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Cloning, Expression, and Pharmacological Characterization of a Human α_{2B}-Adrenergic Receptor

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SUMMARY

An α_2-adrenergic receptor subtype has been isolated from a human genomic spleen library using the human 5-hydroxytryptamine$_{1A}$ receptor gene (also known as G-21) as a probe. This adrenergic receptor gene encodes a protein of 450 amino acids and does not contain any consensus sequences for *N*-linked glycosylation in its amino terminus or extracellular loops. This receptor is also distinguished by the presence of 12 consecutive glutamic acid residues in the region of its third intracellular loop. The deduced amino acid sequence shows greatest homology to previously cloned human α_2-adrenergic receptors and has structural similarities to other guanine nucleotide-binding protein-coupled receptors. The DNA encoding the human α_2 receptor was stably transfected into mouse fibroblast Ltk$^-$ cells and radioligand binding studies were performed using the α_2 antagonist [^{3}H]rauwolscine. [^{3}H]Rauwolscine bound with high affinity (K_d = 0.33 nM) and in a saturable manner (B_{max} = 1.4 pmol/mg of protein). Pharmacological characterization of this receptor indicated a rank order of potency of yohimbine > prazosin > oxymetazoline. Additionally, 100 μM 5′-guanylylimidodiphosphate, produced a rightward shift in the epinephrine competition curve, with resultant increases in both the K_i value and Hill coefficient, suggestive of a functional interaction of the cloned receptor with native guanine nucleotide-binding protein(s) of Ltk$^-$ membranes. The data presented here are consistent with previous biochemical and pharmacological studies on α_2 receptors and are supportive of the designation of this receptor as an α_{2B} subtype.

Radioligand binding studies strongly point toward the existence of at least three α_2-adrenergic receptor subtypes, designated α_{2A}, α_{2B}, and α_{2C} (1). These receptors belong to the family of G protein-coupled receptors that are distinguished by their seven transmembrane-spanning-region configuration and their functional coupling to effector mechanisms via distinct G proteins (2). All three subtypes display a high affinity for the α_2 antagonists yohimbine and rauwolscine but differ in their affinities for various drugs, which can differentiate between α_2-adrenergic subtypes (1). Oxymetazoline exhibits high affinity for the α_{2A} subtype, whereas prazosin displays high affinity for the α_{2B} subtype (3). The α_{2C} subtype is pharmacologically similar to α_{2B}, but α_{2C} has a higher affinity (~10-fold) for [^{3}H] rauwolscine than the α_{2B} subtype (4).

DNA sequences coding for two human α_2-adrenergic receptors have been isolated. A receptor from human platelet has been localized to chromosome 10, having clear pharmacological characteristics of an α_{2A} receptor (5), and has been designated α_2-C10 (6). Another receptor, cloned from human kidney, has been localized to chromosome 4 and is called α_2-C4 (6). The presence of a third α_2 subtype residing on human chromosome 2 has been implied from Southern blot analysis (5) and has not been previously cloned.

We have previously reported preliminary results of the molecular cloning and expression of a genomic sequence encoding a third human α_2-adrenergic receptor subtype (7). In this communication, we describe the deduced amino acid sequence of this receptor, its unique structural features, and the pharmacological binding profile of the expressed clone, which are consistent with the assignment of this receptor as a human α_{2B}-adrenergic receptor subtype. Subsequent to our initial report (7), Zeng *et al.* (8) reported the cloning of a third α_2-adrenergic subtype from rat brain, which exhibits an α_{2B} pharmacology. The deduced amino acid sequence of this rat receptor does not contain any consensus sequences for *N*-linked glycosylation, an unusual feature for G protein-coupled receptors.

Experimental Procedures

Cloning and Sequencing. A human spleen genomic library, in the λ vector Charon 28 provided by Dr. Jeffrey V. Ravetch (Sloan-Kettering Institute, New York), was screened using the 1.6-kb *Xba*I-*Bam*HI fragment from the human 5-HT$_{1A}$ receptor gene (also known as G-21) as a probe (9). The probe was labeled with ^{32}P by the method of random priming (10). Hybridization was performed at 40° in a solution containing 50% formamide, 10% dextran sulfate, 5× SSC (1× SSC is 0.15

ABBREVIATIONS: G protein, guanine nucleotide-binding protein; kb, kilobase; SSC, standard saline citrate; 5-HT, 5-hydroxytryptamine; Gpp(NH)p, 5′-guanylylimidodiphosphate.

M sodium chloride, 0.015 M sodium citrate), 1× Denhardt's (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), and 200 μg/ml sonicated salmon sperm DNA. The filters were washed at 50° in 0.1× SSC containing 0.1% sodium dodecyl sulfate and exposed at −70° to Kodak XAR film in the presence of an intensifying screen. λ-Phages hybridizing to the probe were plaque purified and DNA was prepared for Southern blot analysis (11, 12). For subcloning and further Southern blot analysis, DNA was inserted into pUC18 (Pharmacia, Piscataway, NJ). Nucleotide sequence analysis was done by the Sanger dideoxy nucleotide chain termination method (13) on denatured double-stranded plasmid templates, using Sequenase (U.S. Biochemical Corp., Cleveland, OH).

Expression. The entire coding region of clone 5A, including 393 base pairs of 5′ untranslated sequence and 11 base pairs of 3′ untranslated sequence, was cloned into the eukaryotic expression vector pcEXV-3 (14). Stable cell lines were obtained by cotransfection with the plasmid pcEXV-3 (containing the α_{2B} receptor gene) and the plasmid pGCcos3neo (containing the aminoglycoside transferase gene) into Ltk$^-$ cells, using calcium phosphate (reagents obtained from Specialty Media, Lavellette, NJ). The cells were grown, in a controlled environment (37°, 5% CO_2), as monolayers in Dulbecco's modified Eagle medium (GIBCO, Grand Island, NY) containing 25 mM glucose and supplemented with 10% bovine calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate. Stable clones were then selected for resistance to the antibiotic G-418 and harvested membranes were screened for their ability to bind [^{3}H]rauwolscine.

