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Receptor reserve analysis of the human α_{2C}-adrenoceptor using [^{35}S]GTPγS and cAMP functional assays

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Abstract

Here we determine for norepinephrine, (5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline) (UK14,304), 5,6,7,8-tetrahydro-6-(2-propenyl)-4*H*-thiazolo[4,5-*d*]azepin-2-amine dihydrochloride (BHT-920), (2-[3-hydroxy-2,6-dimethyl-4-*t*-butylbenzyl]-2-imidazoline) (oxymetazoline), and ((*R*)-3-Hydroxy-α-[(methylamino)methyl]-benzenemethanol hydrochloride) (phenylephrine), affinities using a radiolabeled agonist and antagonist, and potency and efficacy values in membrane [^{35}S]guanosine-5′-*O*-(3-thiotriphosphate) ([^{35}S]GTPγS) binding and cAMP cellular inhibition assays, in Chinese hamster ovary cells (CHO-K1) expressing the human α_{2c}-adrenoceptor. These cells express a high ratio of receptor to G-protein because each agonist, but not several antagonists, displaced [^{3}H]UK14,304 with higher affinity than [^{3}H]rauwolscine. The rank order of potency of high affinity K_i and EC_{50} in both functional assays was norepinephrine ≥ UK14,304 > BHT-920 > oxymetazoline > phenylephrine. The receptor reserve of G-protein activation and cAMP responses was measured with the irreversible antagonist, benextramine; K_A values of norepinephrine or UK14,304 were similar (289, 271 or 150, 163 nM, respectively). A 20-fold greater receptor occupancy was required for agonist-induced half-maximal [^{35}S]GTPγS binding compared to cAMP inhibition, indicating significant signal amplification in cells. Therefore, the G-protein activation assay is better at distinguishing full and partial agonists.

Keywords: α_{2C}-Adrenoceptor; Receptor reserve; [^{35}S]GTPγS binding; cAMP; Efficacy; Signal amplification

1. Introduction

The α_2-adrenoreceptors are a distinct family of G-protein coupled receptors that respond to the endogenous catecholamines, norepinephrine and epinephrine and mediate control of many central and autonomic physiological responses, largely through inhibitory effects. Three homologous subtypes (α_{2A}, α_{2B}, α_{2C}) have been identified and described on various cells and tissues (Docherty, 1998). The human genes have been cloned and expressed in heterologous systems (Kobilka et al., 1987; Regan et al., 1988; Lomasney et al., 1990), greatly facilitating the understanding of G-protein activation and signal transduction mediated by these receptors.

Agonist-induced α_2-adrenoreceptor coupling to heterotrimeric GTP-binding proteins (G-proteins) promotes the release of GDP from the α-subunit of G-proteins followed by the binding and subsequent hydrolysis of GTP. This G-protein activation can be measured in the membranes of cells expressing α_2-adrenoreceptors by the binding of [^{35}S]guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) (Jasper et al., 1998; Peltonen et al., 1998), a radiolabeled poorly hydrolyzable GTP analog. Considerable evidence exists that intracellular effects are mediated through pertussis toxin-sensitive inhibitory G_i-proteins, including inhibition of adenylyl cyclase activity, resulting in decreases in cellular cAMP, an important second messenger molecule. Stimulatory effects of α_2-adrenoreceptor agonists have also been described, including coupling to stimulatory cholera toxin-sensitive G_s proteins with increased cAMP generation (Eason et al., 1994; Pohjanoksa et al., 1997; Jasper et al., 1998), and Ca^{2+} mobilization (Kukkonen et al., 1998).

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Numerous studies have described the α_2-adrenoreceptor subtypes in transfected cells through the definition of the affinities and efficacies of antagonists and agonists at these receptors. The description of agonists in such cell systems may be confounding due to the use of different cell types with varying receptor densities defined by various radioligands, multiple cellular or membrane functional assays, and little quantitation of the receptor to G-protein ratio, receptor coupling or receptor reserve in any one system or study. In addition, the vast majority of these studies have looked for similarities and differences across the three homologous subtypes (α_{2A}, α_{2B}, α_{2C}) and have not comprehensively examined any one receptor with multiple assessments of α_2-adrenoreceptor ligands. In this study we have sought to characterize a series of α_2-adrenoreceptor agonists (norepinephrine, UK14,304, oxymetazoline and phenylephrine) and antagonists (yohimbine, WB 4101, HV 723, ARC239) at the human α_{2C}-adrenoreceptor, expressed in CHO-K1 cells, by parallel examinations of binding affinities, and potencies and efficacies in two commonly used functional assays. Binding affinities were derived using both a radiolabeled antagonist as well as a radiolabeled agonist in order to assess the ratio of receptor to G-protein in this recombinant receptor cell system and to definitively quantitate K_{iH}. The functional assays used were the [^{35}S]GTPγS binding in membranes, a measure of G-protein activation, and agonist-mediated inhibition of cAMP in forskolin-stimulated cells. In order to correlate efficacies and potencies of agonists in these assays, the relationship of receptor occupancy to response (i.e. receptor reserve) in each assay was measured. The comprehensive evaluation of these multiple parameters in one recombinant cell system, CHO-K1 cells expressing the human α_{2C}-adrenoreceptor, represents a unique study that enables conclusions to be drawn that are not hampered by disparate conditions under which the studies were conducted. Importantly, significant, quantifiable, signal amplification was observed in the inhibition of cellular cAMP which was absent in membrane G-protein activation, thus explaining the greater discrimination of partial versus full agonists in the latter compared to former response assays. Lastly, we propose the coordinated use of these assays as a means by which novel α_{2C}-adrenoreceptor compounds can be identified.

2. Materials and methods

2.1. Isotopes and drugs

A CHO-K1 cell line stably expressing the human recombinant α_{2C}-adrenoreceptor was purchased from Euroscreen (Brussels, Belgium). The Swissprot accession number is P18825. [^{3}H]Rauwolscine (71 Ci/mmol), [^{3}H]UK14,304 (74.7 Ci/mmol), [^{3}H]norepinephrine (51.8 Ci/mmol), ^{35}S-GTPγS (1259 Ci/mmol), and Basic FlashPlates® were purchased from NEN Life Science Products (Boston, MA, USA). GF/C unifilter plates and Microscint20 were purchased from Packard (Downers Grove, IL, USA). UK14,304 ((5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline)) and ARC239 (2-[2-(4-2-methoxyphenyl) piperazin-1-yl) ethyl] - 4,4 - dimethlyl-1, -(2*H*,4*H*)-isoquinolindione dihydrochloride) were purchased from Tocris Cookson (Langford, UK). Phenylephrine ((*R*)-3-Hydroxy-α-[(methylamino)methyl]benzenemethanol hydrochloride), prazosin (1-[4-amino-6,7-dimethoxy-2-quinazolinyl]-4-[2-furanylcarbonyl]-piperazine hydrochloride), WB4101 (2-(2,6-dimethoxyphenoxyethyl)aminomethy-1,4-benzodiaoxane hydrochloride) were obtained from RBI (Natick, MA, USA). Benextramine, BHT-920 (5,6,7,8-tetrahydro-6-(2-propenyl)-4*H*-thiazolo[4,5-*d*]azepin-2-amine dihydrochloride), bovine serum albumin, EDTA, EGTA, forskolin, GTPγS, GDP, isobutylmethylxanthine (IBMX), $MgCl_2$, norepinephrine, oxymetazoline (2-[3-hydroxy-2,6-dimtehyl-4-*t*-butylbenzyl]-2-imidazoline), Tris–HCl, and yohimbine (17-hydroxy-yohimban-16-carboxylic acid methyl ester hydrochloride) were purchased from Sigma (St. Louis, MO, USA). HV723 (α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxy)ethyl-amino)-propyl)-benzene acetonitrile fumarate) was synthesized by medicinal chemists at Schering-Plough. Ham's F12 medium, penicillin and streptomycin and Hank's Balanced Salt Solution (HBSS) were purchased from Life Technologies (Rockville, MD, USA). Fetal bovine serum was obtained from Summit Biotechnology (FT Collins, CO) and G418 from Gemini Bioproducts (Calabasas, CA).

