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Characterization of human recombinant α_{2A}-adrenoceptors expressed in Chinese hamster lung cells using intracellular Ca^{2+} changes: evidence for cross-talk between recombinant α_{2A}- and native α_1-adrenoceptors

[1]P.H. Reynen, [1]G.R. Martin, [1]R.M. Eglen & *,[1]S.J. MacLennan

[1]Department of Molecular Pharmacology, Center for Biological Research, Neurobiology Unit, Roche Bioscience, 3401 Hillview Avenue, Palo Alto, California, CA 94304, U.S.A.

1 Human α_{2A}-adrenoceptors expressed in Chinese hamster lung (CHL) fibroblasts have been pharmacologically characterized by measuring intracellular calcium (Ca^{2+}_i) changes using the Ca^{2+}-sensitive dye Fluo3-AM, in conjunction with a fluorometric imaging plate reader (FLIPR).

2 Several α-adrenoceptor agonists were examined including the α_2-adrenoceptor agonists UK-14304, B-HT 920, dexmedetomidine and A-54741, the selective α_1-adrenoceptor agonist phenylephrine and the non-selective adrenergic agonist noradrenaline. Of these only noradrenaline (mean $pEC_{50} = 6.49$) and A-54741 (6.90) evoked changes in Ca^{2+}_i; A-54741 was a partial agonist relative to noradrenaline, achieving only 33% of the noradrenaline maximum.

3 Ca^{2+}_i changes induced by noradrenaline and A-54741 were antagonized by the α_2-selective antagonist rauwolscine (10 nM) and by the α_1-selective antagonists prazosin (0.1 nM) and doxazosin (1.0 nM).

4 Phenylephrine (100 μM) and UK-14304 (10 μM) alone were ineffective in causing Ca^{2+}_i increase. In the presence of a fixed concentration of UK-14304 (3.0 μM), phenylephrine induced concentration-dependent increases in Ca^{2+}_i (mean $pEC_{50} = 5.33$). In the presence of phenylephrine (30.0 μM) UK-14304 induced Ca^{2+}_i release ($pEC_{50} = 6.92$). The effects of phenylephrine were abolished by prazosin (1.0 nM) or rauwolscine (100 nM).

5 In saturation radioligand binding experiments using membranes of parental (non-transfected) CHL cells there was a small, specific binding of [^{3}H]-prazosin ($B_{max} = 24$ fmol mg protein^{-1}; $pK_D = 10.24$).

6 Collectively, these data suggest that α-adrenoceptor agonist-induced Ca^{2+}_i release in CHL fibroblasts transfected with the human α_{2A}-adrenoceptor is dependent upon co-activation of the recombinant receptor and a native α_1-adrenoceptor.
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Abbreviations: CHL, Chinese hamster lung; E/[A], concentration-effect; FLIPR, fluorometric imaging plate reader; PLC, phospholipase C; PI, phosphatidyl inositol; Ca^{2+}_i, intracellular calcium

Introduction

It is now well established that many G protein coupled receptors, particularly those in heterologous expression systems, can activate multiple signal transduction pathways (Kenakin, 1996). An example of such pleiotropic signalling is the α_{2A}-adrenoceptor which has been well characterized and a considerable amount is known regarding its signalling properties. Whether expressed natively or heterologously in different cell lines α_{2A}-adrenoceptors can couple to both $G_{i/o}$ and G_s type G proteins, leading to inhibition and activation of adenylyl cyclase respectively (Bylund & Ray-Prenger, 1989; Cottechia *et al*., 1990; Duzic *et al.,* 1992; Eason *et al*., 1992; 1994; Chabre *et al*., 1994; Jansson *et al*., 1995; Nasman *et al*., 1997). Each of the α_2 adrenoceptor subtypes mediate increases of Ca^{2+}_i and evidence for this is particularly strong in the case of α_{2A}-adrenoceptors (reviewed by Lanier, 1995; Akerman *et al*., 1997). The mechanisms by which α_{2A}-adrenoceptor activation elevates Ca^{2+}_i are principally 2 fold: (i) opening of voltage-dependent L-type Ca^{2+} channels, and (ii) release from intracellular stores *via* stimulation of phospholipase C, probably involving $\beta\gamma$ subunits of G-proteins. Depending on the cell type, these mechanisms may involve pertussis toxin-sensitive and insensitive G proteins.