Membrane preparation. Membranes were prepared from transfected Ltk$^-$ cells, which were grown to 100% confluency. The cells were washed twice with phosphate-buffered saline, scraped into 5 ml of ice-cold phosphate-buffered saline, and centrifuged at 200 × *g* for 5 min at 4°. The pellet was resuspended in 2.5 ml of ice-cold Tris buffer (20 mM Tris·HCl, pH 7.4 at 23°, 5 mM EDTA) and homogenized, and the lysate was centrifuged at 200 × *g* for 5 min at 4° to pellet large fragments. The supernatant was then centrifuged at 40,000 × *g* for 20 min at 4°. The membranes were washed once in the homogenization buffer and resuspended in 25 mM glycylglycine buffer, pH 7.6 at 23°. Membrane preparations not assayed immediately were frozen in liquid nitrogen and stored at −80° for up to 1 month, without significant loss of binding properties.

Radioligand binding studies. [^{3}H]Rauwolscine binding was assayed by the method of Bylund and co-workers (3). The incubation solution contained 25 mM glycylglycine buffer, pH 7.6 at 23°, and [^{3}H] rauwolscine (final concentration of 10 pM to 10 nM for saturation studies or 1 nM for competition studies) in a reaction volume of 200 μl. (−)-Norepinephrine (100 μM) was used to define nonspecific binding. Binding was initiated by the addition of 50 μl of membrane homogenate (30 μg of protein) and the mixture was incubated for 2 hr at 23° to achieve equilibrium during saturation binding studies. Competition studies were conducted at a lower protein concentration (15 μg), using a 90-min incubation time and a 1 nM concentration of [^{3}H]rauwolscine. The reaction was terminated by vacuum filtration through presoaked (0.5% polyethyleneimine) GF/B filter strips, using a Brandel 48R cell harvester (Gaithersburg, MD). Filters were washed for 5 sec with iced buffer (50 mM Tris·HCl, pH 7.6 at 4°), dried, and transferred to scintillation vials to which 5 ml of Ready-Organic were added (Beckman Instruments, Fullerton, CA). Radioactivity was measured by liquid scintillation counting, using a Beckman LS 1701 liquid scintillation counter. The efficiency of [^{3}H]rauwolscine counting averaged 50–55% and was determined by addition of a known amount of calibrated [^{3}H] toluene standard. Specific binding was greater than 90% of total binding at 1 nM [^{3}H]rauwolscine. Protein concentrations were determined by the method of Bradford (15), using bovine serum albumin as the standard. Analyses of saturation and competition data were performed by computer-assisted nonlinear regression (16) (ACCUCOMP and ACCUFIT programs; Lundon Software, Chagrin Falls, OH). IC_{50} values were converted to K_i values by the Cheng-Prusoff equation (17). K_d and K_i values are expressed as geometric means ± standard errors; B_{max} values and Hill coefficients (n_H) are expressed as arithmetic means ± standard errors. Statistical significance was determined by Student's *t* test. Linear regression analysis was utilized to determine correlations between parameters. *p* values less than 0.05 were considered statistically significant. All experiments were conducted a minimum of three times.

Several cell lines were obtained as model systems for α_2-adrenergic receptor binding studies. Ltk$^-$, HT-29, and OK cells were obtained from the American Type Culture Collection (Rockville, MD). NG-108-15 cells were generously donated by Dr. Marshall Nirenberg (National Institutes of Health).

Drugs. Drugs were obtained from the following companies: [^{3}H] rauwolscine (80 Ci/mmol), New England Nuclear (Boston, MA); rauwolscine, Accurate Chemicals (Westbury, NY); corynanthine, oxymetazoline, prazosin, and Gpp(NH)p, Sigma (St. Louis, MO); and clonidine, *p*-aminoclonidine, (−)-epinephrine, (−)-norepinephrine, ketanserin, (±)-mianserin, phenoxybenzamine, WB-4101, and yohimbine, Research Biochemicals, Inc. (Natick, MA). All other chemicals were of the highest purity commercially available.

Results

We screened a human genomic spleen library with the 1.6-kb *Xba*I-*Bam*HI restriction fragment derived from the gene for the 5-HT_{1A} receptor (9). A total of 15 clones were isolated and were characterized by restriction endonuclease mapping and DNA sequence analysis. The 15 clones were categorized into three different sets of overlapping clones by restriction analysis. Using sequence analysis, two sets were identified as previously characterized genes, specifically the β_1- (18) and the β_2-adrenergic (19) receptor genes. Clones from a third set were sequenced and a comparison with the human sequences present in Genbank demonstrated that this set of clones was novel.

Amino acid sequence information obtained from one clone, clone 5A, is shown in Fig. 1 as a model with the seven transmembrane-spanning segments predicted for G protein-coupled receptors. The sequence begins with a methionine, whose codon is flanked by nucleotides matching Kozak's (20) consensus sequence for translation initiation. Clone 5A contains an uninterrupted open reading frame, extending from this methionine, that encodes a protein of 450 amino acids in length, having a relative molecular mass of approximately 50,000. A variety of structural features that are invariant in this family, including the aspartic acid residues of transmembrane regions II and III, the DRY sequence at the end of transmembrane region III, and the conserved proline residues of transmembrane regions IV, V, VI, and VII (21), were present in clone 5A. Of all known G protein-coupled receptor clones (EMBL Data Base), the greatest homology was found between clone 5A and the human platelet α_2-C10 (5) and the human kidney α_2-C4 (6) adrenergic receptors.

