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Ligand Efficacy and Potency at Recombinant α_2 Adrenergic Receptors

AGONIST-MEDIATED [^{35}S]GTPγS BINDING

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ABSTRACT. Alpha-2 adrenergic receptors (α_2 AR) mediate incorporation of guanosine 5′-*O*-(γ-thio)triphosphate ([^{35}S]GTPγS) into isolated membranes via receptor-catalyzed exchange of [^{35}S]GTPγS for GDP. In the current study, we used [^{35}S]GTPγS incorporation to characterize the intrinsic activity and potency of agonists and antagonists at the cloned mouse $\alpha_{2a/d}$ and human α_{2a}, α_{2b}, and α_{2c} ARs. Full agonists increased [^{35}S]GTPγS binding to membranes by 2- to 3-fold. Antagonists did not increase [^{35}S]GTPγS binding but competitively inhibited agonist-stimulated [^{35}S]GTPγS binding. Compounds with intrinsic activities less than that of the full agonists norepinephrine (NE) or epinephrine (EPI) were capable of antagonizing agonist-stimulated [^{35}S]GTPγS binding. The agonistic properties of a number of α_2 AR ligands were characterized at each α_2 AR subtype. The rank order of agonist potency for selected compounds at the human receptors (with intrinsic activity compared with NE, defined as 1.0) was:

α_{2a}: Dexmedetomidine (0.73) > guanabenz (0.38) > UK-14304 (1.02) > clonidine (0.32) > ST-91 (0.63) > NE (1.00).

α_{2b}: Dexmedetomidine (1.10) > clonidine (0.18) > guanabenz (0.71) > NE (1.00) > ST-91 (0.44) > UK-14304 (0.59).

α_{2c}: Dexmedetomidine (1.03) > NE (1.00) > UK-14304 (0.75) > ST-91 (0.32) ≥ clonidine (0.23) ≫ guanabenz (0).

This report provides a functional characterization of adrenergic receptor ligands at human and mouse $\alpha_{2a/d}$ AR. It also illustrates the utility of [^{35}S]GTPγS incorporation as a functional marker of receptor activation.
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KEY WORDS. alpha-2 adrenoceptor; adrenergic receptor; [^{35}S]GTPγS; agonist; partial agonist; efficacy; potency; HEK 293 cell

Conventionally, α_2 ARs§ have been described as G_i-coupled receptors that decrease adenylyl cyclase activity [1]. However, α_2 AR also couple to G_s and increase adenylyl cyclase activity [2–7]. Indeed, α_2 ARs can couple to multiple G-proteins, including G_i, G_o, G_s, G_q/$G_{\alpha 11}$, and G_z [8–10]. In both endogenous and recombinant systems, α_2 AR activation decreases cellular cAMP levels at low agonist concentrations, and increases cAMP at higher agonist concentrations [3, 4]. Because of this biphasicity, it is difficult to define the maxima and the potency for α_2 AR agonists.

Agonist stimulation of G-protein-coupled receptors induces conformational changes in their cognate G-proteins, leading to activation of the G-protein and, subsequently, effector activation. Essential for G-protein activation is the exchange of guanine nucleotide, specifically GTP for GDP. This biochemical step can be quantified by the use of a GTP analog, [^{35}S]GTPγS. Activation of G-protein-coupled receptor increases the rate of binding of [^{35}S]GTPγS to G-proteins [11]. The amount of [^{35}S]GTPγS incorporation into membranes at maximal concentrations of agonist is related to the intrinsic activity of various agonists.

This technique affords the opportunity to study α_2 AR activation proximal to adenylyl cyclase, and consequently may avoid those ambiguities arising from promiscuous receptor/G-protein coupling. To directly compare the intrinsic activity and potency of α_2 AR agonists at different α_2 AR subtypes,

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§ *Abbreviations:* AR, adrenergic receptor; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; NE, norepinephrine; EPI, epinephrine; DMEM, Dulbecco's modified Eagle's medium; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; GDPβS, guanosine-5′-*O*-(2-thio)diphosphate; and cAMP, cyclic 5′-adenosine monophosphate.

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agonist-mediated [^{35}S]GTPγS binding assays using membranes of HEK 293 cells expressing the cloned human α_{2a}, α_{2b}, and α_{2c} and mouse $\alpha_{2a/d}$ AR were developed.

MATERIALS AND METHODS

Expression Vector Construction

The human α_{2a}, α_{2b}, and α_{2c} receptor genes were licensed from Dr. Marc Caron (Duke University). The human α_{2a} and α_{2c} receptor genes were isolated from the pBCα2-C10 and the pBCα2-C4 plasmids [12], respectively, with *Nco*I and *Sal*I digests. The *Nco*I site was filled-in using the Klenow fragment of DNA polymerase I to create a blunt end. These cassettes containing the α_2 genes were ligated between the *Eco*RV and *Xho*I sites of pcDNA3 (Invitrogen). The α_{2b} gene was isolated from the pBCα2-C2 plasmid [13] in two pieces; the 5′ fragment was isolated with *Nco*I and *Apa*I digests. The *Nco*I was again filled-in with Klenow. The 3′ fragment of the α_{2b} gene was isolated with *Apa*I and *Hin*dIII digests. The *Hin*dIII site was partially filled with only dATP and dGTP, leaving a 2-base overhang. The pcDNA3 was then cleaved with *Xba*I and *Eco*RV. The *Xba*I site was partially filled with Klenow using only dTTP and dCTP so as to make the end compatible with the partially filled-in *Hin*dIII site of the α_{2b} fragment. The two pieces of the α_{2b} gene were then ligated into this site in pcDNA3. The mouse $\alpha_{2a/d}$ AR gene was isolated from pBC-Ma2-10H plasmid as described by Link *et al.* [14] with *Hin*dIII and *Bam*HI digests and ligated into this corresponding site in pcDNA3.

