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Characterization of human recombinant α_{2A}-adrenoceptors expressed in Chinese hamster lung cells using extracellular acidification rate changes

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1 Human α_{2A}-adrenoceptors heterologously expressed in Chinese hamster lung (CHL) fibroblasts have been characterized pharmacologically using a cytosensor microphysiometer to measure ligand-induced extracellular acidification rate changes.

2 In untransfected CHL cells, noradrenaline had no effect at concentrations up to 100 μM. In α_{2A}-adrenoceptor transfected cells the rank order of agonist potency was A-54741 (mean $pEC_{50} = 8.96$) > dexmedetomidine (8.88) > UK-14304 (8.42) > B-HT 920 (7.05) > noradrenaline (6.92). A-54741, UK-14304 and noradrenaline had the same maximum response while dexmedetomidine and B-HT 920 behaved as partial agonists.

3 The selective α_2-adrenoceptor ligand rauwolscine antagonized acidification rate changes with an affinity independent of the agonist used; the affinity (mean pK_B) against noradrenaline was 8.43.

4 The selective α_1-adrenoceptor ligands prazosin and doxazosin (each 3 μM) had no effect on noradrenaline responses.

5 Acidification rate changes induced by each agonist were abolished by pre-treatment of cells with pertussis toxin.

6 These data suggest that agonist-induced acidification rate responses in CHL cells transfected with the human α_{2A}-adrenoceptor are mediated exclusively by the recombinant protein, *via* pertussis toxin sensitive $G_{i/o}$ proteins.

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Abbreviations: CHL, Chinese hamster lung; E/[A], concentration-effect

Introduction

The primary signal transduction pathway of α_2-adrenoceptors is through pertussis toxin sensitive $G_{i/o}$ G proteins which leads to inhibition of adenylyl cyclase and a reduction of intracellular cyclic AMP. This has been shown for each subtype (α_{2A}, α_{2B}, α_{2C}) in recombinant systems (Fraser *et al*., 1989; Cottechia *et al*., 1990; Duzic *et al*., 1992; Eason *et al*., 1992; 1994; Chabre *et al*., 1994) and in native tissues (Bylund & Ray-Prenger, 1989; Wright *et al*., 1995). α_2-adrenoceptors may also transduce other effects through G_i proteins. Thus, α_{2A} and α_{2C} receptors heterologously expressed in Chinese hamster lung fibroblasts stimulate phosphatidyl inositol production as well as inhibit adenylyl cyclase through G_i (Cottechia *et al*., 1990). A consistent observation in recombinant and native systems, is that α_2-adrenoceptors can elevate cyclic AMP through activation of G_s proteins (Ullrich & Wollheim, 1984; Jones *et al*. 1987; Fraser *et al*., 1989; Cottechia *et al*., 1990; Duzic *et al*., 1992; Eason *et al*., 1992; 1994; Chabre *et al*., 1994; Mhaouty *et al*., 1995; Jansson *et al*., 1995; Nasman *et al*., 1997). Although this latter effect is generally only seen at higher concentrations of agonist, and in the presence of forskolin to raise cyclic AMP levels, such promiscuous coupling to $G_{i/o}$ and G_s G proteins results in biphasic agonist concentration-effect curves and is a major problem when trying to establish agonist or antagonist affinities.

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Both inhibition and augmentation of cyclic AMP formation was observed in preliminary studies using human recombinant α_{2A}-adrenoceptors expressed in CHL fibroblasts (unpublished data) and thus we wished to explore alternative assays to provide rigorous quantitative data. In this study we have employed a Cytosensor microphysiometer, an instrument that measures the extracellular pH of cells superfused with physiological medium. This instrument has been used to study receptors coupled to G_i, G_s as well as G_q type G proteins, since activation of receptors linked to each results in a net increase in the acidification rate of the extracellular medium (see McConnell *et al*., 1992 for references). Our results suggest that in CHL cells transfected with the human α_{2A}-adrenoceptor, agonist responses are mediated exclusively by the recombinant receptor. Furthermore, our results demonstrate a general utility of the Cytosensor microphysiometer for the operational characterization of cell receptors using agonists and antagonists.

Some of these results have been published in abstract form (MacLennan *et al*., 1997b; Reynen *et al*., 1997).