Fig. 2 shows a comparison between the deduced amino acid sequence of clone 5A and the sequences of clones α_2-C10 (5) and α_2-C4 (6). In both cases, an overall homology of approximately 45% amino acid identity was observed (44% with α_2-C10, 45% with α_2-C4), whereas the homology within transmembrane regions was approximately 75% (74% with α_2-C10, 76% with α_2-C4). The amino terminus was the least homologous among all three receptors, whereas significant identities were present in the carboxyl terminus. The third cytoplasmic loops, although lacking sequence identity, were similar with regard to their sizes, with that of clone 5A being slightly larger (175 amino acids) than those of clones α_2-C4 and α_2-C10 (~150 amino acids each). A high content of charged residues is char-

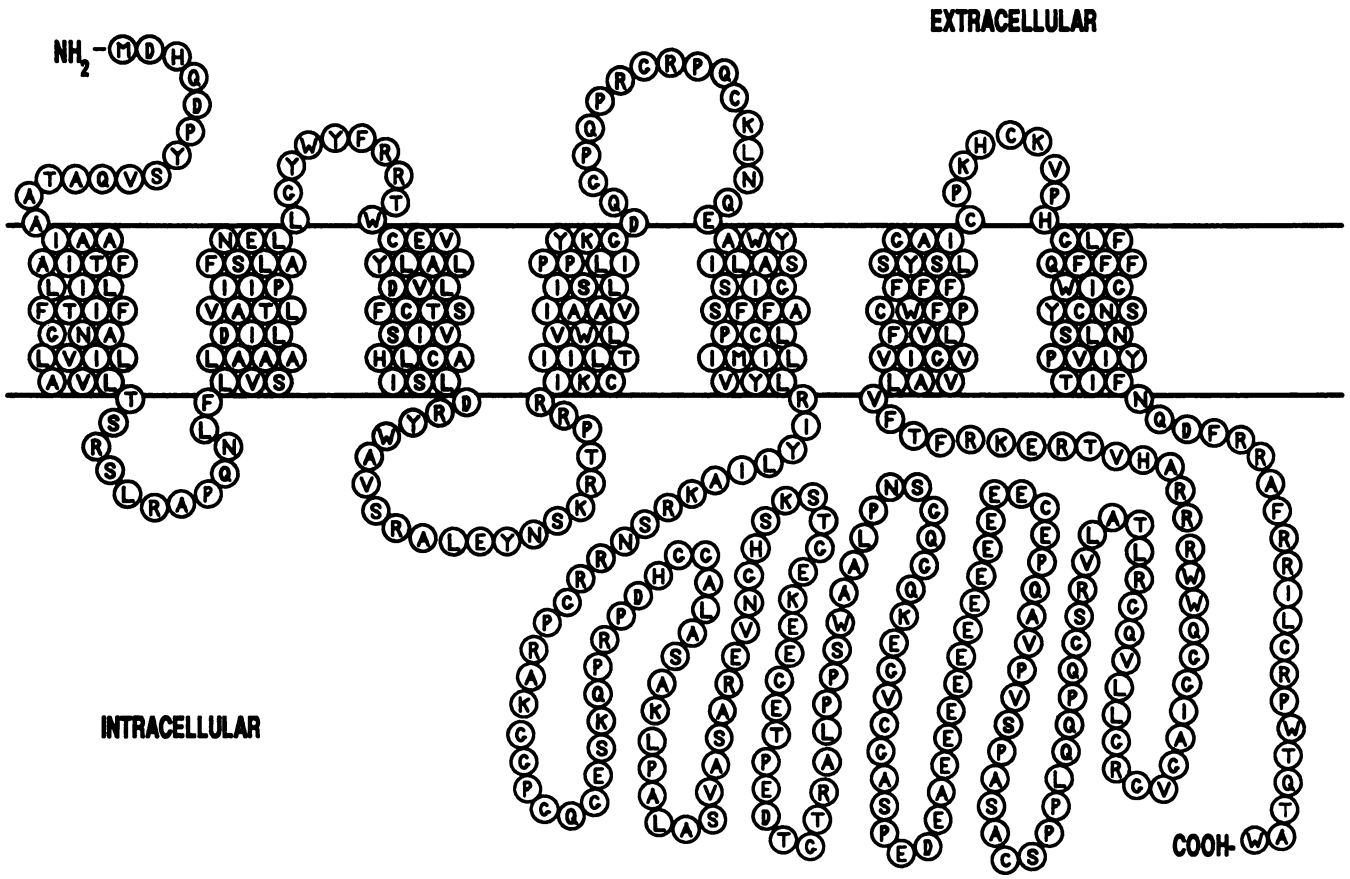

Fig. 1. Seven transmembrane-spanning-region model of the deduced amino acid sequence of the human α_{2B}-adrenergic receptor. The lack of a consensus sequence for *N*-linked glycosylation in the amino terminus of the protein is shown by the absence of the traditional arrows.

acteristically found in the large cytoplasmic loop of G protein-coupled receptors, particularly with respect to the basic amino acids. Interestingly, the large cytoplasmic loop of clone 5A contained a significantly higher content of glutamic acid residues (13%), as compared with clones α_2-C4 (3%) and α_2-C10 (6%), with one region displaying a stretch of 12 consecutive glutamic acid residues. An additional distinguishing feature of clone 5A is the lack of a consensus sequence for *N*-linked glycosylation in its amino terminus. Both α_2-C4 and α_2-C10 contain two such sites each, suggesting that these α_2 receptors are glycosylated with *N*-linked complex oligosaccharides, but a third α_2 receptor (clone 5A) is not.

In order to determine the nature of the newly isolated gene, clone 5A was inserted into a mammalian expression vector, pcEXV-3, and transfected into mouse fibroblast Ltk$^-$ cells. All radioligand binding studies were performed on membranes prepared from a stably expressing cell line. Preliminary experiments were conducted to determine the incubation period and buffer composition that would maximize the binding of the α_2-adrenergic antagonist [^{3}H]rauwolscine. Association studies showed that 1 nM [^{3}H]rauwolscine attained equilibrium only after a lengthy (90 min) incubation at 23° using a glycylglycine buffer. [^{3}H]Rauwolscine bound with high affinity (K_d = 0.33 ± 0.08 nM; three experiments) and in a saturable manner (B_{max} = 1.19 ± 0.34 pmol/mg of protein; three experiments) in this preparation (Fig. 3). The Hill coefficient for [^{3}H]rauwolscine binding did not significantly deviate from unity (n_H = 0.98 ± 0.03), consistent with the labeling of an apparently homogeneous population of noninteracting binding sites. No specific [^{3}H]rauwolscine binding was observed in membranes prepared from untransfected Ltk$^-$ cells.

