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Cloning and expression of a rat brain α_{2B}-adrenergic receptor

(α_2-adrenergic receptor subtypes/cDNA/[^{3}H]rauwolscine/antisense mRNA)

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ABSTRACT **We have isolated a cDNA clone (RBα_{2B}) and its homologous gene (GRα_{2B}) encoding an α_{2B}-adrenergic receptor subtype by screening a rat brain cDNA and a rat genomic library. Nucleotide sequence analysis showed that both clones code for a protein of 458 amino acids, which is 87% homologous to the human kidney glycosylated adrenergic receptor (α_2-C4) and divergent from the rat kidney nonglycosylated α_{2B} subtype (RNGα_2). Transient expression of RBα_{2B} in COS-7 cells resulted in high-affinity saturable binding (K_d = 0.25 nM) for [^{3}H]rauwolscine and a high receptor number (B_{max} = 7.7 pmol/mg of protein) in the membranes of transfected COS-7 cells. Pharmacological analysis demonstrated that the expressed receptor bound adrenergic ligands with the following order of potency: rauwolscine > yohimbine > prazosin > oxymetazoline, with a prazosin-to-oxymetazoline K_i ratio of 0.34. This profile is characteristic of the α_{2B}-adrenergic receptor subtype. Blotting analysis of rat brain mRNA gave one major (3.0-kilobase) and two minor (4.6- and 2.3-kilobase) mRNA species, and hybridization with strand-specific probes showed that both DNA strands of GRα_{2B} may be transcriptionally active. These findings show that rat brain expresses an α_{2B}-adrenergic receptor subtype that is structurally different from the rat kidney nonglycosylated α_{2B} subtype. Thus the rat expresses at least two divergent α_{2B}-adrenergic receptors.**

Pharmacological studies have classified α_2-adrenergic receptors as α_{2A} and α_{2B} on the basis of their different ligand-binding properties and, in particular, their relative affinities for oxymetazoline and prazosin (1, 2). The α_{2A} subtype, which exhibits high affinity for oxymetazoline and low affinity for prazosin, is the sole α_2 subtype found in human platelets, whereas the α_{2B} subtype, which has high affinity for prazosin and low affinity for oxymetazoline, is the only α_2 subtype found in neonatal rat lung (2). Biochemical analysis of partially purified α_2-adrenergic receptors from human platelets and neonatal rat lung showed that the differences in their ligand-binding properties are due to differences in their primary structure (3). This has been substantiated by the molecular characterization of different DNA clones encoding different α_2-adrenergic receptor subtypes (4–8).

Recent molecular cloning data suggest that there are at least three distinct α_2 receptor genes in humans: α_2-C10, encoding the human α_{2A} receptor expressed in human platelets, maps to chromosome 10 (4, 7); α_2-C4, encoding a human glycosylated α_{2B}, maps to chromosome 4 (5); and α_2-C2, encoding a nonglycosylated α_2 that has some of the pharmacological characteristics of α_{2B} (8), maps to chromosome 2.

RNGα_2, a cDNA isolated from a rat kidney library, is highly divergent from the human α_2-C10 and α_2-C4 subtypes and encodes, like the human α_2-C2, a nonglycosylated receptor with some α_{2B} subtype properties (6). This receptor may be the equivalent of the rat α_{2B} adrenergic receptor previously studied in neonatal rat lung (3).

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The contribution of various α_2-adrenergic receptor subtypes to the overall α_2-adrenergic receptor activity in rat tissues and, in particular, rat brain is not known. Pharmacological studies (3) have suggested that the rat brain contains approximately equal amounts of the α_{2A} and α_{2B} subtypes (1). Previous work in this laboratory has shown that there are several α_2-specific transcripts in the rat brain (9). In this study we describe the molecular characterization of a rat brain cDNA encoding an α_{2B}-adrenergic receptor‡ that is different from the previously described nonglycosylated rat α_{2B} subtype RNGα_2 (6).