Stable Transformation of Cells and Cell Culture

Human embryonic kidney (HEK) 293 cells (from ATCC) were grown to 50–60% confluence on 100-mm dishes and were washed twice with PBS; then the medium was changed to prewarmed OPTI-MEM (GIBCO). DNA transfection solution [5 μg of plasmid containing α_2 AR gene in pcDNA3 (Invitrogen)/100 μg of lipofectamine (GIBCO) in 200 mL of water] was freshly mixed, allowed to sit at room temperature for 15 min, and then added to the cells. The cells were incubated at 37° in 5% CO_2 for 5–6 hr. The transfection reaction was quenched by the addition of DMEM with 10% fetal bovine serum (Life Technologies). On the following day, the transfected cells were split 1:5, 1:10, 1:20, and 1:40 and grown for 1 additional day. Selection of the transfected cells was by the addition of 500 μg/mL G-418 to the media. After 7–10 days, distinct colonies of stably transfected cells were formed. Each colony was trypsinized individually using hollow plastic cylinders and grown in successively larger flasks. Once a sufficient number of cells were available, receptor expression levels were determined by radioligand binding. Cells were maintained in 90% DMEM (4.5 g/L of glucose, without sodium pyruvate) with 10% fetal bovine serum and 250 μg/mL of G-418. Cells were grown in a 5% CO_2 environment at 37°. Cells were harvested using 2 mM of EDTA in PBS and centrifuged at 500 *g* for 5 min. Cell pellets were stored at −70°.

Intracellular cAMP Assay

Cells were grown in DMEM (high glucose) with 10% fetal bovine serum and 250 μg/mL of G-418 in a 5%:95% CO_2:O_2 incubator. The cells were harvested with 2 mM of EDTA in PBS (9.0 g/L of NaCl, 0.21 g/L of KH_2PO_4, 0.73 g/L of $Na_2HPO_4 \cdot 7H_2O$) and centrifuged at 500 *g* for 5 min. Cells were washed once with DMEM and brought-up at 2.5×10^6 cells/mL in DMEM containing 25 mM of HEPES, pH 7.4, and 2 mM of isobutylmethylxanthine (to inhibit phosphodiesterase activity). From this cell suspension, 5×10^5 cells/tube were incubated in a final volume of 300 μL at 30° for 15 min with 20 μM of forskolin in the presence or absence of agonist. In some experiments, 1 μM of propranolol was included in the reaction mixture to test for β-adrenergic receptor activity. The reaction was terminated with 50 μL of ice-cold perchloric acid (20%). Samples were allowed to sit on ice for 20 min, and then samples were neutralized with KOH. A precipitate was allowed to form at room temperature after 10–15 min. An aliquot of the supernatant from each tube was pipetted into a Packard OptiPlate for cAMP measurement by the Scintillation Proximity Assay (SPA, Amersham). After overnight incubation, plates were counted in a Packard Topcount scintillation counter.

GTPγ[^{35}S] Binding Assay

MEMBRANE PREPARATION. Cell pellets were suspended in cold homogenization buffer [5 mM of Tris–HCl, 5 mM of EDTA, 5 mM of EGTA, 0.1 mM of phenylmethysulfonyl fluoride (PMSF), pH 7.5 at 4°] and lysed using a Polytron P10 disrupter (setting 6, 5-sec burst). The membranes were centrifuged at 34,000 *g* for 15 min at 4° and the supernatant was removed. This wash procedure was repeated, and aliquots of membranes were resuspended in 50 mM of Tris, 1 mM of EDTA, 5 mM of $MgCl_2$, 0.1 mM of PMSF, pH 7.4, at 4° and frozen in aliquots at −70°.

[^{35}S]GTPγS BINDING. The methods of Tian *et al.* [15] were modified as follows: Membranes were thawed and diluted with buffer (50 mM of Tris, pH 7.4 at 25°, 5 mM of $MgCl_2$, 100 mM of NaCl, 1 mM of EDTA, 1 mM of dithiothreitol, 1 μM of propranolol, 2 μM of GDP). Membrane protein (4–10 μg) was incubated with 0.3 nM [^{35}S]GTPγS and agonist for 60 min at 25°. Reactions were terminated by vacuum filtration over GF/B filters pretreated with 0.5% BSA. Filters were washed with ice-cold wash buffer (50 mM of Tris, 5 mM of $MgCl_2$, 100 mM of NaCl, pH 7.5, at 4°), and incorporated radioactivity was determined using liquid scintillation counting.

Radioligand Binding Assay

MEMBRANE PREPARATION. HEK 293 cells or Chinese hamster lung 1610 cells expressing the α_{2a} AR were maintained in DMEM (Life Technologies) with 5% fetal bovine serum (Life Technologies) and 250 μg/mL of G418 (Life Technologies) in 5% CO_2 at 37°. Cells were harvested using 2 mM of EDTA in PBS and centrifuged at 500 *g*. The cells were broken with a Polytron P10 disrupter (setting 5, 5-sec bursts) and centrifuged in a Sorvall/DuPont RC5C centrifuge with an SS34 at 19,500 rpm (30,000 *g*). The cell pellet was resuspended in homogenization buffer (10x, v/v), using the cell disrupter, and centriftiged at 30,000 *g* for 15 min. This procedure was repeated twice, and the membranes were separated into 1-mL aliquots and stored at −70°.

[^{3}H]MK-912 BINDING ASSAY. Membranes (see above) were thawed at room temperature and diluted in assay buffer [50 mM of Tris, 1 mM of EDTA, 150 mM of NaCl, 2 mM of $MgCl_2$, and 100 μM of Gpp(NH)p, pH 7.4, at 25°] to a final concentration of 5–10 μg of protein/assay tube. For binding isotherms, membranes were incubated with 0.1 to 5 nM of [^{3}H]MK-912 (DuPont NEN) in the presence or absence of 1 μM of RX-821002 for 60 min at room temperature. Binding was terminated by vacuum filtration over glass fiber filtermats (GF/B) using a Packard Top Count 96-well cell harvester. The tubes were rinsed three times with 1 mL of ice-cold 50 mM of Tris, pH 7.4, and bound radioactivity was determined by liquid scintillation counting. For radioligand displacement assays, various concentrations of test ligand were incubated with 0.3 to 0.5 nM of [^{3}H]MK-912 and binding was performed as above.