2.2. Binding studies

CHO-K1 cells stably expressing the human α_{2C}-adrenoreceptor were grown in complete Ham's F12 media containing 10% fetal bovine serum, 50 IU/ml penicillin and 50 μg/ml streptomycin and 400 μg/ml G418. Membranes were prepared by homogenizing the cells in buffer containing 15 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$, 0.3 mM EDTA and 1 mM EGTA, followed by two consecutive centrifugation steps at $40{,}000 \times g$ for 25 min separated by a wash in the same buffer. Membranes were resuspended in buffer containing 7.5 mM Tris–HCl pH 7.5, 12.5 mM $MgCl_2$, 0.3 mM EDTA, 1 mM EGTA and 250 mM sucrose. Protein was quantitated using the Bio-Rad protein assay (Hercules, CA, USA). For saturation analyses, 10 μg membrane protein ([^{3}H]rauwolscine) or 20 μg membrane protein ([^{3}H]UK14,304 and [^{3}H]norepinephrine) was incubated with radioligand in a final volume of 200 μl binding buffer (75 mM Tris–HCl pH 7.4, 12.5 mM $MgCl_2$, 2 mM EDTA) per well of 96 well plates for 1 h at room temperature. The ranges of radioligand tested were 0.1 to 6 nM, 0.15 to 15 nM and 0.2 to 24 nM for [^{3}H]rauwolscine,

[^{3}H]UK14,304, or [^{3}H]norepinephrine, respectively. Total and nonspecific binding were determined in triplicate. Nonspecific binding was defined in the presence of 10 μM yohimbine. Competition studies were performed using either 1 nM [^{3}H]rauwolscine (2 × the K_d value) and 16 concentrations of cold competitor ligand as indicated or 3 nM [^{3}H] UK14,304 (3 × the K_d value) and eight concentrations of cold competitor ligand, in triplicate or quadruplicate. To determine the GTP dependence of agonist binding, 100 μM of GTPγS was included in a parallel set of wells. Assays were terminated by rapid filtration through GF/C unifilter plates, presoaked with 0.3% polyethylenimine, with five washes with 0.5 ml cold 50 mM Tris–HCl pH 7.4 buffer, using a Packard Filtermate Harvester. After drying, bound radioactivity was determined by liquid scintillation counting (Packard TopCount) with Microscint 20, 50 μl/well. All binding data were analyzed using GraphPad Prism (GraphPad Software, San Diego CA). Binding data were analysed on the assumption of a one-site model followed by that of a two-site model and the preferred model selected by the *F*-test.

2.3. Agonist-induced [^{35}S]GTPγS binding

Membranes from CHO-K1 cells expressing the α_{2C}-adrenoreceptor were prepared as described above. Initial experiments evaluated GDP and NaCl concentrations for their effects on potency and efficacy of agonists. Concentrations of GDP and NaCl were chosen to minimize the differences between binding K_i values and EC_{50} values in the [^{35}S]GTPγS binding assay, while maintaining a measurable percent increase of binding over basal [^{35}S]GTPγS binding. The final [^{35}S]GTPγS assay buffer was identical to the binding buffer described which lacked NaCl but included 1 μM GDP. For receptor reserve studies, cells at 90% confluency were pretreated for 1 h at 37°C with the indicated concentration of the alkylating agent benextramine. Cells were washed, followed by cell harvest and membrane preparation. Unless otherwise stated, each reaction was set up in quadruplicate wells by adding the reagents in the following order to NEN Basic FlashPlate® microplates: membranes (20 μg protein/well in 160 μl assay buffer); 20 μl serial dilutions of compounds or 1 μM cold GTPγS (non-specific binding); and 20 μl 0.1 nM [^{35}S]GTPγS for a total volume of 200 μl per well. After 30 min at room temperature including 2 min of slow shaking on a titer plate shaker, the plates were centrifuged for 5 min at 2500 rpm at 4°C in a tabletop Sorvall centrifuge, and counted immediately with a Packard TopCount. The percent increase over basal binding of [^{35}S]GTPγS was calculated as follows: 100 × [[mean total sample cpm − mean basal cpm]/mean basal cpm]. Basal cpm was defined as the mean cpm in the absence of agonist compound minus the mean non-specific binding cpm. Half-maximal effective concentrations (EC_{50}, the concentration of agonist required to give 50% of its own maximal stimulation) were calculated using nonlinear regression with GraphPad Prism. The maximal increase over basal binding of [^{35}S]GTPγS (E_{max}) achieved for each drug is expressed as a percentage of the maximal UK14,304 response tested in the same experiment.

2.4. Cell culture

CHO-K1 cells expressing the α_{2C}-adrenoreceptor were grown according to the protocol provided by Euroscreen, with the exception that fungizone was not included in the culture medium. Cells were cultured as a monlayer in tissue culture flasks in complete Ham's F12 culture medium at 37°C with 5% CO_2 and were recultured every 2–3 days; cells were harvested from the culture flasks by using Dulbecco's PBS (without Ca/Mg^{2+}) containing 5 mM EDTA.