Although pleiotropic receptor signalling can lead to differential pharmacology depending on which transduction pathway is examined, as in the case of receptors for PACAP (Spengler *et al*., 1993), the octopamine-tyramine receptor (Robb *et al*., 1994) and 5-$HT_{2A/2C}$ receptors (Berg *et al*., 1998), such signalling may be exploited in order to establish alternative assays for receptor characterization purposes. Thus, in a previous study (MacLennan *et al*., 1999) we used a Cytosensor microphysiometer, an instrument that measures the extracellular pH of cells, to pharmacologically characterize human α_{2A}-adrenoceptors expressed in Chinese hamster lung (CHL) fibroblasts. We found that the pharmacological profile of agonists and antagonists was consistent with interactions at the α_{2A}-adrenoceptor. In the present study we set out to determine first, if α_{2A}-adrenoceptor

*Author for correspondence; E-mail: stephen.maclennan@roche.com

activation in CHL cells gave rise to $Ca^{2+}{}_i$ elevations and second, to pharmacologically characterize the response. To measure $Ca^{2+}{}_i$ we have used a fluorometric imaging plate reader (FLIPR), an instrument recently introduced for the high throughput screening of cell-based fluorescent assays, and which has been successfully used to measure $Ca^{2+}{}_i$ changes in conjunction with the dye Fluo-3 (see Schroeder & Neagle, 1996; Watson *et al*., 1998; Coward *et al*., 1998; Porter *et al*., 1999; Smart *et al*., 1999). In the course of these studies we obtained evidence of a synergistic cross-talk between the recombinant α_{2A}-adrenoceptor and an endogenous α_1-adrenoceptor, such that α-adrenoceptor agonists only increase $Ca^{2+}{}_i$ in CHL cells when both receptors are simultaneously activated.

Methods

Stable cell line construction and cell culture

The human α_{2A}-adrenoceptor was transfected into CHL fibroblast cells (R 1610) as previously described (MacLennan *et al*., 1997). The clone used for these studies has a specific binding capacity (B_{max} for [^{3}H]-MK-912) of 1.50 pmol mg^{-1} protein (MacLennan *et al*., 1997). 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).

FLIPR studies

Cells were seeded into 96 well plates (1×10^5 cells per well) 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. For measurement of changes in cytosolic calcium, the cells were washed ($\times 2$) with buffer (HBSS without $CaCl_2$, $MgCl_2$, $MgSO_4$, or phenol red), supplemented with 10 mM HEPES, 2.0 mM $CaCl_2$, and 2.5 mM probenicid, then incubated at 37°C for 60 min with 2.2 μg ml^{-1} FLUO-3 AM. Extracellular dye was washed ($\times 2$) from the plates and vehicle or antagonist was added for 20 min at 37°C prior to placing the plates in the FLIPR. Concentration-effect (E/[A]) curves to agonists were constructed by adding different concentrations to different wells. Three minutes after each agonist exposure, cells were challenged with 10 μM ionomycin to assess cell viability. Relative fluorescence is measured by subtracting basal from peak fluorescence after addition of drug.

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 effect, upper-asymptote, mid-point location and slope parameters respectively. Location parameters were actually estimated as logarithms ($-\log_{10}EC_{50}$).

Radioligand binding studies

Membranes of the parental cell line R 1610 were prepared as previously described (MacLennan *et al*., 1997). Aliquots of membranes were thawed and briefly homogenized in assay buffer (mM; Tris-base 50, EDTA (free acid) 1, NaCl 150, $MgCl_2$ 2, pH 7.4) using a Polytron. Estimates of pK_D and B_{max} for [^{3}H]-prazosin were made in saturation binding experiments. Assay tubes contained 300 μg protein, 4 pM – 1 nM [^{3}H]-prazosin (specific acivity 77.2 Ci mmol^{-1}) and 10 μM phentolamine to define specific binding, in a final volume of 500 μl. Following a 90 min incubation at 37°C, the tubes were filtered over GF/B glass fibre filtermats (Whatman, NJ, U.S.A.) using a Packard Top Count 24 well cell harvester. The tubes were rinsed three times with ice cold 50 mM tris-base, pH = 7.4 (3×1 ml/sample). Radioactivity was determined using liquid scintillation counting (Topcount, Packard Instrumentation Co., CT, U.S.A.).

Analysis of radioligand binding data

The pK_D and B_{max} values of [^{3}H]-prazosin were determined from binding isotherms using non-linear regression (Prism; GraphPad Software, CA, U.S.A.).

Materials

R 1610 cells were obtained from ATCC (#CRL-1657). Lipofectamine, Opti-MEM, DMEM, HBSS, HEPES, phosphate-buffered saline, G-418, phenol red and foetal bovine serum were purchased from Gibco Life Technologies (NY, U.S.A.). Fluo-3 AM was from Teflabs (TX, U.S.A.). The following drugs were purchased: probenecid, (−)-noradrenaline bitartrate, prazosin hydrochloride, (Sigma, MO, U.S.A.); rauwolscine hydrochloride, UK-14304, U73122, U73343, thapsigargin, ω-Conotoxin GVIA; (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 in FLIPR experiments, with the following exceptions: prazosin, 50% EtOH; U73122, U73343, thapsigargin, DMSO; ionomycin, EtOH.