Methods

Stable cell line construction and cell culture

Transfection of the Chinese hamster lung fibroblast cell line, R 1610, with the human α_{2A} gene has previously been described (MacLennan *et al*., 1997a); the clone used for these studies has

a specific binding capacity (B_{max} for [^{3}H]-MK-912) of 1.50 pmol mg protein^{-1}. Cells were maintained in DMEM (without sodium pyruvate) supplemented with 4.5 g l^{-1} glucose, 5% foetal bovine serum (FBS) and 250 μg ml^{-1} G-418 and grown in a 5% CO_2 environment at 37°C. In experiments with pertussis toxin, cells were incubated with 500 ng ml^{-1} for 24 h. This concentration was chosen as it is sufficient to completely ADP-ribosylate $G_{i/o}$ proteins in CHO cells (Eason *et al*., 1992).

Microphysiometry studies

Cells were plated into capsule cups (12 mm diameter, 3.0 μm pore) at a density of 3×10^5 cells/capsule cup in DMEM including 5% FBS and 250 μg ml^{-1} G-418. The cells were incubated at 37°C in 5% CO_2 for 24 h. The capsule cups were loaded into the sensor chambers of the Cytosensor microphysiometer (Molecular Devices, CA, U.S.A.) and superfused with buffer (DMEM without bicarbonate and supplemented with 44.4 mM NaCl, 100 μM ascorbic acid, pH 7.35). Agonists, antagonists, and other drugs were dissolved in the buffer and pH adjusted to 7.35 using 0.1 N HCl or NaOH as appropriate. The Cytosensor pump superfuses the cells with medium at 100 μl min^{-1} (50% of maximum pump speed) for 55 s, and is then off for 35 s. This 90 s pump cycle is repeated throughout the experiment. The acidification rate of the extracellular medium between 60 and 88 s was calculated by the Cytosoft program (Molecular Devices, CA, U.S.A.). Agonist-induced responses (increases in acidification rates) were calculated by subtracting the basal acidification rate (average of three rate measurements preceding the addition of drug) from the highest rate measurement after drug addition, divided by the basal rate to obtain the per cent increase above baseline value. Non-cumulative concentration-effect (E/[A]) curves to agonists were constructed by increasing the concentration in 0.5 $\log_{10}$ M increments. Cells were exposed to agonists for 6 min, with 39 min between exposures. Antagonists were added to the medium 75 min before constructing E/[A] curves. In each experiment, two E/[A] curves were constructed for each agonist and the mean data used in further analysis. Data presented are the mean of 3–6 such experiments.

Analysis of E/[A] curve data

The Hill equation was fitted to individual E/[A] curves:

$$E = \frac{\alpha \cdot [A]^{n_H}}{[EC]_{50}^{\ n_H} + [A]^{n_H}} \quad (1)$$

in which E, α, EC_{50} and n_H are in effect, upper-asymptote, mid-point location and slope parameters respectively. Location parameters were actually estimated as logarithms ($-\log_{10}$ EC_{50}). If antagonists produced parallel, surmountable displacements of agonist E/[A] curves, as tested by one-way ANOVA of slope and upper-asymptote estimates respectively, Schild analysis of the data was conducted using least squares linear regression of $\log(CR-1)$ *vs* $\log[B]$ where CR is the concentration-ratio between control and antagonist (B) treated tissues (Arunlakshana & Schild, 1959). If the Schild plot slope was not significantly different from unity it was constrained to this value to estimate K_B, the antagonist dissociation equilibrium constant. In experiments using only a single concentration of antagonist, affinity estimates (apparent pK_Bs) were estimated using the equation:

$$pK_B = \log[CR-1] - n.\log[B] \quad (2)$$

provided that the shift produced by the antagonist was parallel and making the assumption of simple competitive antagonism, i.e. that the value of n was unity.

Statistics

Statistical analyses of data were undertaken using either analysis of variance or a two-sample t-test where appropriate, with $P<0.05$ regarded as statistically significant.