Pharmacological characterization of clone 5A was obtained from analysis of competition for [^{3}H]rauwolscine binding. Eleven structurally unique adrenergic ligands totally displaced, in a monophasic manner, specifically bound [^{3}H]rauwolscine (Fig. 4). Apparent K_i values were calculated by computer-assisted nonlinear regression analysis and are summarized in Table 1. The rank order of potency of the adrenergic ligands to compete for the [^{3}H]rauwolscine-labeled binding site was yohimbine > prazosin > corynanthine > oxmetazoline, consistent with either an α_{2B} or an α_{2C} receptor profile. In general, the Hill coefficients did not significantly deviate from unity, indicating that this membrane preparation expresses a homogeneous population of α_2-adrenergic binding sites. However, competition curves for the full agonist epinephrine consistently yielded Hill coefficients significantly less than unity (n_H = 0.45 ± 0.05) and were better fit to a two-site (two-state) model with 45 ± 5% of these receptors existing in the high affinity state (Table 2). The addition of 100 μM Gpp(NH)p produced a rightward shift in the epinephrine displacement curve, as evidenced by a significant ($p < 0.05$) increase in both the K_i (255 nM increased to 405 nM) value and Hill slope (n_H = 0.66 ± 0.06). Even though epinephrine competition curves were better fit by a two-site model in the presence of 100 μM Gpp(NH)p, there was a decrease in the percentage of receptors in the high affinity state (35 ± 3%). In contrast, the agonists clonidine and oxymetazoline displayed steep displacement curves (Fig. 4) and were best modeled as a single class of binding sites in both the

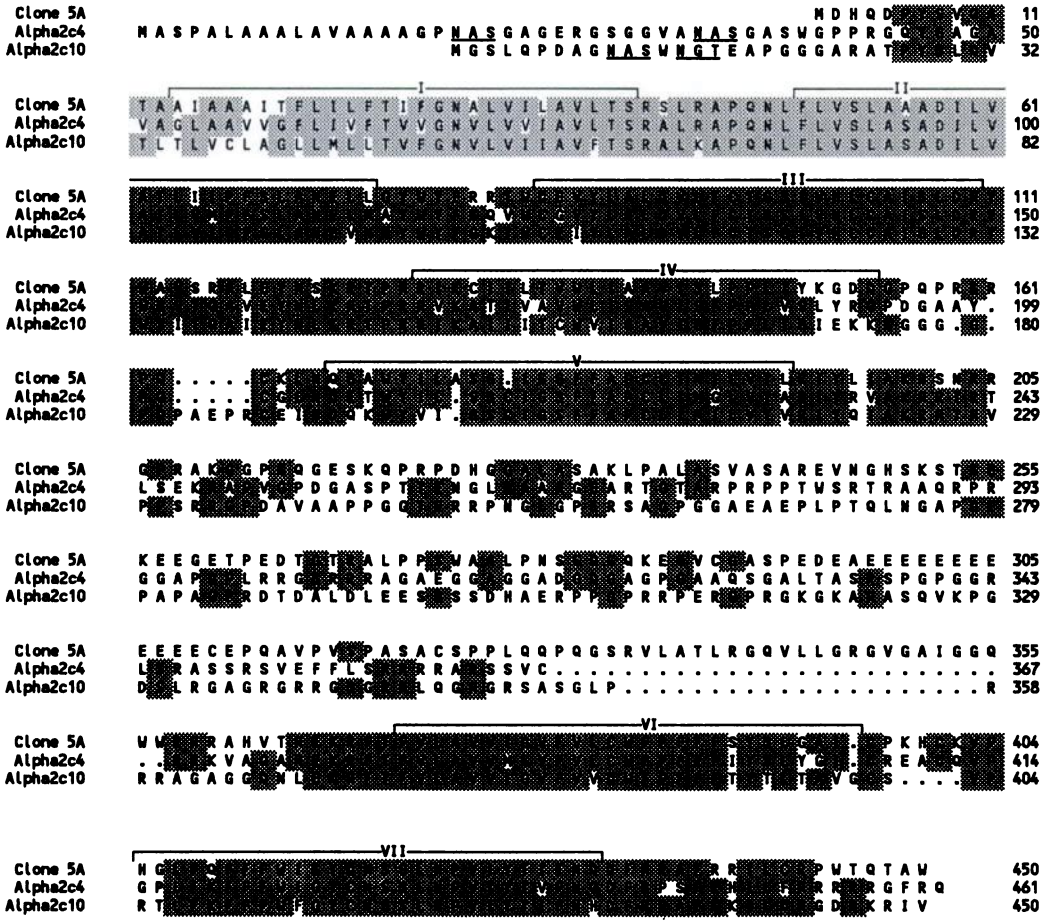

Fig. 2. Comparison of the α_{2B}-adrenergic receptor primary structure with those of α_2-C10 and α_2-C4. The seven putative α-helical membrane-spanning domains (*I–VII*) are highlighted by *solid lines*. In the amino terminal region, consensus sequences for *N*-linked glycosylation sites are *underlined*. Homologies between the three receptors are noted by shading.