MATERIALS AND METHODS

Materials. [^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq), [^{35}S]dATP (1350 Ci/mmol), and [^{3}H]rauwolscine (80 Ci/mmol) were from DuPont/NEN. Rauwolscine was obtained from Accurate Chemicals (Westbury, NY). Phentolamine, prazosin, and oxymetazoline were gifts from CIBA–Geigy, Pfizer, and Schering, respectively.

Isolation and Sequencing of Genomic and cDNA Clones. A 1.6-kilobase (kb) *Nco* I/*Hin*dIII fragment of the human platelet α_2-adrenergic receptor gene (4) was labeled by the random priming DNA method (10) and used to screen 10^6 phage plaques (11) from a partial *Sau*3A-digested Wistar rat genomic library in λDash and a Sprague–Dawley rat whole-brain, oligo(dT)-primed cDNA library in λ-Zap II.

Duplicate nitrocellulose filters were hybridized at 42°C in 50% formamide/5× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate)/1× Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin)/0.1% SDS/salmon sperm DNA (100 μg/ml) and washed at 55°C in 0.5× SSC. Inserts from purified clones were subcloned into pBluescript II SK.

DNA sequences were determined by the dideoxy chain termination method (12) using double-stranded templates and the modified T7 DNA polymerase (Sequenase).

Expression of Rat Brain cDNA. To remove the 5′ untranslated region, the plasmid RBα_{2B} was digested with *Nco* I (see Fig. 1*A*), blunt-ended with Klenow polymerase, and then cleaved with *Eco*RV. An ≈4.6-kilobase-pair (kbp) fragment was gel purified and recircularized by using T4 ligase. Subsequent digestion of this DNA with *Hin*dIII and *Bam*HI allowed for the isolation of a 1.7-kbp fragment that was directionally inserted into the Rous sarcoma virus long terminal repeat expression vector pBC12BI(13). COS-7 cells were grown in Dulbecco's modified Eagle's medium with

Abbreviations: RBα_{2B}, cDNA cloned from rat brain and encoding an α_{2B}-adrenergic receptor subtype; GRα_{2B}, rat genomic homolog of RBα_{2B}; α_2-C4, human kidney cDNA encoding an α_{2B}-adrenergic receptor subtype; RNGα_2, rat kidney cDNA encoding a nonglycosylated α_{2B}-adrenergic receptor subtype; α_2-C10, human genomic clone encoding an α_{2A}-adrenergic receptor; G protein, guanine nucleotide-binding protein; α_2-C2, human genomic clone encoding a nonglycosylated α_2-adrenergic receptor.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M58316).

high glucose (4.5 g/liter) and 25 mM Hepes, supplemented with 10% fetal calf serum, 1% glutamine, 1 mM pyruvate, penicillin (100 units/ml), and streptomycin (100 μg/ml) in 5% CO_2 in an incubator at 37°C. COS-7 cells (3.5–4 × 10^6 per dish) were plated in 100-mm dishes 24–36 hr before transfection, transfected at 80% subconfluence with 5 μg of supercoiled DNA per 100-mm dish by the DEAE-dextran method (13), and harvested 60 hr after transfection.

α_2-Adrenergic Receptor Ligand-Binding Studies. Transfected COS-7 cells were used at 60 hr posttransfection. Membranes were prepared as described (14) by a modification of Wikberg *et al.* (15). Briefly, the cells were washed and scraped into chilled Dulbecco's phosphate-buffered saline/1 mM EGTA. The pelleted cells were lysed at 4°C in 10 ml of a 1:20 dilution of phosphate-buffered saline/1 mM EGTA/0.1 mM phenylmethylsulfonyl fluoride, by freezing and thawing. The membrane pellet obtained after centrifugation at 36,000 × *g* for 10 min was washed, centrifuged again, suspended in 50 mM Tris/1 mM EDTA, pH 7.2, and stored at −70°C.