Materials

The Chinese hamster lung fibroblast cell line R 1610, was obtained from ATCC (#CRL-1657). Lipofectamine, Opti-MEM, DMEM, HBSS, HEPES, phosphate-buffered saline, G-418, phenol red and fetal bovine serum were purchased from Gibco (NY, U.S.A.). The cDNA (in pBC vector) for the human α_{2A}-adrenoceptor was obtained from Duke University (NC, U.S.A.). The following drugs were purchased: (−)noradrenaline bitartrate, prazosin hydrochloride, (Sigma, MO, U.S.A.); rauwolscine hydrochloride, UK-14304, (RBI, MA, U.S.A.). B-HT 920 was a generous gift from Boehringer Ingelheim. Dexmedetomidine hydrochloride, doxazosin and A-54741 (5,6-dihydroxy-1,2,3,4-tetrahydro-1-naphthyl-imidazoline) hydrobromide were synthesized at Roche Bioscience for which Dr R. D. Clark, F. Makra, H. Cai, J.P. Dunn and J.M. Caroon are thanked. All drugs were dissolved in the buffer used for microphysiometer experiments, with the exception of prazosin and doxazosin, which were dissolved in 50% EtOH.

Results

Agonist studies

CHL cells were superfused with medium for 2 h during which time the acidification rate reached a stable level of 30–150 μV s^{-1}. In non-transfected CHL cells, noradrenaline had no effect on acidification rate at concentrations up to 100 μM. In cells expressing the α_{2A}-adrenoceptor, noradrenaline (1 nM–1 mM) produced simple monophasic E/[A] curves with a Hill slope of 0.76 ± 0.01 and mid-point location ($p[A]_{50}$) of 6.92 ± 0.20. The maximum response (increase in acidification rate over basal) was $74\pm12\%$ ($n=6$). The effects of other

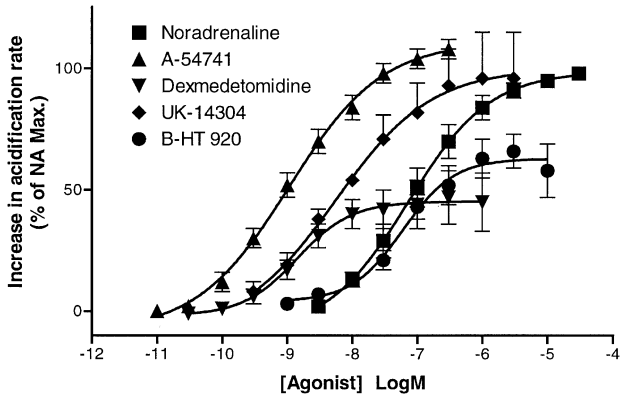

Figure 1 Acidification rate changes evoked by agonists at human α_{2A}-adrenoceptors expressed in CHL cells. In each experiment different chambers of cells were challenged with noradrenaline or other agonists. Data are the mean ± s.e.mean of three to six separate experiments.

Table 1 Potency and intrinsic activity (I.A.) data for agonist activity at human α_{2A}-adrenoceptors expressed in CHL cells, using the Cytosensor Microphysiometer

	Microphysiometer		*Dog saphenous vein*[a]		*Binding*[a]
Agonist	pEC_{50}	*I.A. (NA = 1.0)*	pEC_{50}	*I.A. (NA = 1.0)*	pK_i
Noradrenaline (NA)	6.92 ± 0.20	1.0	6.54 ± 0.06	1.0	5.12 ± 0.10
A-54741	8.89 ± 0.08	1.09 ± 0.03	8.77 ± 0.13	1.01 ± 0.02	7.38 ± 0.03
UK-14304	8.36 ± 0.38	1.06 ± 0.14	7.64 ± 0.09	0.95 ± 0.06	6.71 ± 0.05
Dexmedetomidine	8.84 ± 0.23	0.46 ± 0.10	8.12 ± 0.08	0.67 ± 0.04	7.99 ± 0.04
B-HT 920	7.05 ± 0.23	0.68 ± 0.07	6.56 ± 0.05	0.63 ± 0.05	6.36 ± 0.08

Values are the mean ± s.e.mean of 3–6 individual estimates. For reference potency and intrinsic activity estimates are also given for ligand activity at α_{2A}-adrenoceptor mediated contraction of dog saphenous vein as well as radioligand binding affinity estimates for human α_{2A}-adrenoceptors expressed in CHL cells. [a]Data from MacLennan *et al.* (1997a).