2.5. Measurement of cellular cAMP

CHO-K1 cells expressing the α_{2C}-adrenoreceptor were harvested from the culture flasks as described above. Cells were recultured in 96-well plates at 33,000 cells/well in Ham's F12 medium overnight. Following washing with HBSS (without phenol red), the cells were pretreated with cAMP buffer consisting of HBSS, 10 mM HEPES, 4 mM $MgCl_2$, 0.2% bovine serum albumin, pH 7.4 and 1 mM IBMX for 30 min at 37°C. Agonist compounds in cAMP buffer were added at the indicated final concentrations in the presence of 10 μM forskolin. (This concentration of forskolin was chosen because it induced approximately 50–100 pmol/ml cAMP (10–20 times basal cAMP) and was inhibited by ≥ 85% with ≥ 100 nM of full agonists.) For the receptor reserve studies, the cells were pretreated as above with the inclusion of benextramine (4 or 6 μM) for 60 min followed by two washes with HBSS containing 0.2% bovine serum albumin. All experimental and control conditions were done in quadruplicate. Following incubation at 37°C for 30 min, the HBSS was removed, and ethanol was added to the cell monolayer (75 μl/well). After drying of the ethanol by placing the plate in a 45–50°C water bath, cAMP levels were quantitated by using the cAMP FlashPlate® assay kit (NEN #SMP001A), according to the manufacturer's protocol. Plates were counted in a scintillation counter (Packard). Sample cAMP values were determined by interpolation from a standard curve, corrected for the appropriate dilution factor and expressed as pmol/ml. Agonist mediated inhibition of forskolin-induced cAMP was calculated as follows, where basal or control are the mean cAMP values obtained from quadruplicate culture wells treated with cAMP buffer alone or with 10 μM forskolin, respectively: %inhibition = 100 × [(experimental sample cAMP − basal cAMP)/(control − basal)]. EC_{50} values were calculated using non-

linear regression with GraphPad Prism. The maximal inhibition of cAMP (E_{max}) achieved for each drug is expressed as a percentage of the maximal UK14,304 response tested in the same experiment.

2.6. Receptor reserve analysis

K_A values for UK14,304 and norepinephrine were derived from measurements of agonist-induced inhibition of cAMP and from agonist-induced [^{35}S]GTPγS binding. This was done by plotting the reciprocal values of equipotent concentrations of UK14,304 or norepinephrine before ($1/[A]$) or after ($1/[A']$) pretreatment with benextramine, ensuring that the concentration of benextramine used caused a depression in the maximal agonist response. This concentration of benextramine was determined in preliminary assays where a range of benextramine concentrations was used. From the linear regression of this relationship, the K_A was determined using the equation $K_A = (\text{slope} - 1)/y\text{-intercept}$. The fractional receptor occupancy, at varying agonist concentrations, was then calculated using the equation, receptor occupancy $= ([A]/[A] + K_A)) \times 100$, where $[A]$ = fixed agonist concentration and K_A = dissociation constant for the agonist (Furchgott, 1966). The receptor occupancy was then plotted against the fractional response in order to determine the degree of receptor reserve (Furchgott, 1966; Stanton and Beer, 1997).

3. Results

3.1. Ligand binding studies

Saturation binding was performed using a radiolabeled antagonist, [^{3}H]rauwolscine and two radiolabeled agonists, [^{3}H]UK14,304 and [^{3}H]norepinephrine with membranes from CHO-K1 cells expressing the α_{2C}-adrenoreceptor. The affinities and capacities of the membranes for each radioligand were derived from the binding isotherms and are as follows: [^{3}H]rauwolscine, $K_d = 0.52 \pm 0.04$ nM, $B_{max} = 2.69 \pm 0.88$ pmol/mg ($n = 3$); [^{3}H]UK14,304, $K_d = 1.12 \pm 0.15$ nM, $B_{max} = 1.07 \pm 0.11$ pmol/mg ($n = 3$) and [^{3}H]norepinephrine, $K_d = 2.94 \pm 0.3$ nM, $B_{max} = 0.59 \pm 0.02$ pmol/mg ($n = 3$), respectively. The K_d value of [^{3}H]rauwolscine was similar to values previously reported (Marjamaki et al., 1993; Uhlen et al., 1998). The binding capacity of the membranes was greater (2.4–4.5 fold) for the radiolabeled antagonist compared to the radiolabelled agonists. To determine the GTP dependence of the specific binding of these radioligands, 100 μM GTPγS was included in parallel. As expected, little effect of GTPγS on the K_d or B_{max} was observed with [^{3}H]rauwolscine (K_d in absence and presence of GTPγS = 0.54 and 0.53 nM, respectively; B_{max} in absence and presence of GTPγS = 3.6 and 3.1 pmol/mg, respectively). In contrast, specific binding of both agonist radioligands was ablated by GTPγS, indicating guanine nucleotide sensitivity (data not shown).

Competition binding experiments were performed using five α_2-adrenoreceptor agonists, namely, UK14,304, oxymetazoline, BHT-920, phenylephrine and norepinephrine and the radiolabelled antagonist [^{3}H]rauwolscine. The binding parameters are summarized in Table 1. The competition curves for each agonist were biphasic (Fig. 1) and best modelled by the two-site fit ($n_H \ll 1$). A significant fraction of the total binding sites, ranging from 32.4 to 55.6%, were in the high affinity state. The binding curves of each agonist in the presence of GTPγS (Fig. 1) were best modelled by a one-site fit, as indicated by the Hill coefficient being closer to unity (Table 1). The addition of 100 μM GTPγS induced rightward shifts primarily in the high affinity component of the binding curve of each agonist (Fig. 1). The highest affinity ligand tested was norepinephrine ($K_{iH} = 1.93 \pm 0.66$, $K_{iL} = 339 \pm 235$ nM, Fig. 1A). These values are similar to those previously determined (Pihlavisto et al., 1998) in S115 cells. The rank order of affinities is: norepinephrine > UK14,304 (Fig. 1B) > BHT-920 (Fig. 1C) > oxymetazoline (Fig. 1D) > phenylephrine (Fig. 1E). The α_2 AR antagonists ARC 239, HV 723, yohimbine and WB 4101 yielded monophasic curves with no rightward displacement in the presence of GTPγS (data not shown). The K_i (nM) for these antagonists are: yohimbine, 2.6 ± 0.9; WB 4101, 3.8 ± 1.8; HV 723, 16 ± 7.8; and ARC239, 105 ± 15 (each from four to six independent experiments).

The recombinant system being used is likely to be characterized by a high receptor to G-protein ratio. If so, it

Table 1
Affinities of α_2-adrenoceptor ligands for the α_{2C}-adrenoceptor using a radiolabelled antagonist, [^{3}H]rauwloscine

	K_{iH}	K_{iL}	%H	App. K_i	n_H	App. K_i + GTPγS	n_H
BHT-920	5.67 ± 1.40	316 ± 73	44.9 ± 8.6	52 ± 24	0.55 ± 0.06	389 ± 138	0.87 ± 0.07
Norepinephrine	1.93 ± 0.66	339 ± 235	55.6 ± 3.7	12 ± 3.7	0.42 ± 0.03	646 ± 99	0.78 ± 0.02
Oxymetazoline	9.60 ± 7.51	140 ± 16	32.4 ± 6.3	61 ± 1.0	0.73 ± 0.10	124 ± 6	0.89 ± 0.08
Phenylephrine	42.81 ± 9.16	2944 ± 426	45.0 ± 1.3	287 ± 52	0.49 ± 0.01	9048 ± 635	0.8 ± 0.09
UK14304	3.86 ± 1.0	364 ± 81	38.4 ± 0.3	93 ± 24	0.5 ± 0.03	460 ± 73	0.81 ± 0.02

The competition studies were done with cell membrane preparations and [^{3}H]rauwolscine as radioligand. K_{iH} and K_{iL} are inhibition constants for the high and low affinity sites in a two-site model. %H is the percentage of sites in the high affinity state. Apparent (App.) K_i is the inhibition constant for a one-site model. The n_H indicates the Hill coefficient. All values are means ± SD of three experiments done in triplicate.