Results

Agonist studies

At concentrations up to 1 mM, noradrenaline had no effect in untransfected cells. In CHL fibroblasts expressing human α_{2A}-adrenoceptors noradrenaline (10 μM) induced a rapid, transient increase in $Ca^{2+}{}_i$ which reached maximum ~15 s following agonist addition. A range of structurally distinct adrenergic agonists were examined. In addition to the non-selective catecholamine noradrenaline, we examined selective α_2-agonists including the imidazolines UK-14304 (Cambridge, 1981), dexmedetomidine (Scheinin *et al*., 1989), A-54741 (Hancock *et al*., 1988) and the azepine B-HT 920 (Van Meel *et al*., 1981). Noradrenaline (mean $pEC_{50} = 6.49$) and A-54741 (6.90) were the only agonists to cause $Ca^{2+}{}_i$ changes (Figure 1A; Table 1). Furthermore, A-54741 was a partial agonist relative to noradrenaline, having a relative intrinsic activity of 0.33 (Table 1). $Ca^{2+}{}_i$ responses induced by noradrenaline and

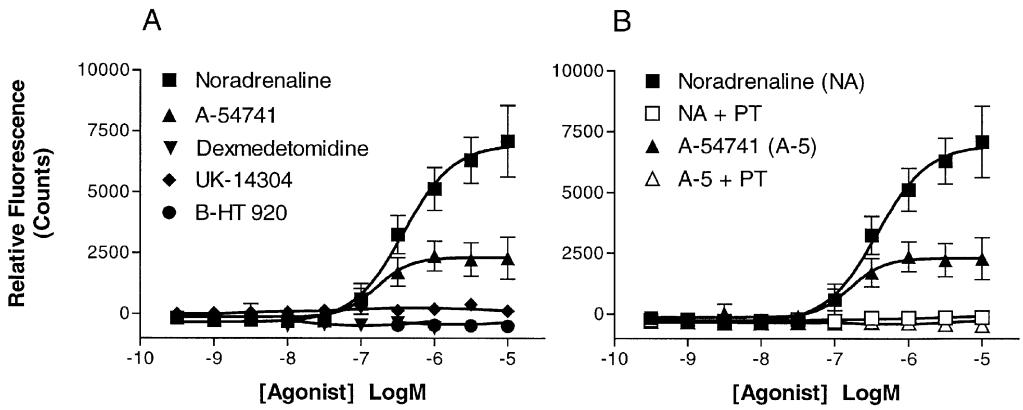

Figure 1 Effect of α-adrenoceptor agonists on $Ca^{2+}{}_i$ of CHL cells expressing the human α_{2A}-adrenoceptor. Cells were loaded with the calcium sensitive dye Fluo-3 AM, before being challenged with agonist. (A) Control concentration-effect curves (B) Cells were treated with pertussis toxin (PT, 500 ng ml^{-1} for 24 h) before agonist exposure. Data are the mean±s.e.mean of three separate experiments.

Table 1 Potency and intrinsic activity (I.A.) data for agonist-induced intracellular Ca^{2+} release in CHL fibroblasts transfected with the human α_{2A}-adrenoceptor, using FLIPR

	FLIPR		*Microphysiometer**		*Binding†*
Agonist	pEC_{50}	*I.A.* (NA = 1.0)	pEC_{50}	*I.A.* (NA = 1.0)	pK_i
Noradrenaline (NA)	6.49±0.12	1.0	6.92±0.20	1.0	5.12±0.10
A-54741	6.90±0.06	0.33±0.02	8.89±0.08	1.09±0.03	7.38±0.03
UK-14304	Inactive	0	8.36±0.38	1.06±0.14	6.71±0.05
Dexmedetomidine	Inactive	0	8.84±0.23	0.46±0.10	7.99±0.04
B-HT 920	Inactive	0	7.05±0.23	0.68±0.07	6.36±0.08

Values are the s.e.mean of 3–6 individual estimates. For reference potency and intrinsic activity estimates are also given for ligand activity at α_{2A}-adrenoceptors mediating acidification rate changes in CHL fibroblasts as well as radioligand binding affinity estimates for α_{2A}-adrenoceptors expressed in CHL cells.*Data from MacLennan *et al.*, 1999. †Data from MacLennan *et al.*, 1997.