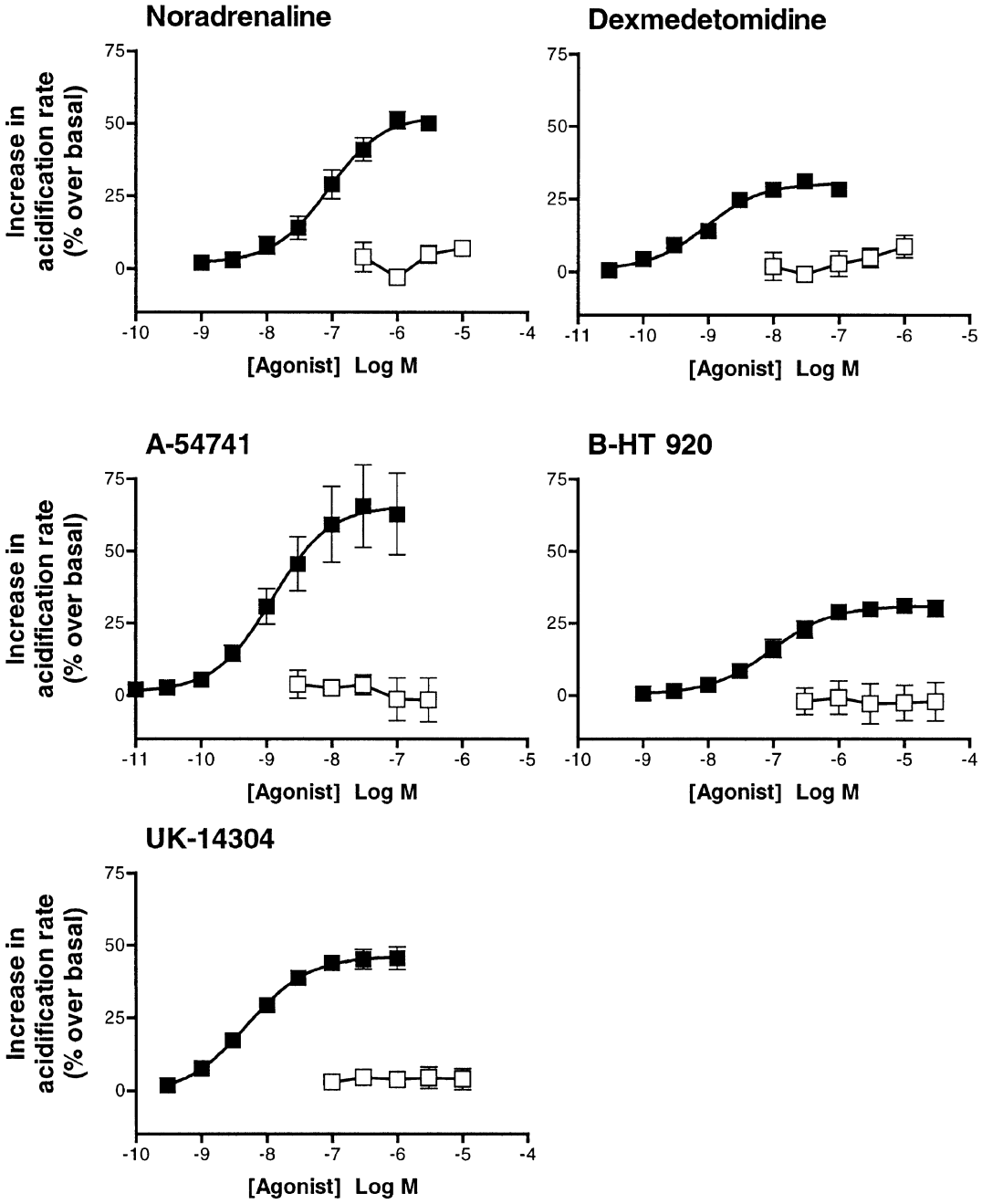

Figure 2 Acidification rate changes evoked by noradrenaline, A-54741, UK-14304, dexmedetomidine and B-HT 920 were abolished by pertussis toxin. Concentration-effect curves to agonists were constructed in control CHL cells expressing the human α_{2A}-adrenoceptor (closed symbols) or in cells exposed to pertussis toxin (open symbols; 500 ng ml^{-1} for 24 h). Data are the mean ± s.e.mean of 4–6 separate experiments.

agonists were assessed and the decreasing rank order of potency was: A-54741 > dexmedetomidine > UK-14304 > B-HT 920 > noradrenaline. With respect to noradrenaline, A-54741 and UK-14304 had similar intrinsic activities while B-HT 920 and dexmedetomidine behaved as partial agonists (Figure 1; Table 1). The effects of each agonist were completely abolished in cells exposed to pertussis toxin, indicating the involvement of $G_{i/o}$ G proteins (Figure 2).