DATA REDUCTION. For each drug tested in radioligand binding studies, the concentration producing 50% inhibition of binding (IC_{50}) and the Hill slope was determined using iterative curve-fitting techniques. The equilibrium dissociation constant (K_i) of each compound was determined according to the method of Cheng and Prusoff [16]. Typically, the negative logarithm of the K_i (pK_i) is presented. In some experiments, membranes were co-incubated with partial agonists and 100 μM of EPI to inhibit EPI-stimulated [^{35}S]GTPγS binding. The affinity (pK_B) of these partial agonists was determined as described by Craig [17]. All data represent the averages of 3–4 separate experiments and are shown as means ± SEM or ± SD (when possible).

RESULTS

Adenylyl Cyclase Studies

Intracellular cAMP accumulation was measured in HEK 293 cells transfected with the human α_{2a} AR, α_{2b} AR, and α_{2c} AR. The cells were stimulated for 15 min with 20 μM forskolin in the presence of increasing concentrations of α_2 AR agonist. These agonists produced biphasic effects on cAMP accumulation through each of the α_2 AR subtypes (Fig. 1 and data not shown). For example, NE reduced forskolin-stimulated cAMP levels at concentrations between 100 pM and 100 nM, while increases in cAMP were observed between 100 nM and 10 μM. Inclusion of propranolol to block potential β-adrenergic receptors did not block the stimulatory effect of agonists (not shown).

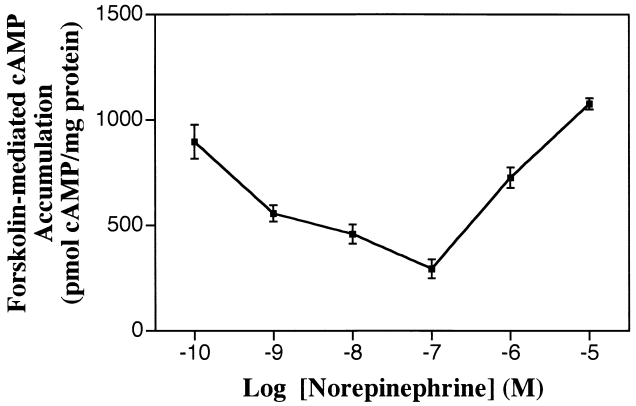

FIG. 1. Human α_{2a} AR-mediated inhibition of 20 μM forskolin-stimulated cAMP accumulation in HEK 293 cells. Whole cells were incubated for 15 min with forskolin in the presence of increasing concentrations of NE. Cells were lysed and tubes were normalized to protein content as described in Materials and Methods. Shown is the representative of four experiments ± SEM for quadruplicate determinations in a single experiment.

Radioligand Binding Studies

Radioligand binding isotherms of [^{3}H]MK-912 to membrane preparations of HEK 293 cells expressing the mouse $\alpha_{2a/d}$ AR yielded a B_{max} of 6.20 ± 1.5 pmol/mg protein and a K_d of 0.60 ± 0.05 nM (N = 3). For the human receptors expressed in HEK 293 cells, values from the [^{3}H]MK-912 binding isotherms were as follows: α_{2a} AR, B_{max} = 1.56 ± 0.4 pmol/mg, K_d = 0.25 ± 0.4 nM; α_{2b} AR, B_{max} = 2.06 ± 0.2 pmol/mg, K_d = 0.33 ± 0.30 nM; and α_{2c} AR, B_{max} = 1.60 ± 0.50 pmol/mg, K_d = 0.057 ± 0.057 nM (N = 3 for each subtype). Consequently, it appeared that the receptor density was similar in all three cell lines.

[^{35}S]GTPγS Binding Studies at the Mouse $\alpha_{2a/d}$ AR

Agonist-stimulated incorporation of [^{35}S]GTPγS in mouse $\alpha_{2a/d}$ AR HEK 293 cell membranes increased linearly with time for up to 2 hr (data not shown). After a 1-hr incubation at room temperature, EPI and NE produced a 2- to 3-fold increase in bound [^{35}S]GTPγS (data not shown). Partial agonists stimulated submaximal levels of [^{35}S]GTPγS binding compared with NE (Fig. 2; Table 1), and when coincubated with full agonists, could also decrease agonist-stimulated [^{35}S]GTPγS binding. The pK_B values for several α_2 AR antagonists and partial agonists were determined in assays competing the ligands against 100 μM of EPI (Fig. 3 and Table 2). The competitive

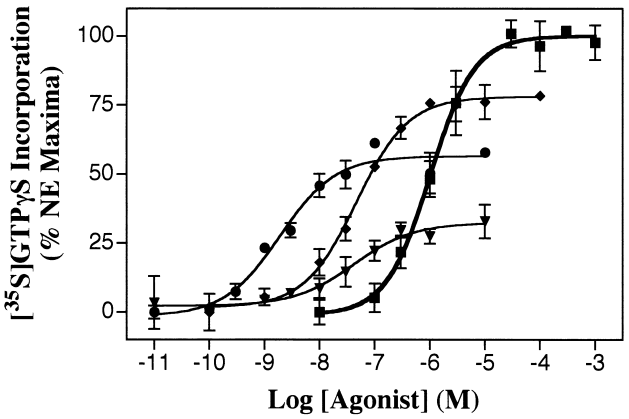

FIG. 2. Alpha-2 receptor-mediated stimulation of [^{35}S]GTPγS binding to G-proteins in HEK 293 cell membranes expressing the mouse $\alpha_{2a/d}$ AR. Membranes were incubated for 60 min at room temperature with increasing concentrations of the agonists NE (■), UK-14304 (◆), dexmedetomidine (●), and clonidine (▼). [^{35}S]GTPγS binding was measured as described in Materials and Methods, and results are the means ± SEM of three experiments.