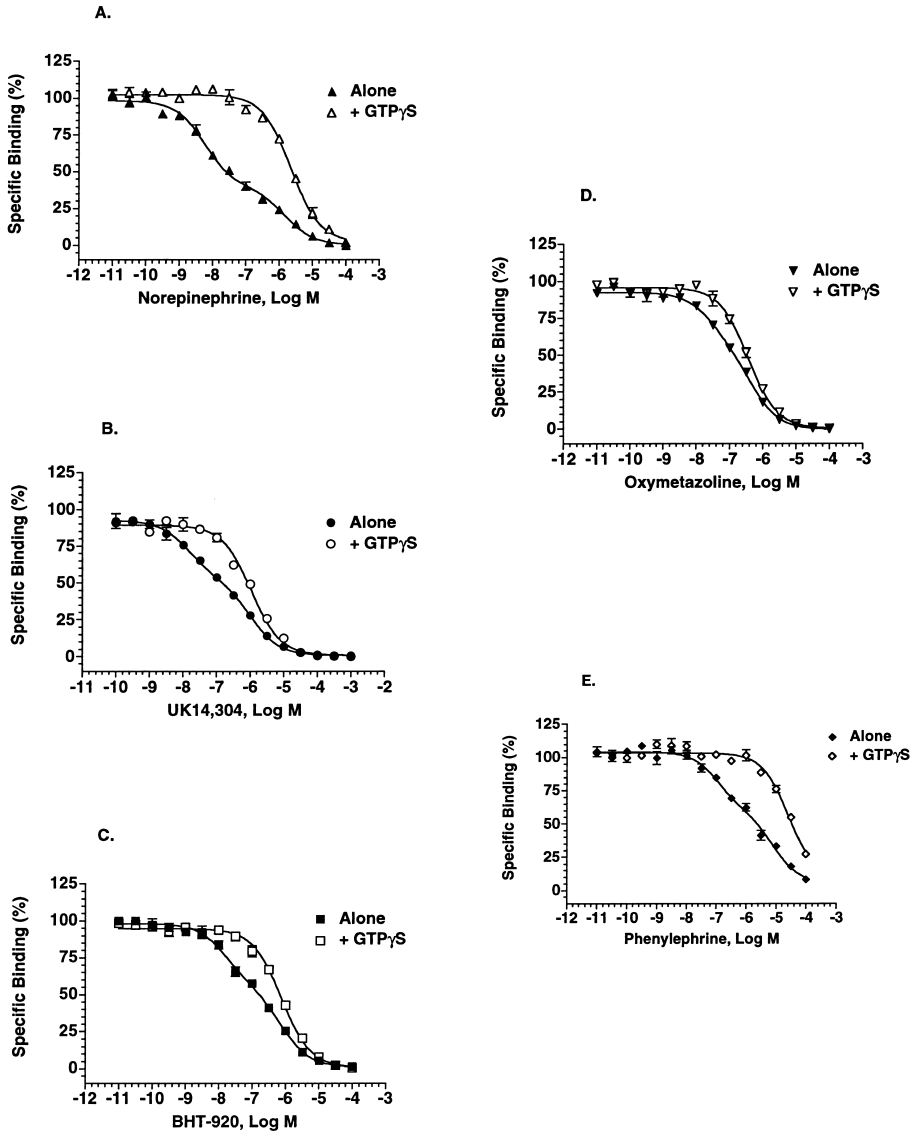

Fig. 1. A comparison of agonist binding curves in the absence (alone) or presence (+GTPγS) of 100 μM GTPγS for (A) norepineprine, (B) UK14,304, (C) BHT-920, (D) oxymetazoline, and (E) phenylephrine in recombinant α_{2C}-adrenoreceptor membranes. The binding curves represent the specific binding of [^{3}H]rauwolscine (mean ± SD of triplicate determinations) in an experiment representative of three independent experiments, which are summarized in Table 1.

is predicted that agonists will displace a radiolabeled agonist with higher affinity than a radiolabelled antagonist and that antagonists would displace these radioligands with equivalent affinity (Kenakin, 1997b). To further explore this, additional competition binding studies were done using the radiolabeled agonist, [^{3}H]UK14,304 and the five α_2-adrenoreceptor agonists evaluated above. The competition curves for each agonist were monophasic (Fig. 2) and did not fit a two-site model ($p \geq 0.4$). Hill slopes were close to unity (Table 2). The rank order of affinities (Table 2) is norepinephrine > UK14,304 > BHT-920 > oxymetazoline > phenylephrine, which is virtually identical to the rank order of K_{iH} obtained with the radiolabeled antagonist, [^{3}H]rauwolscine. In addition, the K_i values for each agonist obtained using [^{3}H]UK14,304 were very similar to the K_{iH} values, but not the K_{iapp} or K_{iL} values obtained with [^{3}H]rauwolscine (Table 1). In contrast, an evaluation of the α_{2C}-adrenoreceptor antagonists ARC 239, HV 723, yohimbine and WB 4101 as competitors versus [^{3}H]UK14,304 yielded K_{iapp} values very similar to the K_{iapp} values derived using the radiolabeled antagonist, [^{3}H]rauwolscine. The K_i (nM) for these antagonists are: yohimbine, 2.5 ± 0.6; WB 4101, 6.5 ± 3.0; HV 723, 13.5 ± 5.7; and ARC239, 88 ± 31 (each from 3 to 7 independent experiments). This pattern of displacement of a radiolabeled-agonist and -antagonist by competitor agonists and antagonists suggests a high ratio of receptor to G-protein in this system (Kenakin, 1997b).

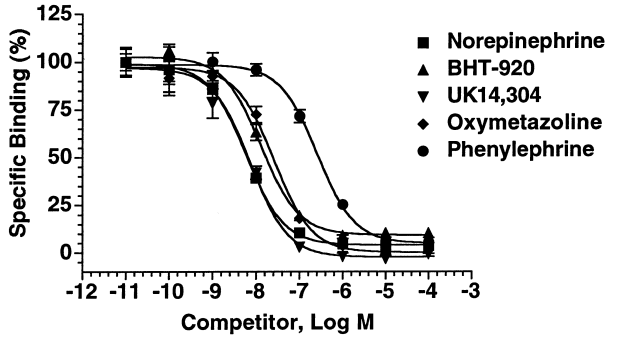

Fig. 2. A comparison of agonist binding curves using the radiolabelled agonist, [^{3}H]UK14,304 in recombinant α_{2C}-adrenoreceptor membranes. The agonists used as competitors were: norepinephrine, BHT-920, UK14,304, oxymetazoline and phenylephrine. The binding curves represent the specific binding of [^{3}H]UK14,304 (mean ± SD of triplicate determinations) in an experiment representative of 3–8 independent experiments, which are summarized in Table 2.