Antagonist studies

The responses to noradrenaline, UK-14304 and dexmedetomidine were competitively antagonized by rauwolscine (10 nM to 3 μM; $n=3$) which caused parallel, surmountable shifts of E/[A] curves (Figure 3, Table 2). A-54741 and B-HT 920 responses were also antagonised by rauwolscine (30 nM) with affinity estimates consistent with an interaction at the α_{2A}-adrenoceptor (Table 2). The α_1 selective antagonists, prazosin and doxazosin (each 3 μM) had no effect on noradrenaline responses (Figure 4). To further characterize the α_{2A}-adrenoceptor, we also employed spiroxatrine which is one of very few ligands which discriminate between α_2-adrenoceptor subtypes (MacLennan *et al*, 1997a). Spiroxatrine (0.3 – 10 μM) caused parallel, surmountable shifts of noradrenaline E/[A] curves with an estimated pK_B of 7.08 ± 0.06 ($n=3$). Noradrenaline responses were not affected by either cocaine or corticosterone (each 30 μM; data not shown) indicating that removal processes were not an influence. None of the antagonists had any effect on the basal acidification rate.

Discussion

The principal finding from this study is that measuring extracellular pH changes, using the cytosensor microphysi-

Table 2 Affinity estimates for rauwolsine at human α_{2A}-adrenoceptors expressed in CHL cells, using microphysiometry

Agonist	*Rauwolscine pK_B*	*Schild slope*
Noradrenaline	8.43 (8.40 – 8.47)	0.95 (0.91 – 1.00)
A-54741	8.62 (8.53 – 8.71)	ND
UK-14304	8.50 (8.20 – 8.80)	1.00 (0.86 – 1.14)
Dexmedetomidine	8.62 (8.53 – 8.71)	1.06 (0.99 – 1.13)
B-HT 920	8.50 (7.73 – 9.27)	ND

Values are the mean of three individual estimates, with 95% confidence limits in parenthesis. ND, not determined as only a single concentration of rauwolscine (30 nM) was examined.