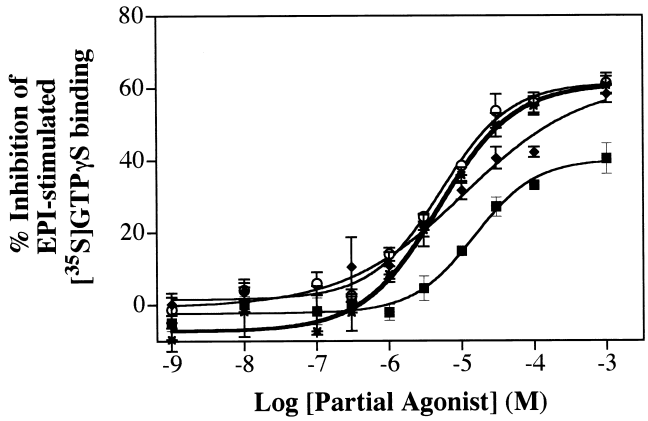

FIG. 3. Partial agonist-mediated inhibition of EPI-stimulated [^{35}S]GTPγS binding to membranes expressing the mouse $\alpha_{2a/d}$ AR. Membranes were incubated for 60 min at room temperature with 100 μM of EPI and increasing concentrations of the partial agonists clonidine (■), xylometazoline (*), oxymetazoline (◆), and naphazoline (○). [^{35}S]GTPγS binding was measured as described in Materials and Methods. The amount of [^{35}S]GTPγS bound is related to the maximal amount induced by 100 μM of EPI in the absence of partial agonists. Shown are the means ± SEM of three experiments.

nature of ligand interaction was assessed by Schild regression analysis [18]. EPI concentration–response curves were constructed as a function of increasing concentration of the antagonist rauwolscine, yielding rightward parallel shifts without a decrease in maximal response (Fig. 4). The Schild slope for rauwolscine was 0.98 and the pA_2 value was 7.53. This value is similar to the radioligand binding dissociation constant (pK_i = 7.27) reported previously for this receptor [14].

Pertussis toxin pretreatment of cells abolished agonist-mediated increases in [^{35}S]GTPγS binding (Fig. 5), demonstrating a predominant coupling of the mouse $\alpha_{2a/d}$ AR to G_i and/or G_o.

TABLE 1. Agonist-mediated increases in [^{35}S]GTPγS binding to HEK 293 cell membranes expressing the mouse $\alpha_{2a/d}$ AR

Ligand	pEC_{50}	Intrinsic activity (relative to NE)
EPI	6.41 ± 0.04	1.10 ± 0.06
NE	5.85 ± 0.13	1.00 ± 0.06
Isoproterenol (N = 2)	4.43 ± 0.19	1.03 ± 0.17
A-54741	8.01 ± 0.14	0.95 ± 0.07
UK-14304	7.36 ± 0.07	0.90 ± 0.08
Dexmedetomidine	8.47 ± 0.17	0.82 ± 0.17
ST-91	6.27 ± 0.12	0.71 ± 0.09
Guanfacine	7.69 ± 0.31	0.71 ± 0.16
Xylazine	5.62 ± 0.18	0.65 ± 0.09
Rilmenidine	6.14 ± 0.15	0.62 ± 0.09
Clonidine	7.39 ± 0.10	0.58 ± 0.04
Oxymetazoline	7.81 ± 0.18	0.57 ± 0.05
Tramazoline	7.81 ± 0.33	0.54 ± 0.07
Naphthalene	8.11 ± 0.18	0.48 ± 0.04
Naphazoline	7.94 ± 0.14	0.46 ± 0.03
Xylometazoline	7.59 ± 0.09	0.42 ± 0.04

Membranes were incubated for 60 min at room temperature with increasing concentrations of the agonists, and [^{35}S]GTPγS binding was measured as described in Materials and Methods. The intrinsic activity of each compound is related to NE (assigned a value of 1.0). Values are means ± SEM of three experiments, except where noted.

Pharmacology and Ligand Selectivity at the Human α_2 ARs

α_2 AR agonists stimulated binding of [^{35}S]GTPγS to HEK 293 cell membranes containing the human α_{2a}, α_{2b}, or α_{2c} ARs (Fig. 6). EPI and NE were full agonists; clonidine and oxymetazoline were partial agonists, while antagonists (e.g. rauwolscine, 5 μM) did not stimulate [^{35}S]GTPγS incorporation (Table 3). Although a few agonists showed a great deal of selectivity for a single receptor subtype, the rank order of agonist potency for selected compounds at the human receptors (Table 3) was:

α_{2a}: Dexmedetomidine > guanabenz > UK-14304 > clonidine > ST-91 > NE

TABLE 2. Antagonism of EPI-stimulated [^{35}S]GTPγS binding to the mouse $\alpha_{2a/d}$ AR receptor

Antagonist	pK_B
RX821002	9.21 ± 0.22
Napthalene imidazole	8.03 ± 0.32
Naphazoline	7.77 ± 0.18
Xylometazoline	7.62 ± 0.11
Oxymetazoline	7.59 ± 0.21
Rauwolscine	7.44 ± 0.15
Yohimbine	7.21 ± 0.12
Clonidine	7.21 ± 0.13

Membranes were co-incubated with 100 μM of EPI and increasing concentrations of partial agonists or antagonists. The pK_B values for each ligand were calculated from the IC_{50} for inhibition of EPI-stimulated [^{35}S]GTPγS binding. Values are means ± SD of three experiments.