3.2. Agonist-induced GTPγS binding and inhibition of cAMP

Some studies suggest that the high affinity state of the α_2-adrenoreceptor represents the form of the receptor that mediates functional responses (Piletz et al., 1996). To evaluate this in the context of recombinant α_{2C}-adrenoreceptor expression in CHO-K1 cells, functional responses mediated by UK14,304, oxymetazoline, BHT-920, phenylephrine and norepinephrine were quantitated such that they could be correlated with the K_i values derived from the binding studies. Two assays were used to measure agonist-induced responses: first, [^{35}S]GTPγS binding in membranes which measures a proximal event of receptor activation that is independent of second messenger formation; and second, agonist-mediated inhibition of cAMP in forskolin-stimulated cells, a more distal event directly quantitating a second messenger molecule.

A study of agonist-induced [^{35}S]GTPγS binding to the α_{2C}-adrenoreceptor is shown in Fig. 3 and potency and efficacy values (E_{max}) of multiple studies are summarized in Table 3. The rank order of potency of the agonists is: norepinephrine > UK14,304 = BHT-920 > oxymetazoline > phenylephrine. In comparison to UK14,304, whose efficacy was set at 100%, norepinephrine demonstrated similar maximal efficacy, while BHT-920 was slightly less efficacious (91.8%); oxymetazoline and phenylephrine were partial agonists (Fig. 3A and B, Table 3). The antagonists ARC 239, WB 4101, HV 723 and prazosin did not induce [^{35}S]GTPγS binding (Fig. 3A).

Table 2
Affinities of α_2-adrenoceptor ligands for the α_{2C}-adrenoceptor using a radiolabelled agonist, [^{3}H]UK14,304

	App. K_i (nM)	n_H
BHT-920	3.2 ± 1.1 (3)	0.91 ± 0.12 (3)
Norepinephrine	1.7 ± 0.4 (3)	0.85 ± 0.12 (3)
Oxymetazoline	7.4 ± 1.7 (3)	1.06 ± 0.07 (4)
Phenylephrine	57 ± 15 (4)	0.96 ± 0.09 (3)
UK14304	2.2 ± 0.5 (8)	1.08 ± 0.19 (8)

The competition studies were done with cell membranes and [^{3}H]UK14,304 as the radioligand. App. K_i is the inhibition constant for a one-site model, which provided the best fit. n_H is the Hill coefficient. All values are means ± SD of the number of experiments indicated in parentheses.

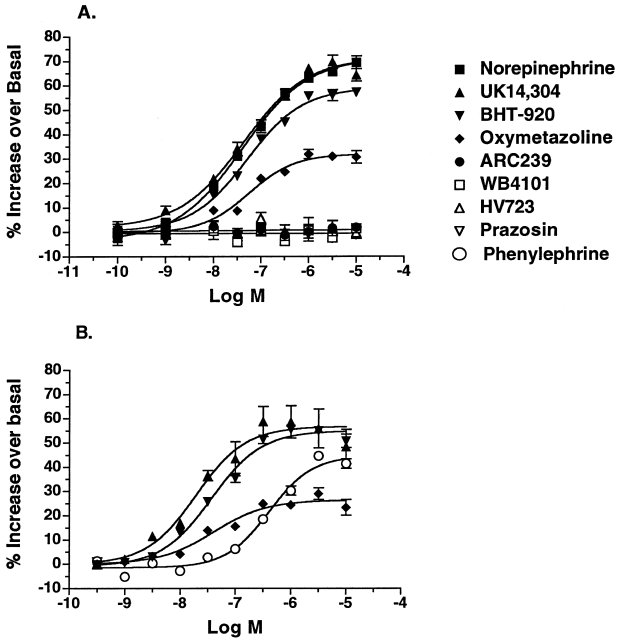

Fig. 3. Recombinant α_{2C}-adrenoreceptor stimulation of [^{35}S]GTPγS binding to CHO-K1 cell membranes. Results are expressed as the percent increase over basal [^{35}S]GTPγS binding. The values shown (mean ± SD of quadruplicate determinations) are of two experiments (A, B) representative of 4–8 independent experiments, which are summarized in Table 3. Basal binding in the experiments shown were approximately: (A) 3200 cpm; (B) 3800 cpm. The agonists tested were norepinephrine (A); UK14,304, BHT-920, oxymetazoline (A, B); and phenyephrine (B). The antagonists tested were ARC 239, WB4101, HV 723 and prazosin (A).

Agonists were also evaluated for their ability to inhibit forskolin-induced cAMP in recombinant α_{2C}-adrenoreceptor-expressing CHO-K1 cells, the same cell line from which membranes were prepared for use in radioligand and [^{35}S]GTPγS binding studies. A representative experiment of agonist-induced cAMP inhibition is shown in Fig. 4 and potency and efficacy values of multiple studies are summarized in Table 3. The rank order of potency of the agonists in inhibiting cAMP is: norepinephrine ≥ UK14,304 > BHT-920 > oxymetazoline > phenylephrine. This rank order is very similar to that obtained in the radioligand binding studies and the [^{35}S]GTPγS assay (Table 3). Norepinephrine, UK14,304, BHT-920 and oxymetazoline were full agonists demonstrating near maximal efficacy of > 94% (Fig. 4A and B, Table 3). Of the ligands tested, only phenylephrine was partially efficacious (56%) in inhibiting cAMP (Fig. 4B, Table 3). Interestingly, of the agonists tested in both functional assays, it was noted that oxymetazoline was a full agonist in the cAMP assay but a partial agonist in the [^{35}S]GTPγS assay.

Table 3
Evaluation of the potency and efficacy of α_2-adrenoceptor agonists in functional assays

	cAMP assay		GTPγS assay	
	EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}
BHT-920	5.4 ± 1.1 (8)	98.1 ± 4.1 (4)	35.0 ± 9.5 (6)	91.8 ± 10.3 (6)
Norepinephrine	1.8 ± 0.2 (5)	97.9 ± 5.6 (7)	28.2 ± 12 (4)	99.3 ± 10 (4)
Oxymetazoline	10.0 ± 6.1 (6)	94.1 ± 7.3 (5)	47.7 ± 12.4 (5)	49.0 ± 8 (5)
Phenylephrine	170 ± 91 (4)	56.0 ± 13 (4)	337 ± 77 (5)	77.8 ± 15.4 (5)
UK14304	2.1 ± 1.5 (18)	100	34.9 ± 12.3 (8)	100

EC_{50} values were calculated using nonlinear regression. The maximal inhibition of cAMP or the maximal % increase GTPγS binding over basal binding (E_{max}) is expressed as a percentage of the maximal UK14304 response which is set to 100%. Values are means ± SD of the number of experiments indicated in parentheses.

