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Solution-Phase Library Screening for the Identification of Rare Clones: Isolation of an α_{1D}-Adrenergic Receptor cDNA

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SUMMARY

α_1-Adrenergic receptor (α_1-AR) subtypes (α_{1A} and α_{1B}) play a critical role in vascular smooth muscle contraction and circulatory homeostasis. Transcripts for these guanine nucleotide-binding protein-coupled receptors are extremely low in abundance, however, and isolation of their cDNAs is difficult. We have developed a novel technique for identifying rare clones in a cDNA library, which has been used successfully to isolate a cDNA clone encoding an α_{1D}-AR. A 564-bp polymerase chain reaction product encoding a region between the third and sixth transmembrane domains of the α_{1D}-AR was first generated using rat brain mRNA as template and highly degenerate primers. The primers corresponded to those domains but contained mismatches to the α_{1B}-AR sequences. A 3-kb transcript was identified with this polymerase chain reaction probe, by Northern analysis of rat hippocampus. However, traditional plaque hybridization failed to identify a cDNA in a rat hippocampus λgt10 library. By solution-phase screening of virtually the entire library, a cDNA containing a 3-kb insert was identified, amplified, and purified. This insert encodes a 560-amino acid protein corresponding to the topology of guanine nucleotide-binding protein-coupled receptors. This receptor has approximately 71% amino acid identity, in the transmembrane regions, to the hamster and rat α_{1B}-ARs. Characterization of the receptor expressed in COS-7 cells, by ligand binding and photoaffinity labeling, revealed some of the characteristics of an α_{1A}-AR. However, unlike α_{1A}-ARs characterized previously in membrane preparations or in solubilized partially purified preparations, the expressed receptor could be extensively inactivated by chlorethylclonidine. In addition, it displays ligand-binding properties that are not consistent with an α_{1A}-AR. This indicates that the cDNA clone that we have isolated encodes a novel α_1-AR subtype, which we classify as the α_{1D}-AR.

The catecholamines epinephrine and norepinephrine mediate their physiological effects by selectively binding and activating different subtypes of integral membrane proteins called ARs (1). These subtypes, as determined by their different pharmacological specificities, physiological effects, and primary structures deduced from molecular cloning studies, are classified as α_1, α_2, β_1, β_2, and β_3 (2). ARs belong to an ever-growing family of G protein-coupled receptors that share in common the structural features of bacteriorhodopsin (3). These features are a single polypeptide chain consisting of seven putative transmembrane domains, interspersed with hydrophilic intra- and extracellular loops, an extracellular amino terminus, and a cytoplasmic carboxyl terminus.

α_1-AR subtypes play a critical role in sympathetic nerve transmission. Two major subtypes (α_{1A} and α_{1B}) have been identified by pharmacological studies (4, 5). The α_{1A} subtype has higher affinity than the α_{1B} subtype for the antagonists WB4101, 5′-methylurapidil, (+)-niguldipine, and phentolamine and for the agonists oxymetazoline and phenylephrine (4–10). Moreover, an alkylating analog of clonidine, CEC, irreversibly inactivates the α_{1B}-AR but not the α_{1A}-AR (5, 10). This has been used as a criterion for differentiating between the α_{1A}- and α_{1B}-AR in various tissues (5, 10). In contrast to CEC, an alkylating analog of prazosin, SZL-49, has been shown to completely inactivate the α_{1A}-AR, while causing only a partial inactivation of the α_{1B}-AR (11–13).

cDNAs for the hamster (14) and rat α_{1B}-AR (15) and for a third subtype (α_{1C}) not previously characterized pharmacologically (16) have been isolated. We now report the cloning of a new and as yet undefined AR. This receptor is encoded by a rare transcript. Isolation of a cDNA for this protein required the development of a novel technique that allows rare clones

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ABBREVIATIONS: AR, adrenergic receptor; HBSS, Hanks' balanced salt solution; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CEC, chlorethylclonidine; bp, base pairs; G protein, guanine nucleotide-binding regulatory protein.