The binding studies were performed as described by Bresnahan *et al.* (14). For saturation binding, ≈10 μg of membrane protein was incubated for 3 hr at 4°C with 0.1–10 nM [^{3}H]rauwolscine in a final volume of 0.5 ml in 50 mM Tris/1 mM EDTA, pH 7.2. Nonspecific binding was determined by including 10 μM phentolamine in parallel assays. The reactions were terminated by vacuum filtration; the filters were washed with 15 ml of buffer at 4°C, dried, extracted overnight with 10 ml of Liquiscint, and assayed for radioactivity. The data were transformed according to Rosenthal (16) and analyzed by linear regression for determination of the equilibrium dissociation constant, K_d, and maximum binding, B_{max}.

Competition studies were performed at a molar concentration of [^{3}H]rauwolscine equal to its K_d and with various concentrations of each competitor. The concentration of drug inhibiting specific binding by 50%, IC_{50}, was obtained from the competition curves and used to calculate the K_i value by the method of Cheng and Prusoff (17).

RNA Extraction and Northern Blotting. Total cellular RNA was extracted from rat tissues by a modification of the guanidinium isothiocyanate procedure (18). Poly(A)$^+$ RNA was selected by passage over an oligo(dT)-cellulose column twice (11). Poly(A)$^+$ RNA (5 μg) was electrophoresed on a 1% agarose/3% formaldehyde gel prepared in 20 mM Mops, pH 7/5 mM sodium acetate/1 mM EDTA, and the fractionated RNAs were transferred to GeneScreen*Plus* membranes.

The filters were prehybridized for 5 hr at 42°C in 50% formamide/1 M NaCl/1% SDS/10% dextran sulfate/sonicated salmon sperm DNA (100 μg/ml). Hybridization was carried out at 42°C for 16 hr in the same buffer by the addition of probe (10^6 cpm/ml). The probe, a 466-base-pair *Nae* I/*Stu* I fragment of RBα_{2B} cDNA containing the third cytoplasmic loop and transmembrane domain VI, was labeled by the random priming method (10). The filters were washed in 0.1× SSC/0.1% SDS at 55°C for 30 min. Autoradiograms were developed after 48 hr at −70°C.

For the strand-specific Northern blot analysis, the *Nae* I/*Stu* I fragment of the RBα_{2B} cDNA was cloned in either orientation into the *Sma* I site of pSP65 (11). Orientation was confirmed by nucleotide sequence analysis. RNA probes were synthesized by *in vitro* transcription with SP6 polymerase from linearized pSP65 constructs (19). The sense and antisense probes were then hybridized to Northern blots at 50°C for 16 hr in a buffer identical to that used for double-

