the high-magnitude Ca^{2+} response when simultaneously co-incubated with (−)-adrenaline. However, the methoxy- and ethoxy-derivatives of idazoxan, respectively RX 821002 and RX 811059, were fully effective as antagonists of both the high and low-magnitude Ca^{2+} response. A similar observation was made with the dexefaroxan analogue atipamezole. Thus, closely related compounds may act differently at the low-magnitude phase of the Ca^{2+} response as mediated by the α_{2C} AR. It is not clear why these observed differential antagonist properties cannot be related to the putative absence or presence of ligand's intrinsic activity. Atipamezole, but not idazoxan, reversed fully the low-magnitude Ca^{2+} response despite both ligands displaying a similar amount of intrinsic activity as assayed in the absence of agonist. Dexefaroxan, though virtually free of intrinsic activity, reversed the low-magnitude Ca^{2+} response only partially.

Whether the apparently silent α_2 AR antagonists are perhaps inverse agonists at the α_{2C} AR cannot be determined by the present Ca^{2+} assay as the ligands did not attenuate the basal Ca^{2+} level. Both RX 821002 and RX 811059 have been shown to act as full inverse α_{2A} AR agonists in some conditions; the magnitude of their negative efficacy was particularly high at the mutant $Thr^{373}Lys$ α_{2A} AR in the co-presence of a $G_{\alpha o}Cys^{351}Ile$ protein (Pauwels *et al.*, 2000b). Positive intrinsic activity for idazoxan, SKF 86466, dexefaroxan and, also atipamezole, has previously been reported at various mutant α_{2A} ARs in contrast to the wt receptor (Wurch *et al.*, 1999; Pauwels *et al.*, 2000b; Pauwels & Colpaert, 2000). Interestingly, these ligands displayed a different profile of positive intrinsic activity at the $Asp^{79}Asn$, $Ser^{200}Ala$, $Ser^{204}Ala$ and $Thr^{373}Lys$ α_{2A} AR mutations; the observed positive efficacy was particularly strong for atipamezole, SKF 86466 and idazoxan at the $Ser^{204}Ala$ α_{2A} AR (Pauwels & Colpaert, 2000). The disparate ligand-mediated Ca^{2+} responses by wt and mutant α_{2A} ARs suggest that multiple activation binding sites exist for these ligands at these receptors, and that their activation may be affected in different ways by the mutations. It has been suggested that each ligand with efficacy may induce a different receptor conformation or set of conformations, but the available evidence to this effect is sparce (see Colquhoun, 1998). Recently, Marjamäki *et al.* (1999) have combined targeted mutagenesis experiments with structural modelling to show that two agonist molecules that covalently link to the α_{2A} AR, chloroethylclonidine and 2-aminoethylmethanethiosulphonate hydrobromide, recognize two different receptor conformations.

Two types of antagonists, those with zero intrinsic activity (neutral antagonists) and those with negative intrinsic activity (inverse agonists) with regard to constitutive receptor activation rely on a lot of attention in molecular pharmacology (see Deaffler & Landry, 2000). However, the concept that putative antagonists may be partial agonists instead of neutral antagonists or inverse agonists should not be underestimated. The view that neutral antagonists at G protein-coupled receptors are rare, suggests careful functional analysis of ligands before it can be defined as an antagonist. Thus, the data suggest that ligands with minor structural modifications may display distinct pharmacological properties at α_{2C} ARs. The underlying mechanisms of the molecular actions of these ligands will offer a better understanding of their physiological effects.

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