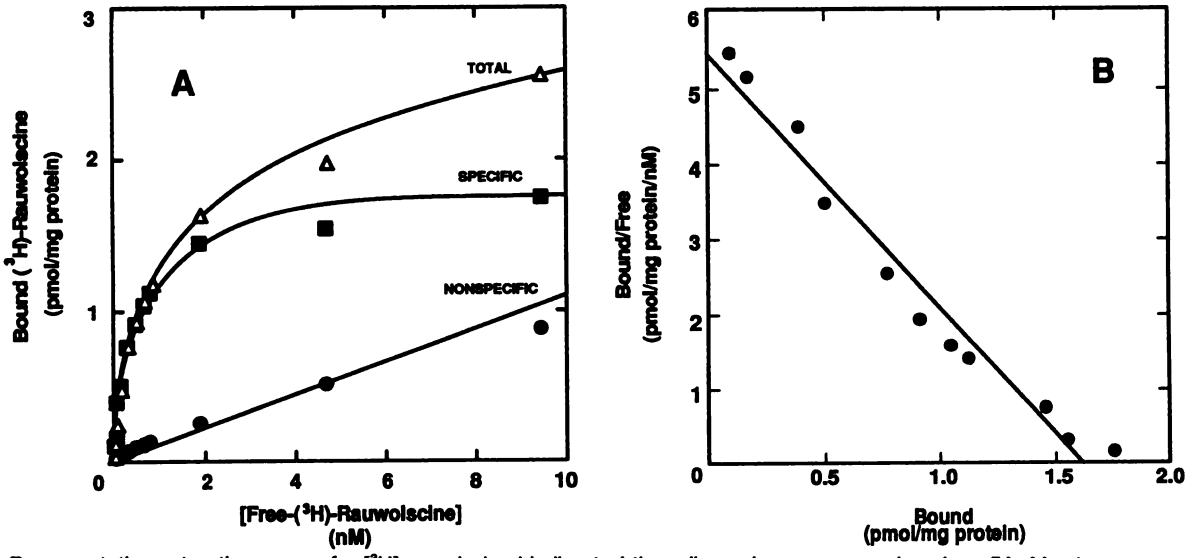

Fig. 3. A, Representative saturation curves for [^{3}H]rauwolscine binding to Ltk$^-$ cell membranes expressing clone 5A. Membranes prepared from stably transfected Ltk$^-$ cells were incubated with increasing concentrations (10 pM to 10 nM) of [^{3}H]rauwolscine, in the absence and presence of 100 μM (−)-norepinephrine, for 2 hr at 23°. Specific binding was greater than 90% of total binding at 1 nM [^{3}H]rauwolscine. Each *data point* represents the mean of triplicate determinations and standard deviations averaged less than 5% of the mean. B, Estimates of K_d and B_{max} values were obtained by nonlinear regression analysis of specific [^{3}H]rauwolscine binding and were inserted into the Scatchard plot. K_d and B_{max} values from this experiment were 0.40 nM and 1.40 pmol/mg of protein, respectively. These results were replicated an additional two times with similar results.

absence (Table 1) and presence of 100 μM Gpp(NH)p, with no significant change in K_i values or Hill coefficients (data not shown). The effects of Gpp(NH)p on agonist competition curves were similar to those reported previously for the α_{2C} receptor from OK cells (4).

In order to best compare the pharmacological properties of the receptor encoded by clone 5A with those of the three known α_2-adrenergic receptor subtypes, competition experiments were performed on cell lines reported to express a homogeneous population of a single α_2 subtype (3, 4). Competition of [^{3}H]

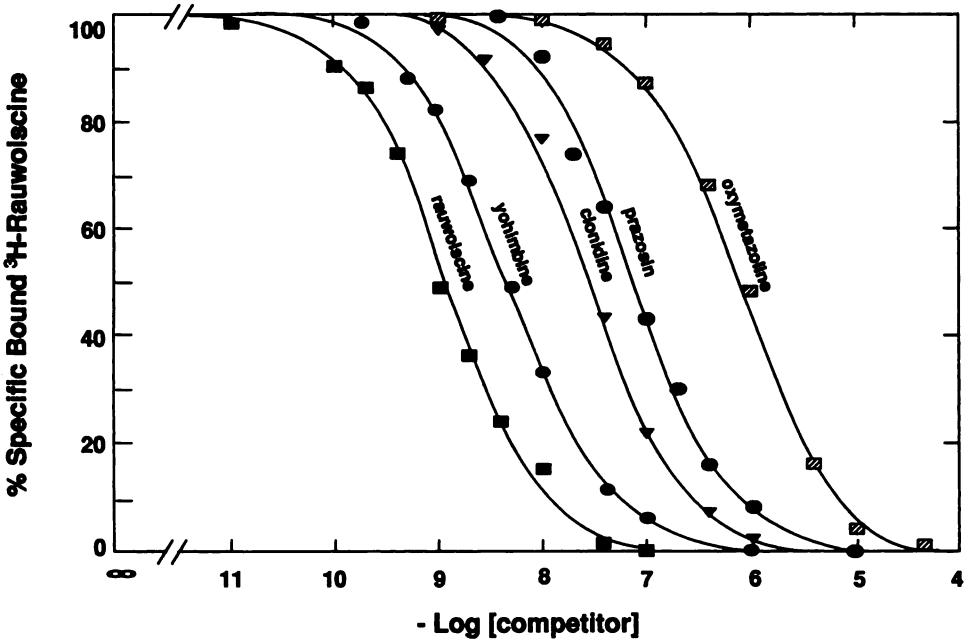

Fig. 4. Representative adrenergic ligand competition curves for the displacement of specific [^{3}H]rauwolscine binding to membranes prepared from stably transfected Ltk$^-$ cells. Membranes were incubated with 1 nM [^{3}H]rauwolscine, in the presence of increasing concentrations of unlabeled competitor, for 90 min at 23°. (−)-Norepinephrine (100 μM) was used to define nonspecific binding. Each *data point* represents the mean of triplicate determinations and standard deviations averaged less than 5% of the mean. K_i estimates were calculated from IC_{50} values using the Cheng-Prusoff equation (17). Mean K_i values are listed in Table 1.

TABLE 1

Potency (K_i values) of adrenergic ligands for displacement of [^{3}H] rauwolscine binding to membranes of Ltk$^-$ cells stably transfected with the human α_{2B} receptor gene

Membranes were incubated with 1 nM [^{3}H+rauwolscine in the presence of increasing concentrations of unlabeled competitor for 90 min at 23°. (−)-Norepinephrine (100 μM) was used to define nonspecific binding. K_i estimates were calculated from IC_{50} values using the Cheng-Prusoff equation (17). K_i values are expressed as geometric means ± standard errors and Hill coefficients (n_H) are expressed as arithmetic means ± standard errors from three to seven independent experiments.