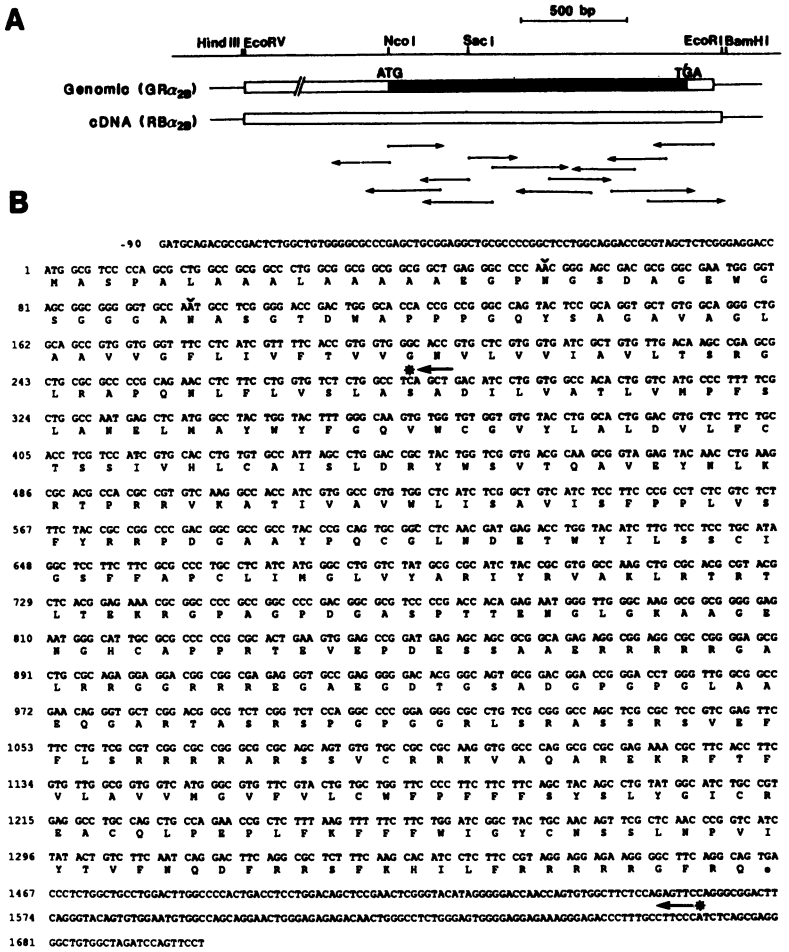

FIG. 1. Nucleotide and deduced amino acid sequence of the rat brain α_{2B}-adrenergic cDNA clone RBα_{2B}. (*A*) Restriction sites used in sequencing and expression studies, the alignment of RBα_{2B} and GRα_{2B}, and the sequencing strategy. The black bar represents the protein coding region. (*B*) Numbers on the left and right margins indicate the nucleotides and the amino acid residues, respectively. The sites for potential N-glycosylation are shown by carets. The termination codon of the α_2-specific open reading frame is indicated by a filled circle. Asterisks at positions 1671 and 285 indicate the beginning and the end of the open reading frame on the complementary strand of the clone.

stranded DNA probe and then were washed in 0.1× SSC/0.1% SDS at 65°C.

RESULTS

Isolation and Sequencing of Rat Brain α_2-Adrenergic Receptor cDNA and Its Homologous Genomic Clone. A rat brain cDNA library and a rat genomic library were independently screened with a probe derived from the human platelet α_{2A}-adrenergic receptor gene (4). This resulted in the isolation of three positive cDNA clones and two positive genomic clones. One of the genomic and one of the cDNA clones were found to be related; both contained inserts that hybridized to a 3.0-kb mRNA species on Northern blots of rat brain mRNA. This mRNA had been detected in a previous study as a major α_2-related mRNA species in the rat brain (9).

The inserts of these two clones were subcloned in pBluescript II SK and further characterized. The cDNA subclone, designated RBα_{2B}, contains an ≈1.4-kbp coding region and ≈1.0 kbp of 5′ and 0.25 kbp of 3′ untranslated sequences. The genomic subclone, designated GRα_{2B}, contains the entire coding region and ≈4.0 kbp of upstream and 0.3 kbp of downstream sequences. Nucleotide sequence analysis of the genomic and cDNA clones showed that they contain an identical protein-coding region encoded in a single exon (Fig. 1*A*).

RBα_{2B} contains two open reading frames, one on each strand. One starts at position 90 with an initiator methionine occurring in an optimal sequence context (20) and terminates with a TGA codon at position 1374. This reading frame encodes a protein of 458 amino acids with α_2-specific sequences and an estimated molecular mass of 49,877 Da (Fig. 1*B*). The other (found on the complementary strand) codes for 432 amino acids, starts at position 1671, and terminates at position 285 (Fig. 1*B*). In this study, we have analyzed the α_2-specific sequence. Hydropathy analysis (21) of the deduced amino acid sequence displayed seven hydrophobic domains separated by hydrophilic regions.

The comparison of the RBα_{2B} deduced amino acid sequence with those of the previously described α_2-adrenergic receptors is presented in Fig. 2*A*. The analysis showed that the most conserved amino acid sequences are those of the putative transmembrane domains. Within these regions, RBα_{2B} has 98.8% homology to the α_2-C4 human kidney cDNA clone (5), 77.3% homology to the RNGα_2 rat kidney α_{2B} clone (6), and 78.6% homology to the α_2-C10 human platelet α_{2B} clone (4). Similar to the human kidney α_2-C4, RBα_{2B} has two potential N-glycosylation sites near the amino terminus of the protein (residues 19 and 33). In contrast, the rat kidney RNGα_2 clone lacks potential N-glycosylation sites (6).