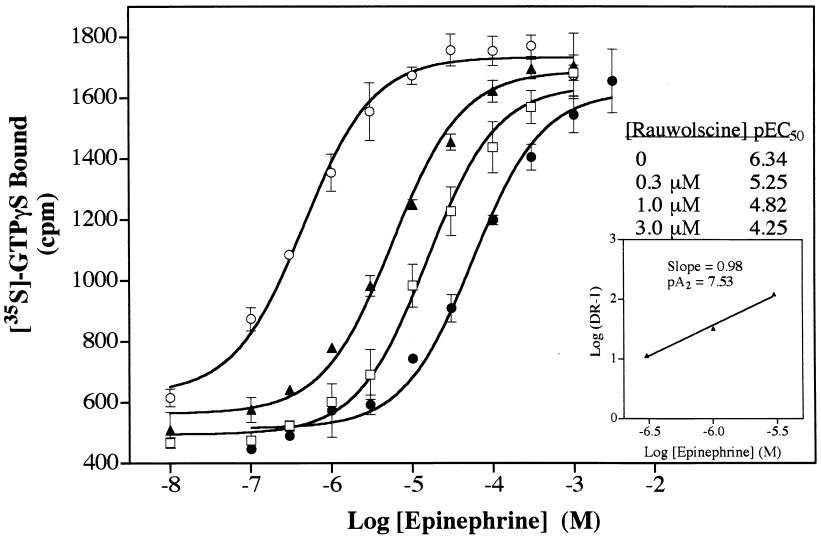

FIG. 4. EPI concentration–response curves for stimulated [^{35}S]GTPγS binding to membranes expressing the mouse $\alpha_{2a/d}$ AR in the presence of various concentrations of rauwolscine and a Schild plot (inset) for the antagonist. Membranes were incubated for 60 min at room temperature with increasing concentrations of the agonist EPI; the amount of rauwolscine was varied between each concentration–response curve. [^{35}S]GTPγS binding was measured as described in Materials and Methods. *Inset*: Data are plotted according to the method of Schild [18], yielding a slope of 0.98 and a pA_2 of 7.53. Data (means ± SD) shown are representative of three experiments.

α_{2b}: Dexmedetomidine > clonidine > guanabenz > NE > ST-91 > UK-14304

α_{2c}: Dexmedetomidine > NE > UK-14304 > ST-91 ≥ clonidine ≫ guanabenz

The rank order of intrinsic activity for the above compounds was:

α_{2a}: UK-14304 ≥ NE > dexmedetomidine > ST-91 > guanabenz ≥ clonidine

α_{2b}: Dexmedetomidine > NE > guanabenz > UK-14304 > ST-91 > clonidine

α_{2c}: Dexmedetomidine ≥ NE > UK-14304 > ST-91 > clonidine ≫ guanabenz

Specific binding of the α_2 AR antagonist [^{3}H]MK-912 was tested in membranes from both HEK 293 cells (Table 3) and Chinese hamster lung 1610 cells expressing each of the human α_2 ARs (data not shown). The pK_i values for a large number of ligands at receptors expressed in either cell line were almost identical; comparison of pK_i values from 293 cells and 1610 cells yielded r^2 values of 0.98 or higher for each receptor subtype. Thus, the affinity of the receptors for ligands was not cell line-specific. None of the antagonists tested showed greater than 15-fold selectivity for a single α_2 AR subtype. The pEC_{50} values for stimulated [^{35}S]GTPγS binding of various agonists were compared to the pK_i values determined from radioligand binding in HEK 293 cells (Table 3). The pEC_{50} values and pK_i values were linearly related between the two assays (Fig. 7).

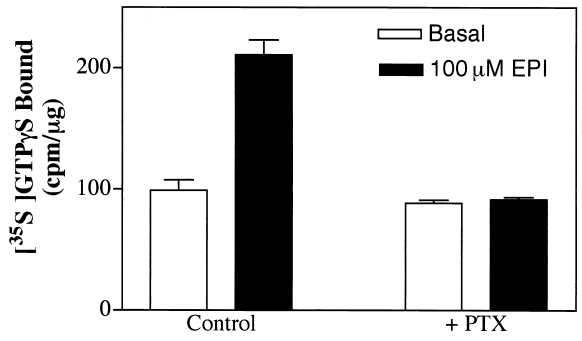

FIG. 5. Pertussis-toxin blockade of EPI-stimulated [^{35}S]GTPγS binding to membranes of HEK 293 cells expressing the mouse $\alpha_{2a/d}$ AR. Whole cells were incubated with pertussis toxin (or vehicle) for 24 hr, membranes were prepared, and either basal or 100 μM EPI-stimulated [^{35}S]GTPγS binding to membranes was measured as described in Materials and Methods. Values are the means ± SEM of three experiments.

DISCUSSION

The pleiotropic coupling of α_2 ARs to various G-proteins complicates the use of adenylyl cyclase activity as a measure of agonist efficacy and potency in recombinant systems. Agonist-mediated [^{35}S]GTPγS binding to G-proteins, therefore, appears to be a more general and robust method for analyzing activation of α_2 ARs.