Drug	K_i	n_H
	nM	
Rauwolscine	0.32 ± 0.05	0.91 ± 0.03
Yohimbine	1.2 ± 0.1	0.91 ± 0.09
Phenoxybenzamine	3.5 ± 1.1	0.84 ± 0.08
p-Aminoclonidine	4.5 ± 1.9	0.83 ± 0.14
Clonidine	7.2 ± 0.9	0.98 ± 0.10
WB-4101	17 ± 6	0.96 ± 0.24
Prazosin	23 ± 4	0.94 ± 0.06
(±)-Mianserin	27 ± 6	1.02 ± 0.24
Corynanthine	77 ± 9	0.95 ± 0.09
Ketanserin	199 ± 6	1.21 ± 0.08
Oxymetazoline	213 ± 13	0.83 ± 0.08
(−)-Epinephrine	255 ± 68	0.45 ± 0.05[a]

[a] Value is significantly different ($p < 0.01$) from unity.

rauwolscine binding by oxymetazoline, prazosin, and yohimbine was examined in membranes prepared from HT29 (α_{2A}), NG108-15 (α_{2B}), and OK (α_{2C}) cells. These results were compared with results obtained with Ltk$^-$ cells expressed clone 5A. The K_i values for these ligands are listed in Table 3. The K_i ratio values of prazosin/oxymetazoline and prazosin/yohimbine, predictors of the α_2 subtype (1), were found to be in good agreement in NG-108-15 cell membranes and Ltk$^-$ membranes expressing clone 5A, indicating that clone 5A encodes a receptor that most closely resembles the α_{2B}-adrenergic pharmacology.

Correlations were also calculated between previously published data on the affinities (pK_i values) of seven adrenergic ligands in the model cell lines and values determined in our experiments for Ltk$^-$ cells stably expressing clone 5A (Fig. 5). Strong correlations were obtained between clone 5A and both the α_{2B} ($r = 0.95$) and α_{2C} ($r = 0.90$) pharmacologies, whereas a much lower correlation ($r = 0.56$) was observed with the α_{2A} subtype. The strong correlation of clone 5A with both the α_{2B} and α_{2C} subtypes is expected, because these two pharmacologically defined subtypes are very closely related. As previously reported, the ligand binding affinities of the α_{2B} and α_{2C} subtypes are strongly correlated ($r = 0.85$) with each other (1).

Discussion

To date, at least three α_2 receptor subtypes have been identified pharmacologically and have been designated α_{2A}, α_{2B}, and α_{2C} (1). The molecular biological studies have now converged with the pharmacology and support the presence of at least three distinct human α_2 receptor genes. Previously, Lefkowitz and colleagues (5, 6) described the human genes encoding two α_2-adrenergic receptor subtypes. Although the chromosomal localizations of these genes have previously been assigned, a

TABLE 2

Effect of Gpp(NH)p on epinephrine competition of [^{3}H]rauwolscine binding

Assays were performed on membranes of Ltk$^-$ cells stably transfected with the human α_{2b} receptor gene. Membranes were incubated with 1 nM [^{3}H]rauwolscine and increasing concentrations of (−)-epinephrine, in the absence and presence of 100 μM Gpp(NH)p, for 90 min at 23°. (−)-Norepinephrine (100 μM) was used to define nonspecific binding. Apparent K_i estimates were calculated from IC_{50} values using the Cheng-Prusoff equation (17) or, alternatively, high and low affinity components (K_H and K_L) were calculated by two-site analysis of the data. K_i, K_H, and K_L values are expressed as geometric means ± standard errors. Hill coefficients (n_H) and the percentage of receptors in the high affinity state (percentage of R_H) are expressed as arithmetic means ± standard errors from three separate determinations.

Condition	K_i	K_H	K_L	n_H	R_H
		nM			%
(−)-Epinephrine	255 ± 68	14 ± 4	804 ± 99	0.45 ± 0.05	45 ± 5
(−)-Epinephrine + Gpp(NH)p	405 ± 25[a]	32 ± 7[a]	1510 ± 123[a]	0.66 ± 0.06[a]	35 ± 3[a]

[a] Values statistically different ($p < 0.05$) from respective (−)-epinephrine control values.

TABLE 3

Potency (K_i values) of oxymetazoline, prazosin, and yohimbine for displacement of [^{3}H]rauwolscine binding to cell membranes expressing only one subtype of α_2-adrenergic receptor

Membranes were incubated with 1 nM [^{3}H]rauwolscine, in the presence of increasing concentrations of unlabeled competitor, for 90 min at 23°. (−)-Norepinephrine (100 μM) was used to define nonspecific binding. K_d values of [^{3}H]rauwolscine for the specific α_2 receptor subtype in HT-29 (0.66 nM), NG-108-15 (0.68 nM), and OK (0.074 nM) cell membranes were obtained from the literature (3, 4), so that K_i values could be calculated from IC_{50} values using the Cheng-Prusoff equation (17). Ltk-5A cells were mouse fibroblast Ltk$^-$ cells stably transfected with clone 5A. K_i values are expressed as geometric means ± standard errors from three separate determinations.