in a cDNA library to be identified, amplified, and purified. While our work on this project was near completion, Lomasney *et al.* (27) reported the isolation of a cDNA that encodes a putative α_{1A}-AR. Our cDNA and that of Lomasney *et al.* (27) are virtually identical, except for two codons. Based on extensive pharmacological characterization, we have demonstrated that our receptor is not the α_{1A}-AR but a novel subtype, which we refer to as an α_{1D}-AR. Potential reasons for the discrepancies between our conclusions and those of Lomasney *et al.* (27) are discussed.

Materials and Methods

PCR cloning. Highly degenerate oligonucleotide primers [primer A, 5′-GAATTCGAATTCGAATTCTGGGC(C/T)GCIGTIGA(T/C)G-T(G/A)CTITGTGCAC(A/C)GC(A/T)TCIAT(T/A)CT-3′; primer B,-5′-GAATTCGAATTCGAATTCAA(A/G)AA(T/C/A)GGIAGCC-AGCA(T/C)AA(T/A)AT(G/A)AACAT(A/C/G)CCIACIACGAT-3′], corresponding to the third and sixth transmembrane domains of the hamster α_{1B} or bovine α_{1C}, were synthesized on an Cyclone (Milligen) DNA synthesizer and purified on 15% denaturing polyacrylamide gels. These primers contained mismatches to the hamster α_{1B}-AR DNA sequence (indicated by dots) and are 83–88% identical to the hamster α_{1B}-AR or bovine α_{1C}-AR sequences, 73% identical to α_2-ARs, and 71% identical to β_2-ARs. The primers were designed with three *Eco*RI restriction sites at the 5′ ends to facilitate subcloning. Single-stranded cDNA was prepared using 1 μg of poly(A)$^+$ mRNA from rat brain that was incubated with deoxynucleotide triphosphates (1 mM of each base), hexameric primers (100 pmol), 50 units of RNasin, 10 mM, dithiothreitol, 50 mM Tris·HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 200 units of Moloney murine leukemia virus reverse transcriptase, in a reaction volume of 20 μl, for 2 hr at 42°. PCR was then performed on one half of the samples, with 40 pmol of each primer (A and B) and 10 mM Tris·HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, deoxynucleotide triphosphates (200 μM of each base), and 2.5 units of *Thermus aquaticus* DNA polymerase. The amplification profile, run for 35 cycles, consisted of 1 min at 95°, 2 min at 45°, and 3 min at 73°, followed by a 15-min extension at 73°. The resulting DNA (564-bp fragment) was separated and isolated using a GTG-agarose low melting point gel (FMC, Rockland, ME), treated with reverse transcriptase, digested with *Eco*RI endonuclease, and cloned into M13. Clones that hybridized under low stringency (30% formamide, 37°) to a random-primed 300-bp fragment, corresponding to a region from the fourth transmembrane region to the middle of the 5–6 loop of the human α_{1B}-AR that we had previously isolated, were identified on plaque lifts and used to prepare single-stranded template for sequencing by the dideoxy chain-termination procedure (Sequenase; United States Biochemical Co., Cleveland, OH).

cDNA library screening. A random-primed rat hippocampus λgt10 library (1.5 × 10^6 recombinants; Clontech, Palo Alto, CA) was screened with the 564-bp PCR fragment labeled with ^{32}P by random priming. A total of 1.8 × 10^6 total plaques were screened, using duplicate nitrocellulose filters, and hybridized in 50% formamide, 5× SSC, 50 mM sodium phosphate, pH 7.0, 2× Denhardt's, 50 μg/ml single-stranded DNA, 0.1% SDS, for 24 hr at 42°. Filters were washed twice at room temperature for 5 min in 2× SSC, 0.1% SDS, and then in 0.1× SSC, 0.1% SDS, at 42° for 15 min, and were exposed at −70° to Kodak X-OMAT film, using Cronex Lighting Plus intensifying screens.