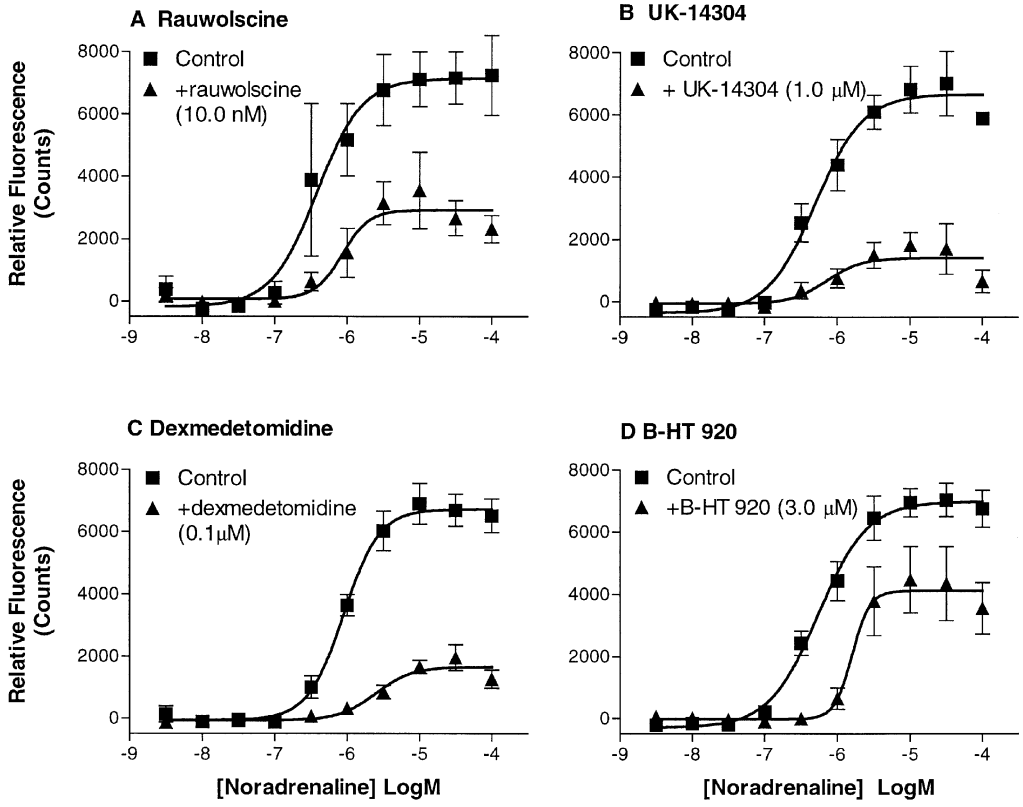

Figure 2 Antagonism of noradrenaline-induced $Ca^{2+}{}_i$ responses in CHL cells expressing the human α_{2A}-adrenoceptor. Cells were exposed to noradrenaline (control) or noradrenaline in the presence of (A) rauwolscine (10.0 nM), (B) UK-14304 (1.0 μM), (C) dexmedetomidine (0.1 μM) or (D) B-HT-920 (3.0 μM) which were added 20 min prior to noradrenaline addition. The concentrations of antagonist were calculated to achieve 50–90% occupancy of α_{2A}-adrenoceptors, based on affinities determined in radioligand binding experiments (MacLennan *et al.*, 1997). Data are the mean±s.e.mean of three separate experiments.

A-54741 were abolished by prior treatment of cells with pertussis toxin (Figure 1B) suggesting that $G_{i/o}$ type G proteins played a principal role in the signal transduction.

Antagonist studies

Each of the silent ligands were examined as antagonists of noradrenaline responses. The concentrations of each ligand were chosen to achieve between 50 and 90% receptor occupancy in the absence of agonist, i.e. 3–10 fold greater than their affinities determined from radioligand binding experiments (MacLennan *et al*., 1997). UK-14304, dexmedetomidine and B-HT 920 as well as the classical α_2-adrenoceptor antagonist rauwolscine caused a non-surmountable blockade of noradrenaline-induced $Ca^{2+}{}_i$ increases (Figure 2). Rauwolscine (10 nM) nearly abolished the effects of A-54741 ($n=3$, data not shown).

The selective α_1-adrenoceptor antagonists prazosin and doxazosin were also examined. Low concentrations of these ligands (0.1 and 1.0 nM) also caused a non-surmountable antagonism of noradrenaline (Figure 3) and A-54741 (data not shown) responses. These data suggested the involvement of α_1-adrenoceptors and the following experiments were conducted to address this. Phenylephrine and UK-14304, regarded as selective agonists for α_1- and α_2-adrenoceptors respectively (Ruffolo *et al*., 1995), had no effect alone on $Ca^{2+}{}_i$ (Figures 1 and 4). In the presence of UK-14304 (3.0 μM), phenylephrine produced concentration-dependent $Ca^{2+}{}_i$ increases (pEC_{50} 5.33 ± 0.14), with a maximum of $\sim$50% of the noradrenaline response (Figure 4). In the presence of phenylephrine (30.0 μM), UK-14304 evoked similar $Ca^{2+}{}_i$ increases (pEC_{50} 6.92 ± 0.14). These effects of phenylephrine and UK-14304 were abolished by α_1- or α_2-adrenoceptor-selective concentrations of prazosin (1.0 nM) and rauwolscine (100 nM), respectively (Figure 5).