The sequences corresponding to the third cytoplasmic loop have the greatest overall divergence. In this loop, human kidney α_2-C4 and RBα_{2B} have an overall homology of 74.4% (Fig. 2*B*). Although the amino-terminal and the carboxyl-terminal regions of this loop are highly homologous between these two clones (Fig. 2*B*), the middle of the loop is more divergent. In addition, there are three amino acid deletions in the protein encoded by clone RBα_{2B}, corresponding to residues 294, 295, and 329 in the protein encoded by clone α_2-C4.

Expression of the RBα_{2B} Clone. To determine the ligand-binding properties of the protein encoded by the RBα_{2B}

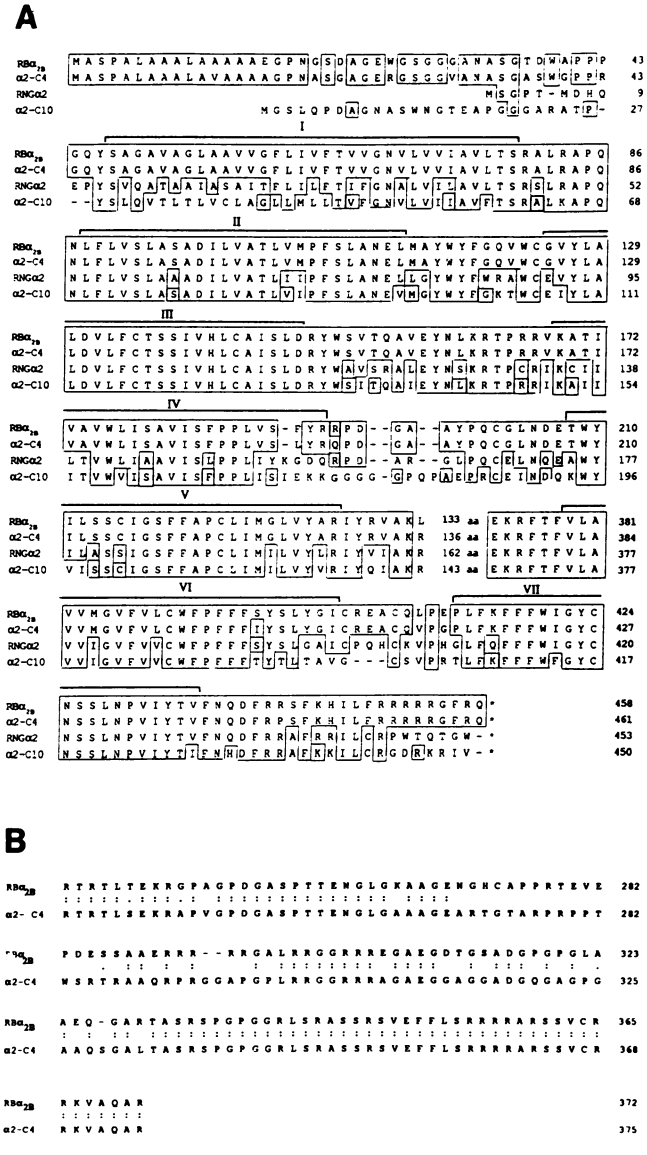

FIG. 2. (*A*) Comparison of the deduced amino acid sequence of the rat brain α_{2B}-adrenergic receptor RBα_{2B} with those of the other cloned α_2-adrenergic receptors. Sequences were aligned using the ALIGN function of Genepro (22). Gaps (indicated by dashes) have been introduced to maximize alignment. Identical amino acids are enclosed in boxes. Brackets I–VII indicate hydrophobic regions postulated to be transmembrane domains. (*B*) Comparison of the amino acid sequences corresponding to the third cytoplasmic loop of the rat brain (RBα_{2B}) and the human kidney (α_2-C4) receptor proteins. RNGα_2 sequences are not compared due to lack of meaningful homology of this domain with RBα_{2B} or α_2-C4. Two dots depict amino acid identity; one dot depicts a conservative amino acid substitution. Dashes indicate amino acid gaps.