3.3. Receptor reserve quantitation

The degree of receptor reserve, with respect to the endogenous ligand, norepinephrine, and another agonist, UK14,304 was determined by comparing the ability of these two agonists to induce [^{35}S]GTPγS binding or inhibition of forskolin-induced cAMP before and after pretreatment of cells with the alkylating agent, benextramine (Furchgott, 1966; Doughty et al., 1990; Stanton and Beer, 1997). A receptor reserve analysis of UK14,304-induced GTPγS binding (Fig. 5) and cAMP response (Fig. 6) is shown while the relevant numerical parameters for both norepinephrine and UK14,304 from multiple experiments are described in the text and summarized in Table 4. Pretreatment of the α_{2C}-adrenoreceptor-expressing CHO-K1 cells with benextramine for 1h caused a reduction of the maximal response to norepinephrine and UK14,304 (Fig. 5A) in the [^{35}S]GTPγS binding assay (Fig. 5A). The EC_{50} values in the absence and presence of benextramine for norepinephrine or UK14,304 were: 61.4 and 177.2 nM or 60.9 and 156.7 nM, respectively. The reciprocal values of equiactive doses of norepinephrine or UK14,304 (Fig. 5B) before and after treatment with benextramine were calculated and yielded a K_A value of 288.5 nM for norepinephrine and 150 nM for UK14,304. A comparison of the receptor occupancy versus the GTPγS binding response demonstrates a larger receptor reserve for norepinephrine than UK14,304 (Fig. 5C). A receptor occupancy of 18.2% by norepinephrine or 28.0% by UK14034 is required to elicit a half-maximal response.

A similar receptor reserve analysis was done for norepinephrine and UK14,304-induced inhibition of cellular cAMP, such that the reserve could be compared for the same agonists in the two functional assays. Pretreatment of the α_{2C}-adrenoreceptor-expressing CHO-K1 cells with benextramine for 1 h caused a reduction in the maximal inhibition of cAMP by norepinephrine and UK14,304 (Fig. 6A). The EC_{50} values in the absence and presence of benextramine for norepinephrine or UK14,304 were: 2.0 and 144.8 nM or 1.9 and 63.1 nM, respectively. K_A values, calculated as described above for the GTPγS binding studies, were 271.3 nM for norepinephrine and 163.2 nM for UK14,304 (Fig. 6B). For each agonist, these K_A values are comparable to those obtained in the [^{35}S]GTPγS binding assay. A comparison of the receptor occupancy versus response also demonstrates a receptor reserve for

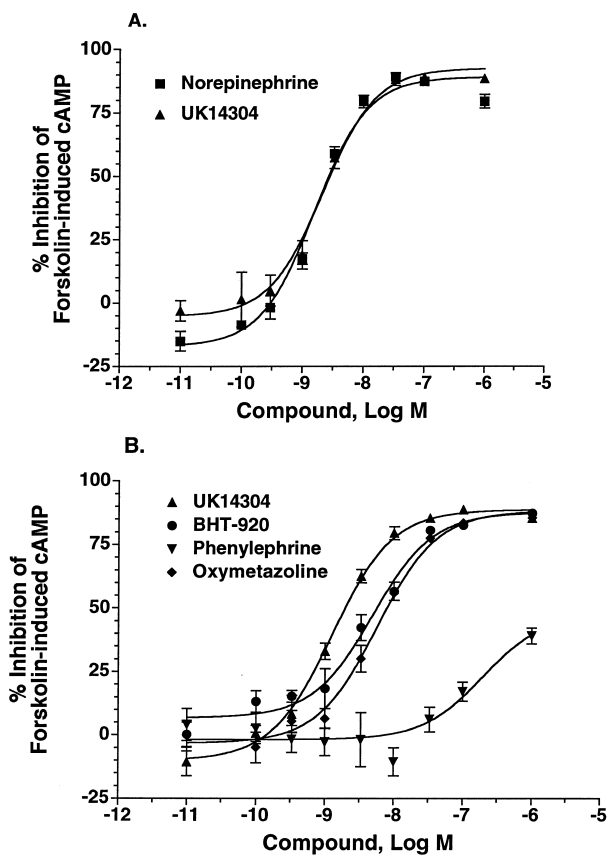

Fig. 4. Recombinant α_{2C}-adrenoreceptor-mediated inhibition of forskolin-induced cAMP in CHO-K1 cells. Results are expressed as the percent inhibition of cAMP induced by 10 μM forskolin in the absence of compound. The values shown (mean ± SD of quadruplicate determinations) are of two experiments representative of 4–18 independent experiments, which are summarized in Table 3. In the experiments shown basal and forskolin-induced cAMP (pmol/ml) were: (A) 6.6 ± 0.82 and 53.4 ± 4.2; (B) 4.3 ± 1.6 and 55.3 ± 7.4. The agonists tested were norepinephrine (A); UK14,304 (A, B), BHT-920, oxymetazoline and phenyephrine (B).

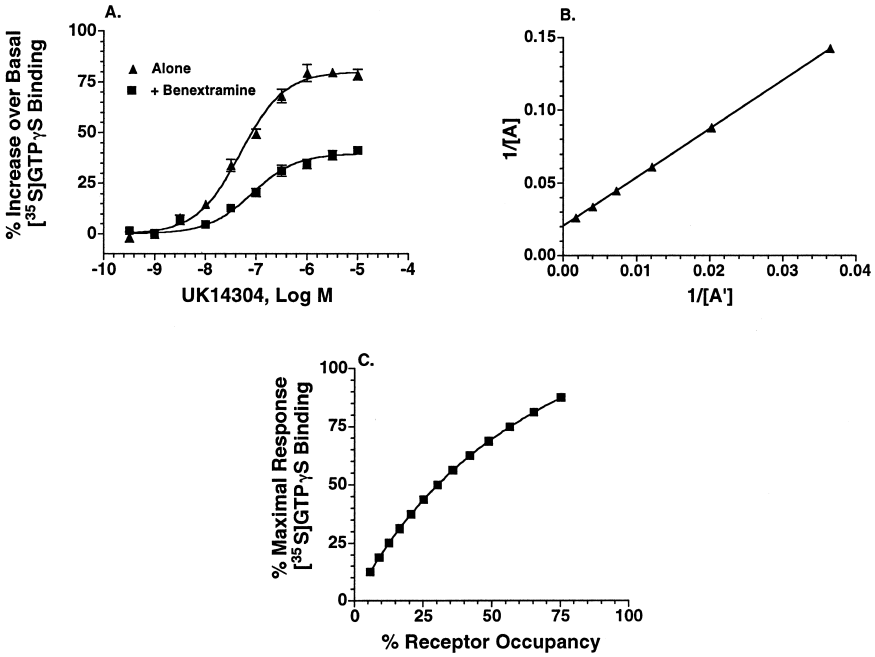

Fig. 5. Receptor reserve analysis of norepinephrine and UK14,304 in the [^{35}S]GTPγS binding assay. (A) Dose response curves of a representative study of agonist-induced binding of [^{35}S]GTPγS to membranes from recombinant α_{2C}-adrenoreceptor expressing CHO-K1 cells, that were pretreated or not with 1 μM benextramine. Values represent mean ± SD of quadruplicate determinations. The EC_{50} values UK14,304 in the absence and presence of benextramine were 50 and 90 nM, respectively in this experiment. (B) A comparison of equiactive doses of UK14,304 with and without benextramine yielded a K_A value for UK14,304 of 114 nM (slope = 3.3 and *y*-intercept = 0.02063). (C) A comparison of receptor occupancy versus response indicated 30.3% receptor occupancy by UK14,304 is required to elicit a half-maximal (50%) response.

norepinephrine that is larger than that of UK14,304 in this assay (Fig. 6C). A receptor occupancy of 0.9% by norepinephrine or 1.4% UK14,034 is required to elicit a half-maximal response. Importantly, it is clear that the