Solution-phase library screening. Nine 1-μl aliquots of the rat hippocampus λgt10 library were each used to infect C600HfL cells (multiplicity of infection, 10^{-2}), in a total volume of 2 ml. After overnight incubation at 37°, one drop of chloroform was added to lyse any remaining cells. The cultures were centrifuged, and the supernatant fraction (phage stock) was kept for future analysis. One milliliter of each culture was used to prepare λ DNA by first addition of 27 units of RNase-free DNase I and then incubation for 20 min at 37°. Three hundred microliters of a 20% polyethylene glycol/2 M NaCl solution were added to each supernatant fraction and incubated on ice for 30 min. After centrifugation for 10 min, the supernatant fraction was removed and a large phage pellet was visible. The pellet was resuspended in 300 μl of TE (10 mM Tris, 1 mM EDTA, pH 8.0) and extracted with phenol/chloroform several times, followed by a final chloroform extraction. Sodium acetate was added to a final concentration of 0.3 M, and then 2 volumes of 100% ethanol were added to precipitate the DNA. The pellet was dried and digested, in a final volume of 17 μl, for at least 1 hr with *Eco*RI endonuclease, to release the inserts. Three microliters (1 × 10^5 cpm total) of the 564-bp PCR fragment, labeled by random priming, were added directly to the digestion, and the salt concentration was adjusted to 3× SSC. The mixture was boiled for 3–5 min and transferred immediately to a 55° water bath for 15 min. Loading buffer was added, and the samples were analyzed on a 4% nondenaturing polyacrylamide minigel and autoradiographed directly. Cultures found to be positive, i.e., containing inserts that hybridized to the 564-bp PCR fragment, were further subdivided into nine 1-μl aliquots and were reamplified and screened as before. A culture that was positive after this second round of screening was again amplified and screened. After a third round of amplification and screening, the strongest positive culture was plated and screened by traditional plaque hybridization.

Transient expression in COS-7 cells. To facilitate subcloning of the cDNA into an expression vector, PCR primers (primer A′, 5′-GAATTCGAATTCCCTCCACTTGCTCGCCCTGTGC-3′; primer B′, 5′-GCGGCCGCGCGGCCGCTTCCCCAGCACCTTCCCCAGCCT-GAGT-3′), corresponding to the 5′ and 3′ untranslated regions of the receptor cDNA, were synthesized and used to generate a PCR product that encoded the restriction sites for *Eco*RI at the 5′ end and *Not*I at the 3′ end. This PCR product was ligated into the *Eco*RI/*Not*I sites of the modified eukaryotic expression vector pMT2′ (17). The resulting construct contained 53 bp of 5′ untranslated region, the entire 1680 bp of coding region, and 39 bp of 3′ untranslated region. The construct was transfected into COS-7 cells by the DEAE-dextran method (18). A cDNA encoding the hamster α_{1B}-AR in the plasmid pSP65 (19) was digested with *Eco*RI, and the 2.1-kb fragment containing 14 bp of 5′ untranslated region, the entire 1545 bp of coding region, and 555 bp of 3′ translated region was inserted into the *Eco*RI site of the pMT2′ expression vector. The correct orientation of the plasmid was confirmed by nucleotide sequencing. The resulting construct was also transfected into COS-7 cells.