Signal transduction studies

The involvement of PLC-dependent pathways in α-adrenoceptor-induced $Ca^{2+}{}_i$ increases was investigated by employing the inhibitor U73122 (10 μM) which blocked noradrenaline responses to a greater extent than its less active analogue U73343 (Figure 6). Thapsigargin (0.1 μM), an inhibitor of Ca^{2+}-ATPase in the endoplasmic reticulum, abolished noradrenaline responses (Figure 6). The L- and N-type calcium channel blockers nitrendipine (0.32 μM) and ω-conotoxin GVIA (0.1 μM) had no effect on noradrenaline responses ($n=3$, data not shown). U73122, thapsigargin, nitrendipine and ω-conotoxin had similar effects on A-54741-induced $Ca^{2+}{}_i$ responses (data not shown), as on noradrenaline responses.

Radioligand binding

Saturation binding with [^{3}H]-prazosin to membranes of the parental CHL cell line R 1610, revealed a saturable, specific,

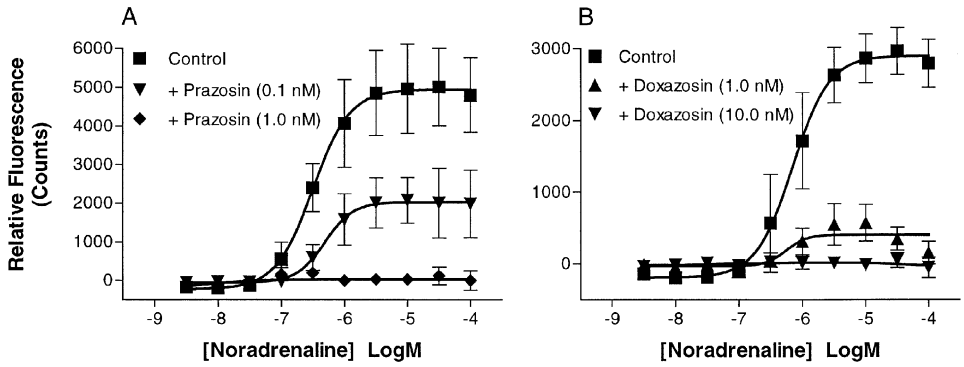

Figure 3 Effect of α_1-adrenoceptor antagonists on noradrenaline-induced $Ca^{2+}{}_i$ responses in CHL fibroblasts expressing the human α_{2A}-adrenoceptor. Cells were exposed to noradrenaline (control) or noradrenaline in the presence of (A) prazosin and (B) doxazosin which were added 20 min prior to noradrenaline challenge. Data are the mean$\pm$s.e.mean of three separate experiments.

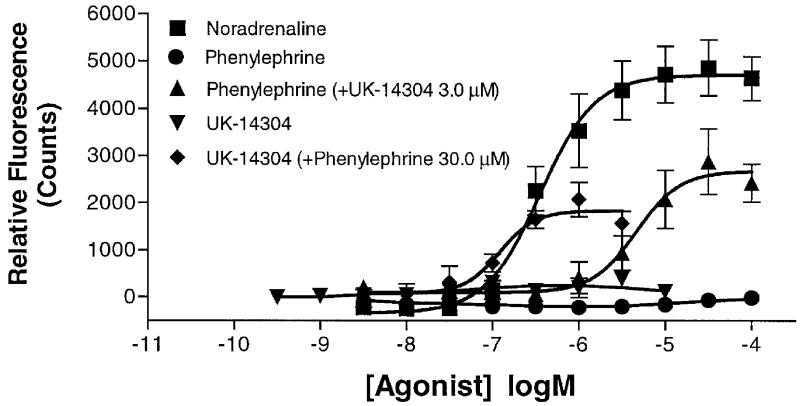

Figure 4 Effect of α-adrenoceptor agonists on $Ca^{2+}{}_i$ in CHL fibroblasts transfected with the human α_{2A}-adrenoceptor. Cells were challenged with noradrenaline, phenylephrine or UK-14304, or a combination of the two latter agonists. A fixed concentration of either UK-14304 (3.0 μM) or phenylephrine (30.0 μM) was added simultaneously with different concentrations of phenylephrine or UK-14304 respectively. Data are the mean$\pm$s.e.mean of three separate experiments.