Adenylyl Cyclase Studies

We expressed murine and human α_2 ARs in HEK 293 cells in order to study the potency and efficacy of a number of α_2 AR agonists. Attempts to measure α_2 AR-mediated inhibition of adenylyl cyclase activity were complicated by biphasic concentration–response curves (see Fig. 1). At lower agonist concentrations, α_2 AR agonists inhibited forskolin-stimulated cAMP accumulation in cells, while at higher concentrations these same agonists potentiated forskolin-stimulated cAMP accumulation. The biphasic agonist concentration–response curves for intracellular cAMP accumulation agreed with those reported previously for cloned α_2 AR expressed in various cell lines [2, 5, 8, 19]. The dual regulation of adenylyl cyclase by α_2 ARs expressed in HEK 293 cells was also observed in Chinese

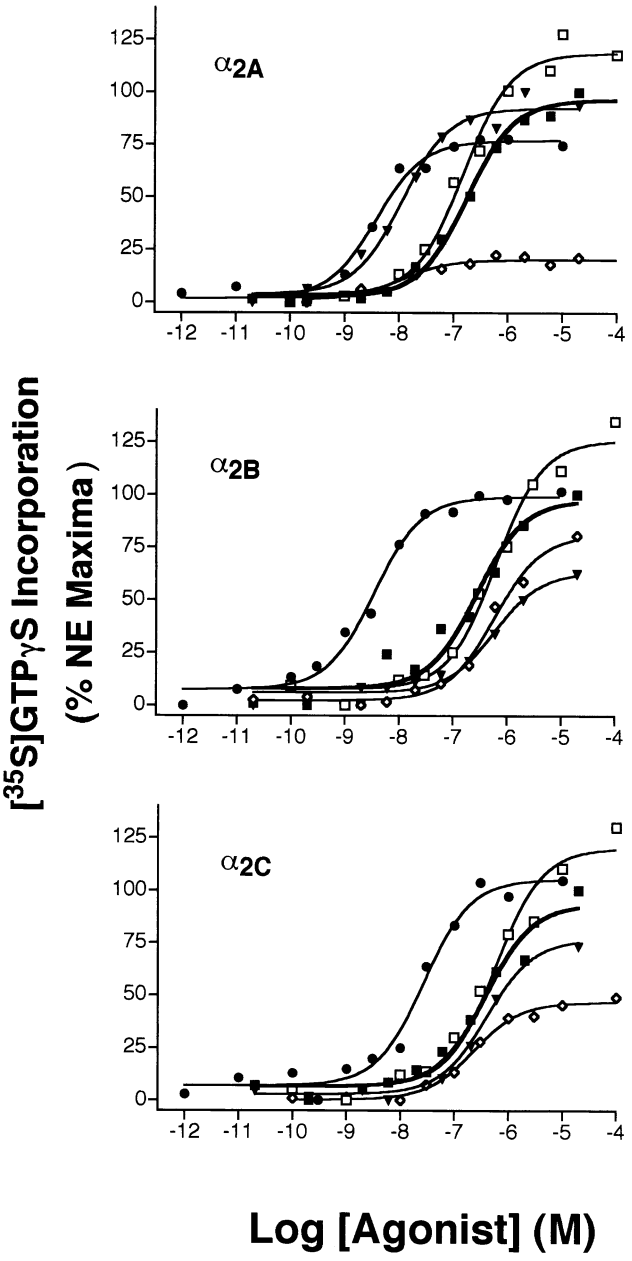

FIG. 6. Alpha-2 receptor-mediated stimulation of [^{35}S]GTPγS binding to G-proteins in HEK 293 cell membranes expressing the human α_{2a} AR, α_{2b} AR, or α_{2c} AR. Membranes were incubated for 60 min at room temperature with increasing concentrations of the agonists NE (■), EPI (□), UK-14304 (◇), dexmedetomidine (●), and oxymetazoline (▼). Shown is the representative of at least three experiments per curve.

hamster lung 1610 cells expressing the human α_2 ARs (data not shown). For each α_2 AR subtype, the stimtilatory effect of agonists was more pronounced in the higher expressing clones; we could not find clones with low enough receptor expression levels to avoid adenylyl cyclase stimulation while still eliciting a measurable agonist-mediated decrease in forskolin-stimulated cAMP levels. Therefore, these biphasic responses precluded accurate determination of agonist potency and efficacy.

[^{35}S]GTPγS Studies

Agonists and partial agonists are characterized by their ability to activate a receptor and induce a response. The intrinsic activity of an agonist is influenced by (a) receptor expression levels, (b) G-protein coupling, and (c) the second messenger assayed in any given biological system. Since release of prebound GDP appears to be the rate-limiting step for GTP binding and subsequent G-protein activation [20], agonist-stimulated [^{35}S]GDPβS release or [^{35}S]GTPγS binding are measures of ligand intrinsic activity, independent of second messenger generation. Lorenzen and coworkers [11] have found that partial agonists induce [^{35}S]GDPβS release more slowly than do full agonists. Thus, the rate of GDP/GTP exchange may determine the extent of signal amplification by ligands of varying intrinsic activity. Alpha-2 AR-mediated G-protein activation measured by [^{35}S]GTPγS binding likely reflects a composite response of α_2 AR interactions with a number of G-proteins rather than merely activation of G_i and adenylyl cyclase inhibition. Since α_2 AR activation appears to be linked to several second messenger systems, including inhibition of adenylyl cyclase [21], inhibition of voltage-dependent Ca^{2+} currents [22], and increases in inwardly rectifying potassium currents [23, 24], agonist-mediated [^{35}S]GTPγS binding may be a better predictor of the activity of α_2 AR agonists.

Agonist-induced [^{35}S]GTPγS binding was used to characterize ligand interactions at the mouse $\alpha_{2a/d}$ and human α_{2a}, α_{2b}, and α_{2c} ARs. Similar to previous findings by Lorenzen *et al.* [11] and Traynor and Nahorski [25], we found that agonist-mediated incorporation of [^{35}S]GTPγS into membranes continues to increase for at least 2 hr (data not shown). Thus, the amount of ligand-stimulated [^{35}S]GTPγS bound at any given time is directly related to the intrinsic activity of a ligand for that system; the relative intrinsic activity of partial agonists and full agonists remains essentially constant over a 2-hr time period [11]. Agonists concentration-dependently increased membrane incorporation of [^{35}S]GTPγS via the mouse $\alpha_{2a/d}$ AR (see Fig. 2). The catecholamine agonists EPI and NE, as well as the imidazoline agonist UK-14304, were full agonists at the mouse $\alpha_{2a/d}$ AR. Partial agonists such as clonidine and oxymetazoline stimulated only a fraction of the NE-induced [^{35}S]GTPγS binding. Antagonists such as rauwolscine did not stimulate [^{35}S]GTPγS binding above basal levels. To further confirm the partial agonist nature of clonidine and oxymetazoline, they were co-incubated with the full agonist EP[I] to confirm their ability to decrease agonist-stimulated [^{35}S]GTPγS binding (see Fig. 3). In fact, their affinities as "antagonists" were similar to their potencies for stimulation of [^{35}S]GTPγS binding (see Tables 1 and 2).