Cell type	Receptor subtype	K_i			K_i ratio	
		Yohimbine	Oxmetazoline	Prazosin	Prazosin/oxymetazoline	Prazosin/yohimbine
			nM			
HT29	α_{2A}	0.76 ± 0.32	2.5 ± 0.2	473 ± 30	189	622
NG108-15	α_{2B}	1.7 ± 0.2	69 ± 11	7.7 ± 1.0	0.11	4.5
OK	α_{2C}	0.42 ± 0.17	35 ± 3	24 ± 6	0.69	57
Ltk-5A	α_{2B}	1.2 ± 0.1	213 ± 13	23 ± 4	0.11	19.2

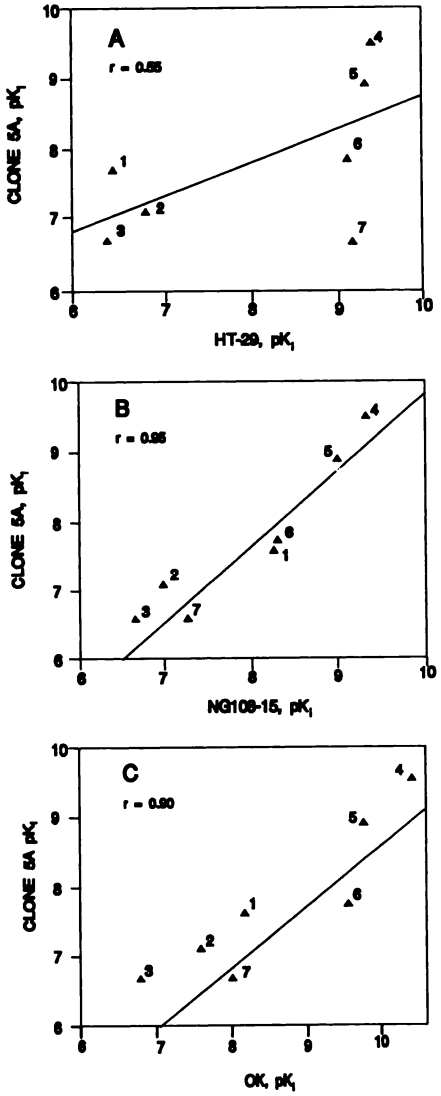

Fig. 5. Correlations between the pK_i values of adrenergic ligands in cells that express a homogeneous population of only one α_2 subtype (HT-29 cells for α_{2A} NG-108-15 cells for α_{2B}, OK cells for α_{2C}) and in Ltk$^-$ cells expressing clone 5A. K_i values were taken from Bylund *et al.* (3) and Murphy and Bylund (4). The correlation coefficient (r) is listed in each panel. *1*, Prazosin; *2*, corynanthine; *3*, ketanserin; *4*, rauwolscine; *5*, yohimbine; *6*, WB 4101; *7*, oxymetazoline.

putative third gene, represented by a 1.8-kb *Pst*I fragment localized to chromosome 2, has not yet been identified (5).[1] The size of the *Pst*I fragment derived from clone 5A, as measured by both sequence and Southern blot analysis, corresponds to the size of the *Pst*I fragment localized to chromosome 2 (data not shown). Although definitive assignment awaits chromosomal mapping, the cloning of a third human α_2-adrenergic receptor subtype distinct from the other two previously cloned is consistent with the notion that clone 5A represents α_2-C2.

Pharmacological criteria that have been used to subclassify the α_2-adrenergic receptors into α_{2A}, α_{2B}, and α_{2C} subtypes (1) are 1) the equilibrium dissociation constant (K_d) of [^{3}H]rauwolscine, 2) the rank order of potency of ligands in competing for ^{3}H antagonist-labeled binding sites, and 3) the K_i ratio values of prazosin/oxymetazoline and prazosin/yohimbine. The data presented in this paper examine each of these criteria and are largely consistent with the designation of this receptor as an α_{2B}-adrenergic receptor. The first point, the K_d for [^{3}H] rauwolscine, deserves some discussion. We have observed that the experimentally observed equilibrium dissociation constant for [^{3}H]rauwolscine is highly dependent on the assay conditions. In glycylglycine buffer, we observed a dissociation constant of 0.33 nM for the transfected human α_{2B}-adrenergic receptor. When Tris buffer was substituted for glycylglycine buffer, we observed a 5-fold decrease in the affinity (increase of the K_d to ~1.6 nM) of [^{3}H]rauwolscine for the transfected receptor (data not shown). The transfected rat α_{2B} receptor recently isolated by Lynch and co-workers (8) was reported to exhibit a dissociation constant of 2 nM for [^{3}H]rauwolscine in a Tris buffer, which is, thereby, in good agreement with our results.

The incubation time may also influence the apparent dissociation constant of a system for [^{3}H]rauwolscine. As mentioned above (see Results), we have found that long incubation times are needed to reach equilibrium in our assay system. Shorter incubation times may produce significantly different results. Bylund *et al.* (3) used incubation periods of 30 min at 23° and obtained a 2.5-fold higher K_d value for [^{3}H]rauwolscine binding in rat tissues than the value we observed for the transfected human clone. It is unclear whether incubation conditions or species effects underlie these different experimental results.

When these effects of incubation conditions are taken into consideration, both the [^{3}H]rauwolscine binding constant and

[1] Recently, Lomasney, Lefkowitz, Caron, and collaborators have isolated and characterized a gene that represents this chromosome 2 locus (personal communication).

pharmacological binding properties of the transfected human α_{2B} receptor are in good agreement with previous data on the α_{2B}-adrenergic receptor from neonatal rat lung and NG-108-15 cells (1). Furthermore, the binding data clearly rule out the assignment of this clone as the α_{2A}-adrenergic receptor subtype, based upon the rank order of drug potencies as well as the K_i ratio values of prazosin/oxymetazoline and prazosin/yohimbine. In contrast, the pharmacological properties of α_{2B} and α_{2C} receptors are very similar and retain the same rank order of potency, rauwolscine > yohimbine > prazosin > oxymetazoline (3, 4). Pharmacological criteria used to discriminate between these adrenergic receptor subtypes include a lower K_d value for [^{3}H]rauwolscine and higher K_i ratio values of prazosin/oxymetazoline and prazosin/yohimbine for α_{2C} receptors, relative to α_{2B} receptors (1). In the present study, the K_i ratio of prazosin/oxymetazoline for clone 5A (0.11) is in excellent agreement with the value from the α_{2B} receptor model tissue, NG108-15 cells (0.11), and quite different from the values for the α_{2A} (189) or α_{2C} (0.69) receptors (Table 3). The K_i ratio of prazosin/yohimbine for clone 5A does not precisely conform to the α_{2B} designation, because this ratio value (19.2) is intermediate between α_{2B} (4.5) and α_{2C} (57) values. The reason for this discrepancy is unknown at present but may be the result of a species difference between the human α_{2B} receptor and the model tissues. Although the rank orders of drug potencies are very similar between α_{2B} and α_{2C}, both the K_i ratio of prazosin/oxymetazoline and the affinity of [^{3}H]rauwolscine derived from saturation studies indicate that clone 5A differs in pharmacological properties from the α_{2C} subtype.