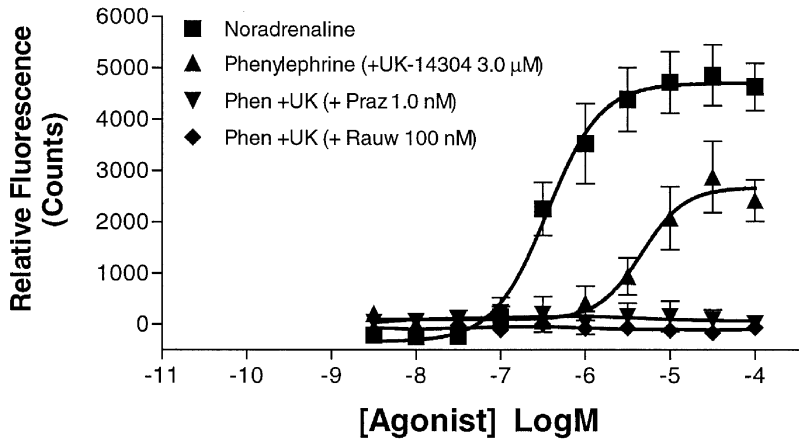

Figure 5 Effect of α-adrenoceptor agonists on $Ca^{2+}{}_i$ in CHL fibroblasts transfected with the human α_{2A}-adrenoceptor. Cells were challenged with noradrenaline or phenylephrine plus UK-14304 (3.0 μM) which were added simultaneously. Cells were also challenged with phenylephrine plus UK-14304 in the presence of either prazosin (1.0 nM) or rauwolscine (100 nM) which had been added 20 min previously. Data are the mean ± s.e.mean of three separate experiments.

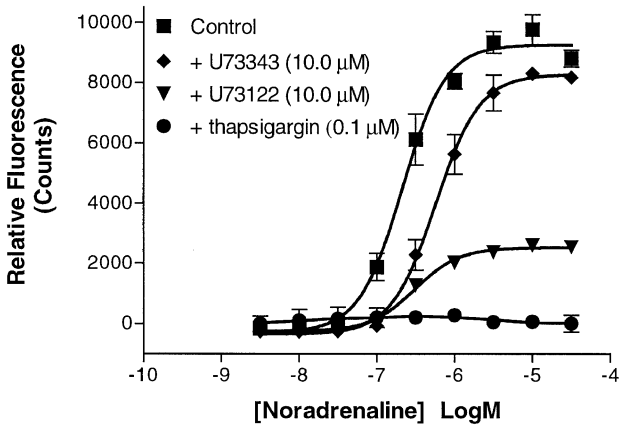

Figure 6 Effect of U73122 (10 μM), U73343 (10 μM) and thapsigargin (0.1 μM) on noradrenaline-induced $Ca^{2+}{}_i$ increases in CHL cells transfected with the human α_{2A}-adrenoceptor. Drugs were added to the cells 10 min prior to the noradrenaline challenge. Data are the mean ± s.e.mean of three separate experiments.

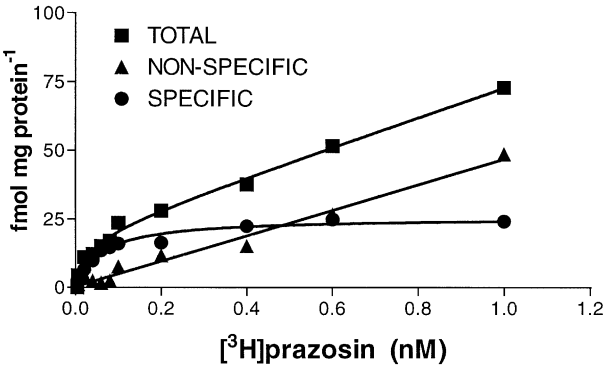

Figure 7 Radioligand ([^{3}H]-prazosin) binding to membranes of the parental (non-transfected) cell line R 1610. Shown are representative data from one of four experiments which were conducted. Non-specific binding was defined using 10 μM phentolamine.

high affinity binding site (Figure 7). The pK_D was 10.24 ± 0.08 with a B_{max} of 24 ± 1 fmol mg protein^{-1} ($n = 4$).

Discussion

The objective of this study was to pharmacologically characterize human α_{2A}-adrenoceptors expressed in CHL fibroblasts by studying receptor mediated effects on $Ca^{2+}{}_i$. To do this we employed a set of well characterized α_2-adrenoceptor agonists comprising noradrenaline, UK-14304, A-54741, dexmedetomidine and B-HT 920 (Cambridge, 1981; Hancock *et al*., 1988; Scheinin *et al*., 1989; Jasper *et al*., 1998; MacLennan *et al*., 1999). Our observations are not consistent with an interaction at α_{2A}-adrenoceptors only however. Instead, the data suggest that co-activation of both the recombinant receptor and an endogenous α_1-adrenoceptor is necessary for $Ca^{2+}{}_i$ release. This conclusion is based on four pieces of evidence from functional experiments: (i) noradrenaline had no effect on $Ca^{2+}{}_i$ release in parental (non-transfected) CHL fibroblasts; (ii) noradrenaline effects were antagonized by α_1-selective concentrations of prazosin and doxazosin and by α_2-selective concentrations of rauwolscine; (iii) phenylephrine and UK-14304, which are regarded as selective α_1- and α_2-adrenoceptor agonists (see Ruffolo *et al*., 1995) had no effect alone but when added together induced a robust release of $Ca^{2+}{}_i$ which was sensitive to prazosin or rauwolscine and (iv) noradrenaline responses were abolished by pertussis toxin which ADP-ribosylates $G_{i/o}$ and not $G_{q/11}$ G proteins which are the principal G proteins utilized by α_2- and α_1-adrenoceptors respectively. Evidence for an endogenous population of α_1-adrenoceptors was obtained in radioligand binding experiments which found a small (24 fmol mg protein^{-1}) but specific binding of [^{3}H]-prazosin to membranes of the parental cell line.