Ligand Characterization

Eason and colleagues [2] previously studied α_2 AR activation by measuring agonist-mediated changes in forskolin-

TABLE 3. Agonist potency (pEC_{50}), intrinsic activity (I.A.), and radioligand binding affinity (pK_i) for α_2 AR ligands at the human α_2-receptors expressed in HEK 293 cells

	Alpha-2a			Alpha-2b			Alpha-2c		
	pEC_{50}	I.A.	pK_i	pEC_{50}	I.A.	pK_i	pEC_{50}	I.A.	pK_i
Agonists									
A-54741	8.34	1.03	6.61	7.46	1.05	6.04	6.88	1.20	6.11
α-Me-NE	5.42	1.28	5.44	6.16	1.40	5.58	5.95	1.20	5.54
Clonidine	7.57	0.32	7.21	7.25	0.18	7.16	6.04	0.23	6.87
Dexmedetomidine	8.50	0.73	7.88	8.48	1.10	7.47	7.54	1.03	7.02
EPI	6.78	1.18	5.83	6.24	1.28	5.20	6.22	1.39	5.77
Guanabenz	8.25	0.38	7.66	7.01	0.71	6.55	<5		6.35
Guanfacine	7.28	0.41	7.03	6.54	0.73	5.86	6.22	0.48	5.41
Levlofexidine	<5		7.84	7.37	0.47	6.90	<5		6.76
Naphazoline	<5		7.68	6.68	0.43	6.39	<5		6.39
Naphthalene	<5		8.34	7.46	0.54	7.77	<5		8.48
NE	6.70	1.00	5.70	6.56	1.00	5.56	6.35	1.00	5.87
Oxymetazoline	7.97	0.25	8.14	6.25	0.75	5.75	6.70	0.22	6.62
p-Aminoclonidine	8.1	0.39	8.10	<5		7.53	<5		7.67
ST-91	6.81	0.63	6.24	6.43	0.44	6.10	6.07	0.32	6.21
Tramazoline	7.84	0.25	7.50	6.83	0.40	6.86	6.87	0.30	6.56
UK-14304	8.06	1.02	6.67	6.24	0.59	6.02	6.24	0.75	5.71
Xylazine	5.73	0.52	5.24	5.72	0.45	5.46	5.90	0.28	4.81
Xylometazoline	<5		7.82	6.81	0.46	5.98	<5		6.89
Antagonists									
Alinidine	<5		5.22	<5		5.19	<5		4.47
B-HT920	<4		6.40	<4		6.29	<4		6.02
Idazoxan	<4		8.01	<4		7.43	<4		7.70
ICI106270	<5		8.10	<5		7.17	<5		6.55
Imiloxan	<4		6.50	<4		7.17	<4		6.82
MK-912	ND		9.07	ND		9.05	ND		10.17
Phentolamine	<4		7.69	<4		7.48	<4		6.59
Prazosin	<4		6.10	<4		7.05	<4		7.51
Rauwoscine	<5		8.92	<5		8.92	<5		9.26
Rilmenidine	<4		5.80	<4		5.76	<4		5.33
RX-821002	ND		9.44	ND		8.79	ND		9.10
Tolazoline	<5		6.64	<5		5.50	<5		5.43

The intrinsic activity values are in relation to NE, which is defined as 1.0. For compounds with an intrinsic activity value of less than 0.20, the values are reported as less than the highest concentration tested, i.e. pEC_{50} < highest concentration of ligand tested. Shown is the average of at least three experiments per datum. ND, not determined.

stimulated adenylyl cyclase activity in membranes of CHO cells expressing the human α_2 ARs. In those studies, CHO cells were incubated with either pertussis toxin to ablate α_2 AR/G_i coupling, or cholera toxin to mask α_2 AR/G_s coupling. Six agonists were studied, all of which showed similar degrees of adenylyl cyclase inhibition but varying levels of cyclase stimulation [2]. The pEC_{50} values of NE and EPI for inhibition of forskolin-mediated adenylyl cyclase activity via the α_{2a} AR were 6.44 and 6.91, respectively, while the pEC_{50} values of NE and EPI for stimulation of forskolin-mediated adenylyl cyclase activity were 4.39 and 4.65, respectively. Although we have reproduced some of their work in our own laboratory (data not shown), these studies are difficult to perform on a large scale, and ranking the degree of receptor activation for each agonist is formidable.

In the [^{35}S]GTPγS studies presented here with the human α_2 ARs, the magnitude of stimulation of [^{35}S]GTPγS binding depended upon the agonist tested. Intrinsic activity was calculated with respect to NE by comparing the maximum amount of [^{35}S]GTPγS incorporation (see Fig. 6). Thus, as with the mouse $\alpha_{2a/d}$ AR, EPI and NE were full agonists; clonidine and oxymetazoline were partial agonists, while antagonists (e.g. rauwolscine, 5 μM) did not stimulate [^{35}S]GTPγS incorporation. The agonist potency of each of these ligands for [^{35}S]GTPγS incorporation was related to their ability to displace [^{3}H]MK-912 from the receptors (see Table 1). Comparison of agonist potencies to radioligand binding dissociation constants (see Fig. 7) indicates that there was little receptor reserve for α_2 receptor-mediated [^{35}S]GTPγS binding. Note that the pEC_{50} values we calculated for NE and EPI stimulation of [^{35}S]GTPγS incorporation via the α_{2a} AR, 6.70 and 6.78, respectively, are similar to the values described above (6.44 and 6.91) for agonist-mediated inhibition of adenylyl cyclase activity reported by Eason and colleagues for the human α_{2a} AR [2]. From our studies it is apparent that there are few available agonists or antagonists that exhibit significant subtype selectivity. The most selective α_{2A} AR agonists are A-54741, guanabenz, oxymetazoline, and UK-14304. The most selective α_{2B} AR agonists are levlofexidine, naphazoline, and naphthalene. The most useful tool to study α_{2c} AR is presumably the antagonist MK-912.