Ligand binding. COS-7 cell membranes were prepared by washing culture plates twice with warm HBSS. One milliliter of HBSS was added, and the plates were scraped and transferred to a 50-ml centrifuge tube. The plates were rescraped with cold HBSS and the cells were pooled. The intact cells were centrifuged at 1000 × *g* in a Sorvall RT6000B rotor for 5 min, and the pellet was resuspended in 5 ml of 0.25 M sucrose. The cell suspension was centrifuged again at 1000 × *g* for 5 min, and the pellet was resuspended in 10 ml of 0.25 M sucrose containing the following protease inhibitors: 20 μg/ml aprotinin, 20 μg/ml leupeptin, 20 μg/ml bacitracin, 20 μg/ml benzamidine, and 17 μg/ml phenylmethylsulfonylfluoride. The cells were disrupted by N_2 cavitation and then homogenized in a Dounce homogenizer by 10 strokes with a loose fitting (B) pestle. The mixture was then centrifuged at 1260 × *g* for 5 min. Buffer containing 50 mM Tris, pH 7.4, 12.5 mM $MgCl_2$, and 5 mM EGTA was added to the supernatant fraction, which was then centrifuged at 30,000 × *g* for 15 min. The resulting pellet was resuspended in 50 ml of buffer and recentrifuged for 15 min. The resulting pellet was suspended in 1 ml of buffer containing 10% glycerol. The protein concentration was measured using the method of Bradford (20).

The ligand-binding characteristics of the cloned expressed receptor, which we initially refer to as rat α_x, were determined in a series of radioligand binding studies using [^{3}H]prazosin as a receptor probe and were compared with those observed with the expressed hamster α_{1B}-AR. Binding reactions (total volume 0.5 ml) contained 20 mM HEPES, pH 7.5, 1.5 mM EGTA, 12.5 mM $MgCl_2$, 0.8 nM [^{3}H]prazosin, COS-7

cell membranes, and increasing amounts of unlabeled ligands known to interact with the ARs. Nonspecific binding was determined in the presence of 100 μM phentolamine. Reactions were allowed to proceed for 1 hr at room temperature. Reactions were stopped by the addition of ice-cold HEPES buffer and were filtered onto Whatman GF/C glass fiber filters with a Brandel cell harvester. Filters were washed five times with HEPES buffer, and bound radioactivity was determined by liquid scintillation counting. Binding data were analyzed by the iterative curve-fitting program LIGAND.

Inactivation by chemically reactive receptor probes. After initial receptor characterization, the ability of CEC and SZL-49 to inactivate the α_1-AR was assessed. Two inactivation protocols (400-μl volume) were carried out. In one protocol, aliquots of either the α_{1X} or the α_{1B} receptors were incubated with various concentrations of CEC or SZL-49, in the HEPES buffer, for 10 min at 37°. In the other protocol, the COS cell membranes were incubated with the inactivating ligand for 24 hr at 4°. After the incubation period, the reactions were diluted by the addition of 1.5 ml of ice-cold HEPES, and the membranes were centrifuged in a desk-top microfuge. Washing and resuspension were repeated three additional times. To control for noncovalently bound CEC and SZL-49 that may not have been removed completely from the membranes, paired reactions were run in which the ligand was added to the native membranes after the incubation period but immediately before the washing steps.

Photoaffinity labeling. Photoaffinity labeling was performed as described previously (21), using the photoaffinity probe [^{125}I]azidoprazosin [2-[4-(4-azido-3-[^{125}I]iodobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline], prepared from the corresponding aryl amine analog of prazosin, ^{125}I-CP 63 789, as previously described (22).

Materials. Drugs were obtained from the following manufacturers: WB4101, 5-methylurapidil, and CEC, Research Biochemicals Inc. (Natick, MA); (−)-epinephrine, (−)-norepinephrine, and (−)-propranolol, Sigma; phentolamine, CIBA-Geigy; rauwolscine, Roth (Germany); prazosin, Pfizer (CT); and [^{3}H]prazosin, New England Nuclear (Boston, MA). (+)-Niguldipine was generously provided by Byk Gulden (Germany). SZL-49 was synthesized by the method of Pitha *et al.* (23).