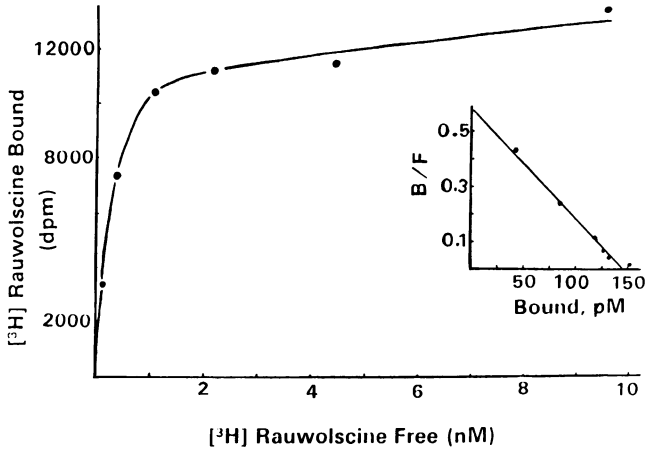

FIG. 3. Saturation isotherm for [^{3}H]rauwolscine-specific binding to membranes of COS-7 cells transfected with the rat brain α_{2B}-adrenergic receptor clone RBα_{2B}. (*Inset*) Rosenthal plot: K_d = 0.25 nM; B_{max} of 145.1 pM was converted to 7.72 pmol/mg of protein based on protein concentration of 18.8 mg/liter. B, bound; F, free.

clone, the entire coding region and 3′ noncoding sequences were inserted into the mammalian expression vector pCB12BI(13) and transfected into COS-7 cells. Sixty hours posttransfection, the cell membranes were prepared for receptor binding and competition experiments. Saturation binding with [^{3}H]rauwolscine and Rosenthal analysis are presented in Fig. 3. This analysis showed high affinity (K_d = 0.25 nM) and high receptor number (B_{max} = 7.7 pmol/mg of membrane protein). Pharmacological analysis in which the specific binding of [^{3}H]rauwolscine at the K_d level was inhibited by adrenergic ligands showed that the expressed protein has the binding characteristics of α_{2B}-adrenergic receptors (Fig. 4). Adrenergic ligands displayed the following order of potency: rauwolscine > yohimbine > prazosin > oxymetazoline. The K_i values were 0.76 nM for rauwolscine, 1.49 nM for yohimbine, 50 nM for prazosin, and 148 nM for oxymetazoline, with a prazosin-to-oxymetazoline K_i ratio of 0.34. This pharmacological profile is characteristic of the B subtype of the α_2-adrenergic receptor (1) (Fig. 4).

Blotting analysis of mRNA isolated from rat tissues using the RBα_{2B}-derived probe showed a tissue-specific hybridization pattern (Fig. 5). In brain, the prominent mRNA species was 3.0 kb in size; minor mRNAs of 4.6 kb and 2.3 kb were also detected. In kidney, a major 4.6-kb mRNA species and two minor species of 3.6 kb and 3.0 kb were detected. In spleen, weakly hybridizing species of 4.6 kb and 2.0 kb were detected.

The multitude of the mRNA species along with the presence of a potential open reading frame on the complementary strand of RBα_{2B} prompted us to investigate whether the antisense strand is transcriptionally active. To test this possibility we used strand-specific RNA probes in Northern blot analysis (Fig. 6). In spleen, brain, and kidney the antisense probe detected transcripts of the same size as those detected by the double-stranded DNA probe. Hybridization with the sense probe detected at least two distinct mRNA species of 4.6 kb and 8.5 kb in all three tissues studied. However, this probe failed to detect the 3.0-kb transcript in brain.