A synergistic interaction between α_1- and α_{2A}-adrenoceptors in CHL fibroblasts was unexpected as in a previous study using this cell line (MacLennan *et al*., 1999) we found that noradrenaline, UK-14304, A-54741, dexmedetomidine and B-HT 920 each increased the extracellular acidification rate *via* activation of α_{2A}-adrenoceptors only, and were insensitive to the selective α_1-adrenoceptor antagonists prazosin and doxazosin. The question arises as to why in microphysiometer experiments agonists such as noradrenaline are not sensitive to selective α_1-adrenoceptor antagonists. The explanation may lie in the very transient nature of the $Ca^{2+}{}_i$ response which reaches maximum ~15 s after addition of a maximally effective concentration of noradrenaline, and returns to baseline after ~60 s. This contrasts with the α_{2A}-adrenoceptor induced effect on extracellular acidification rate, which reflects a net increase in cellular metabolism, which reaches a peak only after

~300 s exposure to noradrenaline. It would appear that the cellular metabolic changes caused by α_1-adrenoceptor activation are not substantial or prolonged enough to contribute to the overall acidification response induced by ligands such as noradrenaline and thus are insensitive to selective α_1-adrenoceptor antagonists.

The presence of endogenous α_1-adrenoceptors in CHL fibroblasts has implications for previous studies which have used this cell line for exploring the signal transduction of α_2-adrenoceptors. Cottechia *et al.* (1990) concluded that α_{2A}-adrenoceptors expressed in CHL fibroblasts (PS120 strain) could directly stimulate PLC, based on the sensitivity of PI hydrolysis to pertussis toxin. The involvement of native α_1-adrenoceptors was not pharmacologically examined using selective antagonists and would clearly be worthy of further investigation.

Amplifying interactions, or synergy, between receptors coupled to $G_{i/o}$ and $G_{q/11}$ proteins is a phenomenon frequently observed in isolated tissues and in cell-based assays (MacLennan *et al*, 1993; Selbie & Hill, 1998) although the molecular mechanisms are not understood. Available evidence points to the involvement of $\beta\gamma$ subunits released from $G_{i/o}$ proteins interacting with α subunits of $G_{q/11}$ which together augment PLC activation with a resultant increase in $Ca^{2+}{}_i$, PKC activity and arachidonic acid production (see Selbie & Hill (1998) for references). The actions of the enzyme inhibitor U73122 support the involvement of PLC in the interaction between native α_1 and recombinant α_{2A}-adrenoceptors expressed in CHL cells which we have observed in the present study.

To our knowledge the synergy which we observed is unique in that selective agonists for both receptors induced a response only when added concommitantly; added alone neither had any effect. We have proposed a theoretical model of amplification among receptors in which the stimulus produced by one receptor is amplified by the stimulus from a second (Martin *et al*., 1996). This model, which is a refinement of the two-receptor:1-transducer model first described by Leff (1987) accounts for amplification in a unidirectional manner only, for example amplification of 5-HT_{1B} mediated contraction of rabbit femoral artery ($G_{i/o}$ coupled) by TP receptor agonists ($G_{q/11}$ coupled) but not *vice versa* (MacLennan *et al*., 1993). It does not describe the data obtained in the present study where we observed amplification of phenylephrine-induced $Ca^{2+}{}_i$ release by UK-14304 and *vice versa*. Another refinement of the two-receptor:1-transducer model can account for bi-directional synergy (Scaramellini *et al*., 1997). In this model the interactions between agonists displaying E/[A] curves of different shapes were analysed by incorporating slope factors into the separate and common parts of the transduction pathways. This model predicts bi-directional amplification of the maximum response to a partial agonist in the presence of a fixed concentration of the other agonist under the scenario where the Hill equation describing the common signal transduction pathway has a steep slope coefficient, i.e. greater than unity. We are presently investigating whether this model can account for the dramatic type of synergy observed in the present study.