Results and Discussion

A method based on the application of the PCR was used to identify an unique α_1-AR. Similar methods have been used previously to clone members of the G protein (24) and G protein-coupled receptor families (25). Oligonucleotide primers used for PCR were synthesized based on the homology among ARs. Regions of the third and sixth transmembrane domains of various receptors were defined, and primers were designed to opposite strands of the target sequences, to allow amplification of the region between the corresponding domains. The 64–144-fold degenerate primers each contained several inosines and, importantly, contained mismatches at the wobble positions of the hamster α_{1B}-AR or bovine α_{1C}-AR sequences. The primers were 83–88% homologous to the α_{1B}- or α_{1C}-ARs, 73% to α_2-ARs, and 71% to β_2-ARs. Mismatching of the primers was crucial to preventing saturation of the PCR product with the α_{1B}-AR sequence. Using these primers, a 564-bp fragment was amplified from rat brain single-stranded cDNA, which upon subcloning into M13 hybridized to a probe made from the fourth transmembrane region to the middle of the 5–6 loop of the human α_{1B}-AR. Sequencing of individual clones indicated that the unique α_1-AR was present, on average, in 1/20th of the recombinant pool, with the remaining clones encoding the α_{1B}-AR.

The PCR product encoding the unique α_1-AR was then used to probe a rat hippocampus λgt10 cDNA library. Traditional plaque hybridization of a total of 1.8×10^6 recombinant plaques failed to identify a cDNA in the library, even though the PCR product identified a 3-kb transcript by Northern blot analysis of rat hippocampus mRNA (data not shown). The presence of at least one copy of the cDNA in the library was verified by performing a Southern blot analysis on 10 μl of the library (data not shown). Alternatively, one could have performed PCR analysis on aliquots of the library. The extremely low abundance of the cDNA in the library prompted us to develop the solution-phase library screening method.

The methodology of solution-phase library screening is analogous to performing a Southern blot analysis on the library, but the key feature is diluting and reamplifying positive cultures. On initial screening, only one 1-μl aliquot of nine was positive for the unique α_1-AR (Fig. 1A). The insert from this culture was approximately 3 kb, similar to the size of the mRNA transcript. Because 1 μl of the library represents 10^7 recombinants, our receptor was present in only one of 9×10^7 recombinants. Theoretically, isolation of this cDNA would require the equivalent of approximately 2250 Petri dishes (150 mm) to be screened by traditional plaque hybridization. The positive culture (Fig. 1B, culture 2) was subsequently divided into 1-μl aliquots, reamplified, and screened. This round produced four positive cultures, with one culture being predominant. Culture 5 (Fig. 1B) was further divided into 1-μl aliquots, reamplified, and screened (Fig. 1C). After this third round of amplification, all cultures were positives. Traditional plaque hybridization screening of culture 5 (Fig. 1C) (300,000 recombinants) produced one positive clone that upon sequencing encoded the unique α_x-AR. Solution-phase library screening enhanced the concentration of the cDNA approximately 300-fold (3×10^5 versus 9×10^7 recombinants). Subsequently, culture 5 (Fig. 1C) was diluted 1/100, and 1-μl aliquots were reamplified and screened (data not shown). All cultures were positive. The strongest positive culture was further diluted 1/1000, and 1-μl