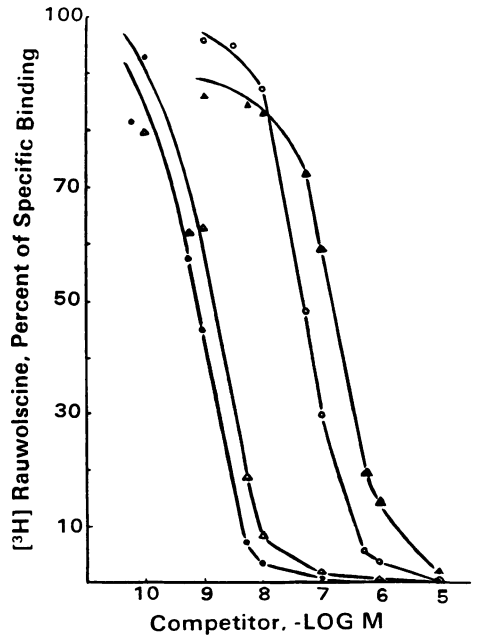

FIG. 4. Specific binding of [^{3}H]rauwolscine (0.27 nM) to membranes of COS-7 cells transfected with rat brain α_{2B}-adrenergic receptor clone RBα_{2B}. Binding was measured in the presence of various concentrations of rauwolscine (●), yohimbine (△), prazosin (○), and oxymetazoline (▲) spanning four orders of magnitude.

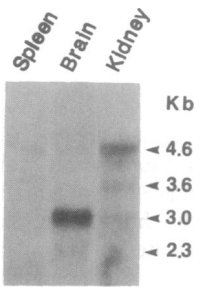

FIG. 5. Blotting analysis of mRNA isolated from rat tissues. Five micrograms of poly(A)$^+$ RNA from rat spleen, brain, and kidney was electrophoretically separated on a 1% agarose/3% formaldehyde gel, transferred to a nylon membrane, and hybridized to the rat brain α_{2B}-adrenergic receptor RBα_{2B} probe. The sizes of the different mRNA species are indicated.

DISCUSSION

Previous ligand-binding studies suggested that both α_2-adrenergic receptor subtypes, α_{2A} and α_{2B}, are present in the rat brain in roughly equal amounts (1). To understand the structural basis of α_2-adrenergic receptor heterogeneity in the rat brain, we have previously analyzed the transcripts of an α_2-adrenergic receptor gene(s) in rat tissues and identified two major mRNA species of 3.0 kb and 3.8 kb in the rat brain (9). In the present study we describe the molecular cloning and characterization of a rat brain cDNA, RBα_{2B}, encoding an α_{2B}-adrenergic receptor. The predicted amino acid sequence of RBα_{2B} indicates that it encodes a membrane protein of 458 amino acids that has the structural features of the guanine nucleotide-binding protein (G protein)-coupled receptors (23). Thus, the protein contains seven stretches of hydrophobic amino acids corresponding to putative membrane-spanning domains. It also contains two potential N-linked glycosylation sites near the amino terminus.

RNA blot analysis shows that RBα_{2B} detects the 3.0-kb mRNA in brain, whereas another clone, RBα_{2A}, characterized in our laboratory detects the 3.8-kb mRNA (C.S.F., D.E.H., & H.G., unpublished data) in rat brain. RBα_{2B} also detects two minor mRNA transcripts of 4.6 and 2.3 kb in brain. In rat kidney, one major (4.6-kb) and two minor (3.6- and 3.0-kb) transcripts are found. These multiple transcripts may represent products of homologous but distinct genes. Alternatively, they may originate from the same gene by the utilization of different transcription initiation or polyadenylylation sites.