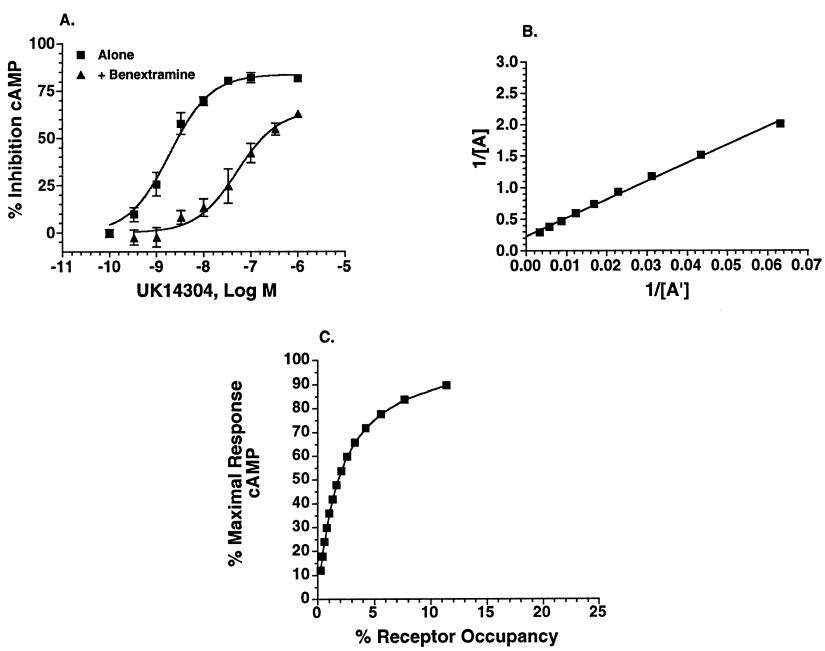

Fig. 6. Receptor reserve analysis of norepinephrine and UK14,304 in the cAMP assay. (A) Dose–response curves of a representative study of agonist-induced inhibition of forskolin-induced cAMP in recombinant α_{2C}-adrenoreceptor expressing CHO cells, that were pretreated or not with 4 μM benextramine. Values represent mean ± SD of quadruplicate determinations. In the experiment shown basal and forskolin-induced cAMP were 4.56 ± 0.73 and 61.44 ± 5.96 pmol/ml, respectively. The EC_{50} values for UK14,304 in the absence and presence of benextramine were 1.92 and 51.8 nM. (B) A comparison of equiactive doses of UK14,304 with and without benextramine yielded a K_A value for UK14,304 of 122 nM (slope = 29.04 and *y*-intercept = 0.2299). (C) A comparison of receptor occupancy versus response indicated that 1.9% receptor occupancy by UK14,304 is required to elicit a half-maximal (50%) response.

Table 4
Receptor reserve analysis

	GTPγS				cAMP			
	EC_{50} (nM)		K_A	% Occupancy	EC_{50} (nM)		K_A	% Occupancy
	Minus	Plus[a]			Minus	Plus[b]		
Norepinephrine	61.4 ± 6.5	177.2 ± 58.6	288.5 ± 111	18.2 ± 4.9	2.0 ± 0.2	144.8 ± 80.5	271.3 ± 96.3	0.9 ± 0.2
UK14,304	60.9 ± 15.8	156.7 ± 94.6	150 ± 50.9	28 ± 3.2	1.9 ± 0.1	63.1 ± 16	163.2 ± 58.3	1.4 ± 0.7

All values represent mean ± SD of 2–3 independent experiments. K_A and % occupancy values required for a 50% response were calculated as described in Section 2.

[a] In the presence of 1 μM benextramine.

[b] In the presence of 4 or 6 μM benextramine.

receptor reserve for each ligand is larger in the cellular cAMP assay than in the membrane [^{35}S]GTPγS binding assay.

4. Discussion

To identify novel α_{2C}-adrenoreceptor agonists, we have compared binding affinities of several standard agonists, using a radiolabeled agonist and antagonist, with potencies and efficacies in two functional responses, a membrane G-protein activation and cellular cAMP inhibition assays. This comprehensive evaluation of these multiple parameters in one recombinant cell system, CHO-K1 cells expressing the human α_{2C}-adrenoreceptor, is a unique study that enables conclusions to be drawn that are not hampered by disparate conditions under which the studies were conducted. First, the recombinant system used has a high ratio of receptor to G-protein as demonstrated by the comparison of K_i values using a radiolabeled agonist and radiolabeled antagonist. The affinities of norepinephrine, UK14,304, oxymetazoline and phenylephrine for displacing [^{3}H]UK14,304 have not been reported previously; only rarely, has a radiolabeled agonist (Piletz et al., 1996) been used to label G-protein coupled α_2-adrenoreceptors in competition binding studies. Second, the rank order of agonist or antagonist (yohimbine, WB 4101, HV 723, ARC239) affinities was identical using either [^{3}H]-UK14,304 or [^{3}H]rauwolscine as the radioligand. Third, the rank order of affinities of agonists was identical to the rank order of potency in each response assay. Fourth, receptor reserve quantitation demonstrated on average a requirement for 20-fold greater receptor occupancy by both norepinephrine and UK14,304 for half-maximal G-protein activation compared to half-maximal inhibition of cellular cAMP. This finding clearly indicates significant cellular signal amplification and explains the greater discrimination of partial versus full agonists in the G-protein activation compared to cAMP response assays.

The K_d and binding capacity of the membranes from the recombinant α_{2C}-adrenoreceptor-expressing CHO-K1 cells determined here using a radiolabeled antagonist, [^{3}H]rauwolscine, and two radiolabeled agonists, [^{3}H]norepinephrine and [^{3}H]UK14,304 as well as in the determination K_i values of a series of α_2-adrenoreceptor agonists using [^{3}H]UK14,304 or [^{3}H]rauwolscine in the absence and presence of GTPγS, clearly demonstrated that less than 50% of the total binding sites were in the high affinity state. The present inability of all α_{2C}-adrenoreceptors to couple simultaneously to G-protein has also been observed in other cell backgrounds (Pihlavisto et al., 1998). For the agonists studied, the K_i values determined using the radiolabeled agonist, were virtually identical to the K_{iH}, not the K_{iapp}, determined with the radiolabeled antagonist. In contrast, the K_{iapp} values of the antagonists did not differ when displacing the radiolabeled antagonist or agonist. One explanation for the observed differences in affinities of agonists using the different radioligands is a high ratio of receptor to G-protein in this recombinant system (Kenakin, 1997b). Of further significance is the observation that the rank order of potency of the agonists (norepinephrine ≥ UK14,304 > BHT-920 > oxymetazoline > phenylephrine) or antagonists (yohimbine > WB 4101 > HV723 > ARC239) was identical using either radioligand.

We then evaluated the potency and efficacy of these α_2-adrenoreceptor agonists in two commonly used functional assays in the same α_{2C}-adrenoreceptor-CHO-K1 cell expression system: first, [^{35}S]GTPγS binding in membranes which measures a proximal event of receptor activation that is independent of second messenger formation, and second, agonist-mediated inhibition of cAMP in forskolin-stimulated cells, a more distal event directly quantitating a second messenger molecule.