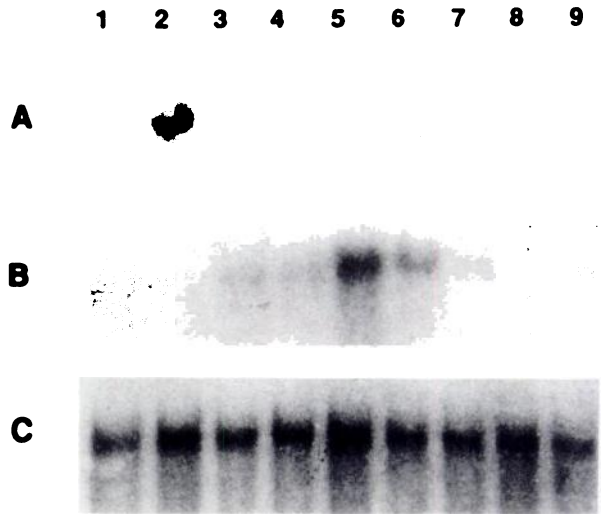

Fig. 1. Solution-phase library screening. On the initial screening, nine 1-μl aliquots of the rat hippocampus λgt10 library were amplified and screened using the 564-bp PCR product, as described in the text. One culture (no. 2) of nine was positive (*row A*). Positive culture 2 (*row A*) was divided into 1-μl aliquots, reamplified, and screened. This produced four positive cultures (*row B*, cultures 3–6). Positive culture 5 (*row B*) was divided into 1-μl aliquots, reamplified, and screened. This produced cultures that were all positive (*row C*). Culture 5 (*row C*) was screened by traditional plaque hybridization and allowed a cDNA clone for rat α_x to be isolated, as detailed in the text.

aliquots were reamplified and screened. Traditional plaque hybridization screening from these cultures produced 15 positive clones from 300,000 recombinants. The unexpected low number of positive clones from the highly diluted cultures is probably due to the unbalanced amplification of phage in a library. This is a common occurrence and is the basis for the practice of not reamplifying libraries, thus preventing the loss of rare clones. We have observed a loss in the signal intensity when highly diluted cultures are reamplified and we suggest that, to concentrate clones, only 10–100-fold dilutions of the cultures be reamplified and screened. This technique can be applied potentially to isolate any rare clone or to provide a rapid alternative to large scale screening. If only one cDNA clone is present in the library, screening of enough large aliquots of the library should allow a hybridizing insert, corresponding approximately to the size of the message, to be identified and will provide a stock in which the clone can be concentrated by subsequent dilution, reamplification, and screening.

The cDNA clone of the rat α_x-AR obtained by solution-phase library screening contains a 1680-bp open reading frame encoding a protein of 560 amino acids. Hydropathy analysis (26) of the protein is consistent with a putative topography of seven transmembrane domains, indicative of the G protein-coupled receptor family. Comparison of the rat α_x deduced amino acid sequence with known α_1-ARs and other members of the AR family (Fig. 2A) shows the largest concentration of identical amino acids to be in the putative transmembrane domains. In these transmembrane regions, the percentage of identity for the α_x-AR is 71.3%, compared with the hamster α_{1B}-AR, which is typical among subtypes, 64% for the bovine α_{1C}-AR, 40.4% for the rat β_1-AR, and 29.8% for the rat M_1 acetylcholine receptor. When considering the full length proteins, the percentage of identity drops and is only 41% for the hamster α_{1B}-AR, 42% for the bovine α_{1C}-AR, 26% for the rat β_1-AR, and 16% for the rat M_1 receptor. Both the alignment and percentage of identity of the α_x amino acid sequence, relative to other members of the AR family, strongly suggest that this new receptor is a member of the α_1 subtype. Other features of the new rat α_{1X} receptor are the presence of two potential sites for *N*-linked glycosylation in the amino terminus (asparagine residues 60 and 76) (Fig. 2A) and the presence of several serines and threonines in the carboxyl terminus and intracellular loops, which may serve as sites for potential phosphorylation by protein kinase C but not by protein kinase A. While this work was in progress, Lomasney *et al.* (27) reported the isolation of a cDNA encoding the putative rat α_{1A}-AR. The DNA sequence of our receptor is identical to theirs, except for two codons (Fig. 2B). Although this results in only two amino acid differences, these changes may be functionally significant, because residue 37 in the amino terminus of α_{1X} is a proline rather than a glycine and residue 306 in the 5–6 loop is an arginine rather than the alanine noted by Lomasney *et al.* (27).

To examine the pharmacological and biochemical characteristics of the rat α_{1X} receptor, a PCR fragment was generated, containing the entire coding region, and inserted into the mammalian expression vector pMT2′. The resulting construct, pMT2α_{1X}, as well as the construct for the hamster α_{1B}-AR, pMT2α_{1B}, were used to transfect transiently COS-7 cells. The level of expression for pMT2α_{1X} was approximately 24 pmol/mg of protein, whereas that of the α_{1B} was 670 pmol/mg. Nontransfected COS-7 cells, or COS-7 cells transfected with a plasmid encoding the β_2-AR, showed no specific [^{3}H]prazosin binding.