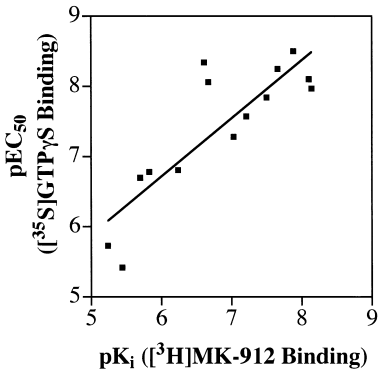

FIG. 7. Comparison of radioligand ([^{3}H]MK-912) binding (pK_i) and ligand-stimulated [^{35}S]GTPγS binding (pEC_{50}) to HEK 293 cell membranes expressing the human α_{2a} AR. The pK_i and pEC_{50} values were taken from Table 3 (average of 3–4 experiments per data point). Comparison plots for the human α_{2b} AR or α_{2c} AR expressed in HEK 293 cells were very similar to that of the human α_{2a} AR (not shown).

Receptor/G-Protein Coupling

The tissue distribution of α_2 ARs, and possibly even cellular distribution of receptors [26], may affect the ultimate response to an α_2 AR agonist due to differential G-protein coupling. For example, the biphasic cAMP concentration responses of α_2 ARs may be partially the result of coupling to multiple G-proteins. Thus, α_2 ARs couple to both G_i and G_s [3, 5, 8], G_i and G_o [8, 9], G_q/G_{11} [5, 9], and G_z [10]. Although most known α_2 AR-mediated physiological effects (including inhibition of adenylyl cyclase) are sensitive to pertussis toxin [8], there are instances of α_2 AR/G_s-coupling in native tissues such as rat islets [7] and rat brain slices [6]. The robust agonist-mediated [^{35}S]GTPγS binding assay allows for measurement of receptor-coupling to several G-proteins, without regard to the effector. Thus, G-protein-coupling depends primarily on the cell type, and to a certain degree, receptor expression levels. Pertussis toxin pretreatment of cells blocked EPI-stimulated [^{35}S]GTPγS binding via the α_2 AR (see Fig. 5). This supports previous reports that α_{2a} ARs preferentially associate with the G_i and G_o family of G-proteins compared with the G_s or G_q family [8] and also confirms other reports that pertussis toxin ablates α_2 AR-mediated [^{35}S]GTPγS binding [15].

These data highlight the paradox that α_2 AR activation stimulates adenylyl cyclase (at high agonist concentration) while apparently acting through pertussis-sensitive (G_i/G_o) proteins. Either an undetectable level of G_s activation (as measured by [^{35}S]GTPγS binding) is adequate to stimulate adenylyl cyclase, or the free βγ subunits from G_i/G_o are activating cyclase isoenzymes in 293 cells and CHO cells. Since direct activation of G_s has been observed previously for α_2 AR expressed in both CHO and 293 cells [3, 8], and since forskolin-stimulated cAMP accumulation is synergistically enhanced by low levels of G_s stimulation [27], direct G_s activation via α_2 ARs is a possibility. Given the fact that G-protein βγ subunits activate type II and type IV adenylyl cyclase in the presence of a small amount of activated $G_s\alpha$ [28], the latter explanation is also tenable. In fact, both mechanisms could act together to enhance adenylyl cyclase activity, even though the bulk of α_2 AR-mediated [^{35}S]GTPγS binding measured in our studies appears to be via G_i/G_o proteins. Furthermore, we have attempted to measure agonist-stimulated [^{35}S]GTPγS binding via G_s-coupled receptors (e.g. 5-HT_7 receptors) and found that these signals can be quite weak (unpublished observation). It is possible that in the current experiments the $G_s\alpha$ subunit dissociates from the membrane when activated by agonist-liganded receptors [29] and, thereby, is not detectable in our [^{35}S]GTPγS membrane-binding assay.

In HEK 293 cells, we observed a one-to-one relationship between agonist potency toward [^{35}S]GTPγS binding and the radioligand binding dissociation constants. The radioligand binding affinity reported in our studies ostensibly reflects binding to the low affinity agonist binding site [11] since Gpp(NH)p was included in the [^{3}H]MK-912 binding assays. Similarly, the [^{35}S]GTPγS functional assay was performed in the presence of high concentrations of guanine nucleotide (GDP), which would presumably also decrease receptor affinity for agonists [30]. Thus, it is not surprising that the functional [^{35}S]GTPγS assay and radioligand binding values were similar. The fact that we see a similar correlation over a range of ligand intrinsic activities indicates the presence of a low receptor reserve, even for the most efficacious agonists. In corroboration of these results, we found that the expression level of mouse $\alpha_{2a/d}$ AR (6.2 pmol/mg) was similar to the total number of [^{35}S]GTPγS binding sites observed in [^{35}S]GTPγS binding isotherm studies (4.2 pmol/mg; unpublished observation). This correlation of receptor to activated G-protein is the likely reason for the similarity between potency for agonist stimulation and affinity for radioligand binding.

In summary, agonist-mediated [^{35}S]GTPγS binding in HEK 293 cells is a sensitive and relatively simple method to study α_2 AR activation of G-proteins. α_2 AR activation is measured at a point preceding second messenger generation and allows one to distinguish compounds of differing intrinsic activity and potency.

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