The strand-specific Northern blot analysis shows that the strand of the GRα_{2B} gene complementary to the α_{2B} coding strand may be transcriptionally active. The biological func-

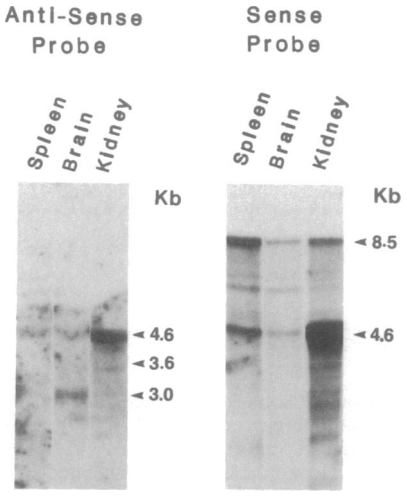

FIG. 6. Strand-specific Northern blot analysis. Five micrograms of poly(A)$^+$ RNA from rat spleen, brain, and kidney was electrophoretically separated on a 1% agarose/3% formaldehyde gel and transferred to a nylon membrane. Single-stranded probes were prepared from either strand of a *Nae* I/*Stu* I fragment of RBα_{2B} (see *Materials and Methods*) and hybridized to the blotted RNA. The sizes of the transcripts are indicated.

tion of the antisense transcripts is unknown. Overlapping transcripts from complementary strands of particular gene loci have previously been found in a few eukaryotic systems (24–27).

Ligand-binding studies of the expressed RBα_{2B} displayed saturable specific binding with high affinity for [^{3}H]rauwolscine as well as pharmacological properties of the α_{2B} subtype, with a prazosin-to-oxymetazoline K_i ratio of 0.34. This ratio is similar to those obtained by Lomasney *et al.* (8) with α_2-C4 (0.5) and α_2-C2 (0.2), both of which possess subtype B-like properties. On the other hand, it differs from the ratio of 169 obtained with α_2-C10, the α_{2B} subtype, or the ratio of 0.05 obtained with cells transfected with RNGα_2 (6), which encodes a nonglycosylated α_{2B} protein.

The derived protein sequence of RBα_{2B} has 87% amino acid homology to the human kidney α_2-C4 protein. This sequence homology and the pharmacological profile of the protein encoded by the RBα_{2B} clone suggest that our clone represents the rat homolog of the human α_{2B} receptor α_2-C4. In contrast, the derived protein sequence of RBα_{2B} is only 55% homologous to the rat kidney nonglycosylated protein (RNGα_2). Nevertheless, these structurally distinct rat proteins are pharmacologically similar, showing α_{2B}-adrenergic receptor binding properties.

The RBα_{2B} and rat kidney nonglycosylated α_2 receptor proteins share 77% homology in their transmembrane domains. These findings suggest that the homology within the transmembrane regions of the proteins encoded by the RBα_{2B}, α_2-C4, and RNGα_2 clones may be sufficient to confer the α_{2B} subtype specificity. It is also possible that, despite the similarity in their ligand-binding properties, these receptors have essential functional differences that cannot be discerned by pharmacological studies.

The third cytoplasmic loop contains the most divergent sequences among different adrenergic receptors (4–8). This region has been found to be important for coupling to G protein (28–32) in the β_2-adrenergic receptor. Thus, sequence variation of this region may permit receptors with similar ligand-binding properties, such as the rat brain α_{2B} receptor described in this study and the rat kidney nonglycosylated α_{2B} receptor (6), to exert diverse functions.

The sequence variability between the human kidney (α_2-C4) and the rat brain (RBα_{2B}) proteins is localized to the middle of the third cytoplasmic loop. Previous deletion analysis of β_2-adrenergic receptors has shown that amino acid residues within conserved amino- and carboxyl-terminal regions of the third cytoplasmic loop are necessary for the effective coupling of those receptors to G_s protein and the subsequent stimulation of adenylate cyclase (29–31). The high homology between the rat brain RBα_{2B} and the human kidney α_2-C4 within the amino-terminal and carboxyl-terminal domains of the third cytoplasmic loop implies a similar critical involvement of these sequences for the coupling of these receptors to the G protein.

In summary, the present study describes the structural and functional properties of an α_{2B}-adrenergic receptor that has similar pharmacological binding properties but is structurally divergent from the rat kidney nonglycosylated α_{2B} subtypes. This suggests that diverse intracellular portions of the α_{2B}-adrenergic receptors could mediate different cellular functions.

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