It is common practice to determine the correlation between K_i and EC_{50} values and in order to make predictions as to whether the high or low affinity site mediates function. This is often problematic given that buffer and/or kinetic conditions may be different in the functional and binding studies. In the measurement of G-protein activation, we specifically chose buffer conditions to maintain affinity, i.e., no NaCl was included. NaCl is known to decrease the affinity of agonists (Deupree et al., 1996), possibly through allosteric effects (Deupree et al., 1996; Pihlavisto et al., 1998) by modulating the receptor affinity for G-protein (Horstman et al., 1990). Under the present conditions, the EC_{50} values of agonist-induced [^{35}S]GTPγS

binding were approximately ten-fold larger than the high affinity K_i and very similar to the K_{iapp}. A contributing factor to this shift is the inclusion of 1 μM GDP in the GTPγS binding buffer; this change caused a three-fold increase in the K_d of [^{3}H]UK14,304 (K_d = 3.2 nM). If NaCl had been included in the buffer, as in other studies (Jasper et al., 1998), a larger apparent disparity in potency and K_{iH} values would most likely have been observed. In another study, a high correlation of EC_{50} in GTPγS binding and K_{iL} values was observed (Jasper et al., 1998), where potency was determined in the presence of high GDP and NaCl and binding values determined using a radiolabeled antagonist in the presence of GTP. As this illustrates, it is difficult to correlate K_i and EC_{50} values across different studies due to the many differences in the conditions under which each measurement is made.

In contrast to the moderate disparity of the high affinity K_i and the EC_{50} values of agonist-induced G-protein activation for the agonists tested, near identity of the high affinity K_i and the EC_{50} values of agonist-induced cAMP inhibition was observed. Other studies (Nathanson, 1983; Jansson et al., 1994) suggest a correlation of the low affinity site and this response. These differences between studies are best attributed to differences in receptor coupling and reserve in the different cell systems used, as described below.

To characterize the pharmacological activity of α_2-adrenoreceptor agonists, quantitating efficacy is extremely valuable. Although some indication of agonist efficacy may be estimated from the GTP shift derived from ligand binding in the absence and presence of G-protein complexation (Kenakin, 1997b), direct determination is definitive. In the cellular assay, of the five agonists tested, all except phenylephrine were full agonists and we observed near complete inhibition of forskolin-induced cAMP with the full agonists. In contrast, others have seen incomplete cAMP inhibition in recombinant α_{2C}-adrenoreceptor expressing cells (Jansson et al., 1994; Pohjanoksa et al., 1997; Parsley et al., 1999), which is correlated with receptor density (Pohjanoksa et al., 1997). Other studies of cellular activation in α_{2C}-adrenoreceptor-expressing CHO cells, such as extracellular acidification (Pihlavisto and Scheinin, 1999) and intracellular calcium mobilization (Kukkonen et al., 1998), indicate that UK14,304 and norepinephrine have similar efficacy, as is shown here. In addition, in CHO cells treated with pertussis toxin to isolate the G_s response (Eason et al., 1994), norepinephrine and UK14,304 demonstrate similar efficacy in α_{2C}-adrenoreceptor activation in stimulating cAMP.

Based upon the limited set of agonists tested, the agonist-induced G-protein activation response provided better discrimination of agonist efficacy compared to the cAMP response, since both oxymetazoline and phenylephrine displayed partial efficacy, while only the latter was a partial agonist in the cellular assay. Other investigations have shown a rank order of efficacy of norepinephrine > UK14,304 > oxymetazoline in recombinant α_{2C}-adrenoreceptor-expressing human embryonic kidney-293 (HEK-293) cell membranes (Jasper et al., 1998) or have shown little efficacy of UK14,304 relative to norepinephrine (Peltonen et al., 1998). The inclusion of 100 mM NaCl (Jasper et al., 1998; Peltonen et al., 1998) and more GDP (Jasper et al., 1998) in these studies probably contributed to detection of differential efficacy of norepinephrine and UK14,304, since relative efficacy differences among agonists are magnified by increasing GDP concentrations (Selley et al., 1996), and affected by NaCl (Horstman et al., 1990; Deupree et al., 1996; Pihlavisto et al., 1998).

To allow for more meaningful conclusions to be drawn as to affinity and potency, we quantified the relationship of receptor occupancy to each response. The method chosen was that of inactivating a portion of the receptor pool by irreversible antagonism using the alkylating agent, benextramine (Furchgott, 1966; Doughty et al., 1990; Stanton and Beer, 1997). This allows for an accurate derivation of the dissociation constant of the agonist-receptor complex (K_A) without the necessity to use K_d values and EC_{50} values derived under different buffer or kinetic conditions. The derived K_A values for both norepinephrine and UK14,304 were very similar in the GTPγS and cAMP response assays, thereby validating the approach. Of importance, 20-fold higher receptor occupancy was needed for norepinephrine and UK14,304-induced half-maximal G-protein activation response compared to the cAMP response. Because the present methods exclude differences in cells or experimental conditions as the explanation of the larger receptor reserve seen in the cAMP response, it is likely that the explanation resides in the fact that agonist-mediated inhibition of cAMP in forskolin-stimulated cells, a more distal event that quantitates a second messenger molecule, involves signal amplification, while agonist-induced [^{35}S]GTPγS binding in membranes, measures a proximal event of receptor activation independent of second messenger formation and involves little signal amplification. Furthermore, the large receptor reserve observed in the cAMP response suggests that most agonists will be fully efficacious. In support, we found that of the five agonists tested, only phenylephrine was a partial agonist. In contrast, the agonist-induced GTPγS response with a lower receptor reserve, better discriminated agonist efficacy, with both phenylephrine and oxymetazoline displaying partial agonism. Taken together, with respect to these two measurements, relative agonist efficacy is better defined using the binding of [^{35}S]GTPγS than the cellular cAMP response in this α_{2C}-adrenoreceptor system.

There are practical implications to the many parameters described here given that development of α_2-adrenoreceptor subtype-selective agonists would be useful in determining the non-redundant function of each subtype. The use of a radiolabeled agonist to define the K_i of novel compounds with high affinity displacement would not underestimate their binding affinity as would the use of a radiola-

beled antagonist (in the absence of GTP) in a cell system with a high ratio of receptor to G-protein, such as was used here. Typically, recombinant systems, which are often used for ligand screening, are characterized by a high ratio of receptor to G-protein. The parallel use of the [^{35}S]GTPγS binding assay using buffer conditions that maximize affinity, will discern agonists of differing efficacy. In addition potencies are likely to be reasonably predictive of the K_{iapp} and within ten-fold of the K_{iH}. The potencies determined in the [^{35}S]GTPγS binding assay will correlate with the rank order of potency of binding affinities. Lastly, agonists identified by G-protein activation, a biochemical assay which lacks amplification mechanisms, are likely to also exhibit agonist activity in intact cell- or tissue-based systems that possess signal amplification mechanisms (Kenakin, 1997a).

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