We have provided evidence of an endogenous population of α_1-adrenoceptors in CHL fibroblasts. Further experiments are required using subtype-selective ligands to elucidate the subtype involved, but on the basis of the high affinity of [^{3}H]-prazosin ($pK_D = 10.24$) it may be similar to the α_{1B}-adrenoceptor (Williams *et al*., 1999). Since the agonists noradrenaline and A-54741 only gave rise to $Ca^{2+}{}_i$ release in cells transfected with the α_{2A}-adrenoceptor it follows from the conclusion discussed above that these ligands must have efficacy at both α_1- and α_{2A}-adrenoceptors to elicit $Ca^{2+}{}_i$ release, whereas UK-14304, dexmedetomidine and B-HT 920 may be ineffective due to low efficacy at one or both receptors. Noradrenaline and A-54741 have similar efficacy at α_{2A}-adrenoceptors mediating contraction of dog saphenous vein (MacLennan *et al*., 1997), as determined by operational modelling, and have greater intrinsic efficacy than UK-14304, dexmedetomidine and B-HT 920. With respect to α_1-adrenoceptors we are not aware of quantitative affinity and efficacy estimates for these ligands. However, dexmedetomidine is a low affinity (~1 μM) partial agonist at α_{1B}- and α_{1A}-adrenoceptors expressed in Hela cells (Schwinn *et al*., 1991); UK-14304, B-HT 920 and A-54741 are agonists at α_1-adrenoceptors mediating contraction of rat and rabbit isolated aorta (Beckeringh *et al*., 1984; DeBernardis *et al*., 1986). Quantitative estimates of the affinity and efficacy of these α_2 agonists at α_1-adrenoceptor subtypes may provide evidence for their failure to induce $Ca^{2+}{}_i$ release in CHL fibroblasts.

In a previous study we obtained affinity estimates for A-54741 at α_{2A}-adrenoceptors mediating contraction of dog saphenous vein ($pK_A = 8.03$) and human recombinant α_{2A}-adrenoceptors expressed in CHL cells ($pK_i = 7.38$). The lower potency of A-54741 to induce intracellular Ca^{2+} release ($pEC_{50} = 6.90$) is presumably related to its lower affinity at α_1-adrenoceptors such that functional effects are only seen at concentrations of the ligand which occupy both receptor subtypes. As mentioned above, we are not aware of affinity estimates for A-54741 at α_1-adrenoceptors.

This study has provided evidence that α-adrenoceptor agonist-induced $Ca^{2+}{}_i$ elevations in CHL cells involves a PLC-dependent release of Ca^{2+} from intracellular stores as noradrenaline and A-54741 responses were blocked by the PLC inhibitor U73122 (Bleasdale *et al*., 1990) and by thapsigargin which inhibits the Ca^{2+}-ATPase pump of the endoplasmic reticulum (Thastrup *et al*., 1990). Although it is well established that both α_1- and α_2-adrenoceptor mediated $Ca^{2+}{}_i$ changes are both subtype and cell specific (see McGrath *et al*., 1989; Lanier, 1995; Akerman *et al*., 1997) there is no evidence to suggest a synergistic cross-talk between α-adrenoceptors as part of the underlying mechanism. In several cell lines including NIH-3T3, S115 or Sf9, α_2-adrenoceptor activation does not elevate $Ca^{2+}{}_i$ (Duzic & Lanier, 1992; Enkvist *et al*., 1996). It would be interesting to determine if these cells lacked native α_1-adrenoceptors. In astrocytes, which express native α_{2A} and α_{2B} subtypes (Enkvist *et al*., 1996), the agonists noradrenaline, UK-14304 and dexmedetomidine each raised $Ca^{2+}{}_i$, and responses were abolished by pre-treatment of the cells with pertussis toxin or U73122 (Salm & McCarthy, 1990; Enkvist *et al*., 1996), indicating a similar mechanism of action to that which we have found in CHL cells. The possible involvement of α_1-adrenoceptors was not examined using selective antagonists. However, phenylephrine-induced $Ca^{2+}{}_i$ release was resistant to pertussis toxin which suggests the presence of native α_1-adrenoceptors. Whether cross-talk between native α_1- and α_2-adrenoceptors contributes to agonist-induced $Ca^{2+}{}_i$ release in astrocytes is therefore worthy of further investigation. Such synergy does not contribute to adrenoceptor agonist-induced $Ca^{2+}{}_i$ release in human erythroleukaemia cells which have endogenous α_{2A}-adrenoceptors since responses to epinephrine and UK-14304 were insensitive to 100 nM prazosin (Michel *et al*., 1989).

Conclusion

We have provided evidence of a synergistic interaction between endogenous α_1- and recombinant α_{2A}-adrenoceptors in CHL fibroblasts, and which may have relevance to other cell types which co-express these receptor subtypes. Vascular smooth muscle cells are one example, many of which (particularly venous smooth muscle) express both α_1- and α_2-adrenoceptors (see McGrath *et al*., 1989, for references). In dog and rabbit saphenous veins it is the α_{2A}-adrenoceptor which mediates contraction, the subtype of α_1-adrenoceptor is not known (MacLennan *et al*., 1997). In rabbit saphenous vein (Daly *et al*., 1988) and rat tail artery (Templeton *et al*., 1989) there is already evidence of post-receptor interactions between the α-adrenoceptor subtypes which supports the notion that synergy between these receptors may have a general importance in the control of cellular responsiveness. Our results have clear implications for the heterologous expression of recombinant α-adrenoceptors and shows that careful screening of host cell lines is necessary to detect low levels of native adrenoceptors.

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