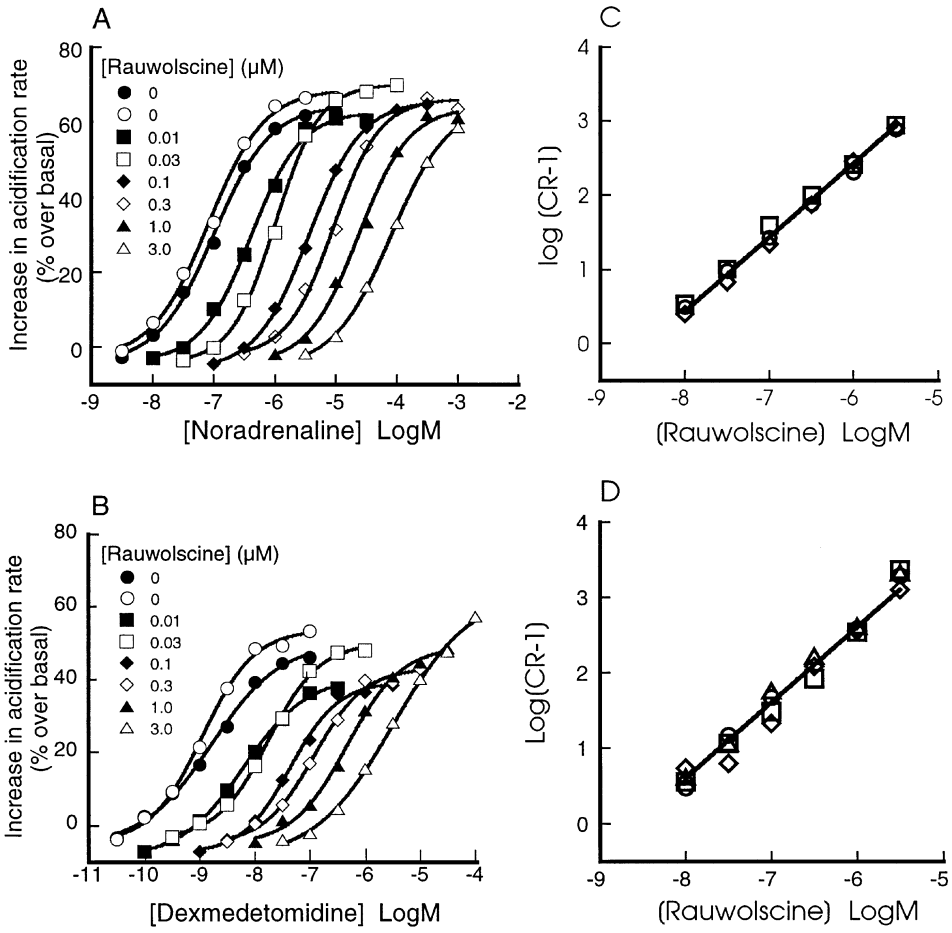

Figure 3 Antagonism by rauwolscine of (A) noradrenaline and (B) dexmedetomidine induced acidification rate increases mediated by human α_{2A}-adrenoceptors expressed in CHL cells. E/[A] curves to agonists were constructed by non-cumulative exposure of the agonist in the absence or presence of rauwolscine which had been added 75 min earlier. (A) and (B) show representative data from one experiment only while the Schild plots (C, *vs* noradrenaline; D, *vs* dexmedetomidine) show the data from all experiments ($n=3$, noradrenaline; $n=4$, dexmedetomidine) and the different symbols represent data from each experiment.

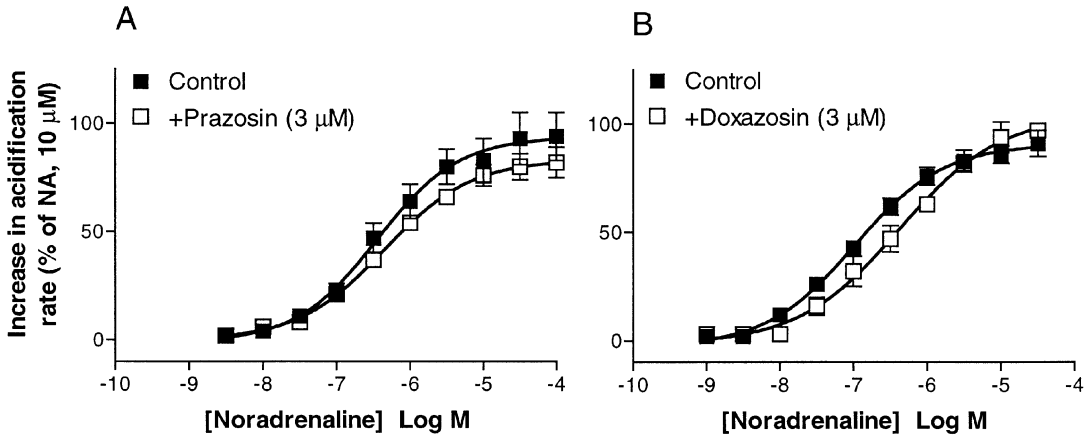

Figure 4 Effect of prazosin (A) and doxazosin (B) on noradrenaline-induced acidification rate increases mediated by human α_{2A}-adrenoceptors expressed in CHL cells. E/[A] curves to agonists were constructed by non-cumulative exposure of the agonist in the absence or presence of antagonist which had been added 75 min earlier ($n=3$).

ometer, has proven to be a valuable functional assay from which we obtained robust estimates of antagonist affinities and of agonist intrinsic activities, at the human recombinant α_{2A}-adrenoceptor expressed in CHL cells. Agonist E/[A] curves were very reproducible, both within an experiment on different cells, and between experiments on different days, thus allowing robust quantitative data to be generated.