Following our preliminary report of the characterization of clone 5A as an α_{2B}-adrenergic receptor (7), we learned of the isolation by Lynch and co-workers (8) of a highly homologous cDNA from rat, which encodes an α_{2B} receptor. The protein encoded by this rat cDNA clone exhibits 83% overall homology to the protein encoded by the human genomic clone of this study, and the pharmacological binding properties of the two clones are very similar, once the effects of incubation conditions are considered. These similarities have led both groups to conclude that the rat and human α_{2B} clones represent species homologues of the same gene (8).

One unusual property shared by the human and rat α_{2B}-adrenergic receptor genes is the lack of *N*-linked glycosylation sites in either the extracellular loops or the amino terminus of this receptor. This is consistent with the biochemical results of Lanier *et al.* (22), who have shown that the α_{2B} receptor purified from neonatal rat lung does not have any associated oligosaccharide moieties. Two other members of the G protein-coupled receptor family, RDC7 and RDC8, have also been described as lacking consensus sequences for *N*-linked glycosylation and containing short amino terminal segments (23). The functional significance of this lack of *N*-terminal glycosylation in this small subfamily of G protein-coupled receptors is not known. Because the human and rat α_{2B}-adrenergic receptors can be stably expressed in a mammalian cell line, *N*-linked glycosylation is apparently not necessary to obtain cell surface expression of G protein-coupled receptors. It is also unlikely that the human α_{2B}-adrenergic receptor is a transient, developmentally regulated form of the receptor. The mRNA for the rat α_{2B} receptor is expressed at significantly higher levels in adult rat kidney than in neonatal rat lung (8). These α_{2B} receptors, as well as RDC7 and RDC8, apparently represent a new subclass of G protein-coupled receptors containing short amino terminal segments and lacking *N*-linked glycosylation. The functional consequences of these structural features remain to be determined.

The previously described α_2-C4 clone was originally proposed to be the human α_{2B} receptor, based on a preliminary pharmacological analysis (6). More recent work on the diversity of α_2-adrenergic receptor subtypes has led to the suggestion that it may, instead, encode a fourth type of α_2-adrenergic receptor distinct from the α_{2A}, α_{2B}, and α_{2C} receptors (1). Alternatively, it may represent an α_{2C} subtype, because the published prazosin/oxymetazoline and prazosin/yohimbine ratios, as well as the presence of glycosylation consensus sequences in its extracellular domains, are in better agreement with known properties of the α_{2C}-adrenergic receptor. Although the exact pharmacological classification of the α_2-C4 clone remains to be determined, it appears to resemble the α_{2B} pharmacology less well than does clone 5A, as described in the present study.

The human α_{2B}-adrenergic receptor gene reported here is another example of a gene encoding a G protein-coupled receptor whose entire coding region is contained within a single exon. Among the known α_2-adrenergic receptor clones, the human platelet α_{2A} receptor (α_2-C10) has been shown to contain an intronless coding region, whereas the genomic structure of the human α_2-C4 receptor and the recently characterized rat α_{2B} receptor (8) await characterization. Although intron-containing genes of this family are less frequently observed, several cases have been described, including the genes for the visual pigment rhodopsin (24), substance K (25), 5-HT$_{1C}$ (25, 26), and human dopamine D$_2$ (27) receptors. The evolutionary relationships between these receptor genes and the mechanism and significance of intron loss or acquisition remain to be determined.

Guanine nucleotides are known to modulate agonist binding to G protein-coupled receptors (28). In the present study, 100 μM Gpp(NH)p produced rightward shifts in epinephrine competition curves, with a resultant increase in both K_i values and Hill coefficients, suggestive of a functional interaction of cloned human α_{2B} receptors with native G protein(s) of Ltk$^-$ cells. Furthermore, only the agonist epinephrine exhibited significant effects of Gpp(NH)p on both Hill slope and K_i values, consistent with the two-state model of agonist effects on G protein-coupled receptors. However, Gpp(NH)p failed to increase the Hill coefficient to unity and to totally convert the receptors to the lower affinity state. These results may be due to the high level of expression of this receptor relative to the amount of endogenous G protein(s) in the murine fibroblast membranes. Previous studies on transfected muscarinic M$_1$ (29) and 5-HT$_2$ (30) receptors demonstrated an inverse relationship between receptor density and the proportion of receptors found in the high affinity state. In such transfection systems, G protein concentrations may become rate limiting, resulting in an apparent loss of functional interaction between the receptor and G protein(s) and an incomplete conversion to the agonist low affinity state.

In summary, we have isolated and characterized a third human α_2-adrenergic receptor gene, which appears to encode an α_{2B} receptor subtype based on six criteria, 1) the rank order of drug potencies at displacing [^{3}H]rauwolscine binding, 2) the K_i ratio of prazosin/oxymetazoline, 3) the low K_d value of [^{3}H] rauwolscine, 4) the absence of glycosylation sites, 5) the high

degree of sequence homology (98% in transmembrane regions) to the rat α_{2B} clone that has been shown to be expressed in the neonatal rat lung (8), and 6) sequence homology (~75% in the transmembrane regions) to the other two known human α_2-adrenergic receptor genes (5, 6). This new human α_{2B}-adrenergic receptor clone is approximately equidistant in sequence divergence from both the human α_2-C10 and α_2-C4 clones previously described. The availability of all three human clones provides an important new tool for the investigation of α_2-adrenergic receptor function and for the further exploration of the diverse family of G protein-coupled receptors.

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