Agonist studies

Each agonist which was examined gave simple monophasic E/[A] curves which were abolished by prior exposure of cells to pertussis toxin. These two observations suggest the involvement of a single receptor only, which is coupled to $G_{i/o}$ type G proteins, and was confirmed using the competitive antagonist rauwolscine whose estimated affinity was independent of the agonist being used. The apparent lack of involvement of G_s type G proteins was an interesting finding but one which meant we had a relatively more simple assay system than that employing inhibition of cyclic AMP production which has been the assay of choice in most other functional studies of α_2-adrenoceptors (see introduction for references). The lack of G_s activation may not be so surprising since most studies only reveal α_2-adrenoceptor activation of G_s type G proteins in the presence of forskolin or other activator of adenylyl cyclase, a necessary requirement when attempting to study inhibiton of cylic AMP *via* $G_{i/o}$ coupled receptors.

This promiscuity of the α_{2A}-adrenoceptor, and the resultant technical complexities of measuring inhibition of cyclic AMP, has resulted in there being only very limited published data with which to compare agonist potencies and intrinsic activities. In one study G_i mediated inhibition of cyclic AMP was isolated by treating cells with cholera toxin to prevent G_s mediated effects (Eason *et al*., 1994). Under these conditions UK-14304 had a potency of 20 nM, while noradrenaline and B-HT 920 had similar potencies but approximately 15 fold lower than UK-14304. This is similar to our data from cytosensor experiments, in which UK-14304 had a potency of 4 nM and was approximately 25 fold more potent than noradrenaline or B-HT 920. In our study and that of Easons group, B-HT 920 had an intrinsic activity of 0.7 with respect to both UK-14304 and noradrenaline. These comparitive data help validate the use of the cytosensor microphysiometer for characterizing α_{2A}-adrenoceptors. We were further intrigued to find that both the absolute potencies of agonists and their intrinsic activities were very similar in the present study employing recombinant α_{2A}-adrenoceptors to that in native α_{2A}-adrenoceptors mediating contraction of dog saphenous vein (Table 1; *c.f.* MacLennan *et al*., 1997a). This may be somewhat fortuitous since both receptor expression level and efficiency of receptor-transducer coupling govern agonist efficacy, and each of these parameters maybe somewhat different in the two systems.

Antagonist studies

We were able to undertake rigorous analyses of antagonist interactions with the α_{2A}-adrenoceptor by employing a 300 fold antagonist concentration range. Our data with the selective α_2-adrenoceptor antagonist rauwolscine is consistent with competition for a single receptor site as Schild plots had slopes not significantly different from unity. A comparison of antagonist affinities with published data utilizing recombinant α_{2A}-adrenoceptors in functional experiments is difficult as little quantitative data is available; in general it is only qualitative effects of antagonists which have been assessed in functional experiments. One study does report a pK_i for yohimbine (a stereoisomer of rauwolscine) of 8.42, calculated from inhibition of agonist-induced intracellular Ca^{2+} elevations (Michel *et al*., 1989). This is in good agreement with the affinity ($pK_B=8.43$) of rauwolscine which we obtained in the present study. We found that antagonist affinity estimates for rauwolscine (8.43) prazosin (<5.5) and spiroxatrine (7.08) are generally lower than those obtained from ligand binding studies (9.02, 6.12 and 7.43 respectively; MacLennan *et al*., 1997a). Spiroxatrine and prazosin were examined as these compounds have the greatest selectivity for α_{2B} and α_{2C} over the α_{2A}-adrenoceptor in radioligand binding studies (MacLennan *et al*., 1997a). Their low affinity at α_{2A}-adrenoceptors was confirmed in these functional experiments. Lower affinity estimates in functional assays may result from methodological differences which include the assay buffer composition and temperature. Such differences are known to influence receptor binding affinity estimates, including those for the α_2-adrenoceptor subtypes (Bylund & Ray-Prenger, 1989). In this latter study affinity estimates for prazosin (and several other antagonists) at the native α_{2A}-adrenoceptor in HT29 cells were considerably lower in functional experiments ($pK_B=5.77$) using hypotonic media at 37°C, than in radioligand binding studies ($pK_i=6.47$) using low isotonic media at room temperature.

Conclusions

We have successfully employed a Cytosensor microphysiometer to characterize human recombinant α_{2A}-adrenoceptors expressed in CHL cells. Rigorous estimates of antagonist affinities, and of agonist potencies and intrinsic activities, have been made and which are in general accord with published data. These data suggest that agonist-induced extracellular rate increases are mediated solely by the recombinant α_{2A}-adrenoceptor.

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