Table 1 shows the K_i values determined for various AR ligands. Prazosin, a highly selective α_1-AR antagonist, exhibited high affinity for the expressed rat α_{1X}-AR, and the K_i determined for this ligand is similar to that reported for endogenous α_1-AR (4, 5), as well as that reported for the expressed putative α_{1A}-AR (27). Furthermore, (−)-propranolol, a nonselective β-AR blocker, interacted with low affinity (3 μM) at the α_{1X}-AR. Rauwolscine, a high affinity α_2-AR antagonist, also exhibited low affinity for the α_{1X}-AR. Finally, norepinephrine exhibited stereoselective binding, with the (−)-isomer being >10-fold more potent than the corresponding (+)-isomer.

These data indicate that the α_x-AR has the properties of an α_1-AR. It is less clear, however, whether the cDNA clone that we have isolated encodes the endogenous α_{1A}-AR, expressed in a variety of tissues, that has been characterized previously on the basis of pharmacological and biochemical studies. On the one hand, several characteristics of the α_{1X}-AR are in keeping with those of an α_{1A}-AR. These include its expression in the hippocampus, cerebral cortex, and vas deferens, as determined by Northern blot analysis (data not shown); its resistance to photoaffinity labeling with [^{125}I]azidoprazosin (Fig. 3), which we have noted previously with solubilized and partially purified α_{1A}-AR preparations (32); its ability to be more completely inactivated by SZL-49 than the α_{1B}-AR (Table 2), a finding that we have reported previously from both *in vitro* and *in vivo* studies (6, 11); and its higher affinity for both WB4101 and 5-methylurapidil than that of the expressed hamster α_{1B}-AR. On the other hand, several features of the α_{1X}-AR are not consistent with those of an α_{1A} subtype. For example, the affinities of the α_{1X}-AR for WB4101 and 5-methylurapidil (1.8 and 14 nM, respectively), although higher than those of the hamster α_{1B}-AR, are nonetheless lower than those of the endogenously expressed α_{1A}-AR. Thus, in several different reports (6, 8, 32–34), the affinity of the α_{1A}-AR for WB4101 has been estimated to be approximately 0.2 nM and for 5-methylurapidil, 0.6 nM. Furthermore, the differences in the affinities of the α_{1X}-AR and hamster α_{1B}-AR for these ligands are small (3-fold) and much less than the 10–100-fold difference reported for the endogenously expressed α_{1A}- and α_{1B}-ARs (6, 8, 32–34). The α_{1A}-AR has also been shown previously to exhibit higher affinity for phentolamine, compared with the α_{1B}-AR (1 nM versus 65 nM, respectively) (4, 32, 34). We show no such difference in affinities of the α_{1X}-AR and hamster α_{1B}-AR for this ligand. Indeed, if one compares the affinity for phentolamine reported by Lomasney *et al.* (27) for their rat receptor with that originally reported for the hamster α_{1B}-AR by Schwinn *et al.* (16), no differences are noted. Finally, the affinity (46 nM) of the α_{1X}-AR for the α_{1A}-selective ligand (+)-niguldipine is >750-fold less (Table 3) than that of the endogenously expressed α_{1A}-AR (0.06 nM). The lack of agreement between the pharmacology for our cloned α_{1X}-AR and that determined in numerous ligand binding studies done on tissue homogenates suggests two possibilities. First, the properties of the cloned α_{1A}-AR when overexpressed in a model cell system differ from its properties when prepared from tissues and organs. This is unlikely, however, because other receptors such as the hamster α_{1B}-AR retain their native ligand-binding activity when overexpressed (compare the K_i values shown in Table 1 for the expressed hamster α_{1B}-AR with the literature K_i values for this subtype shown in

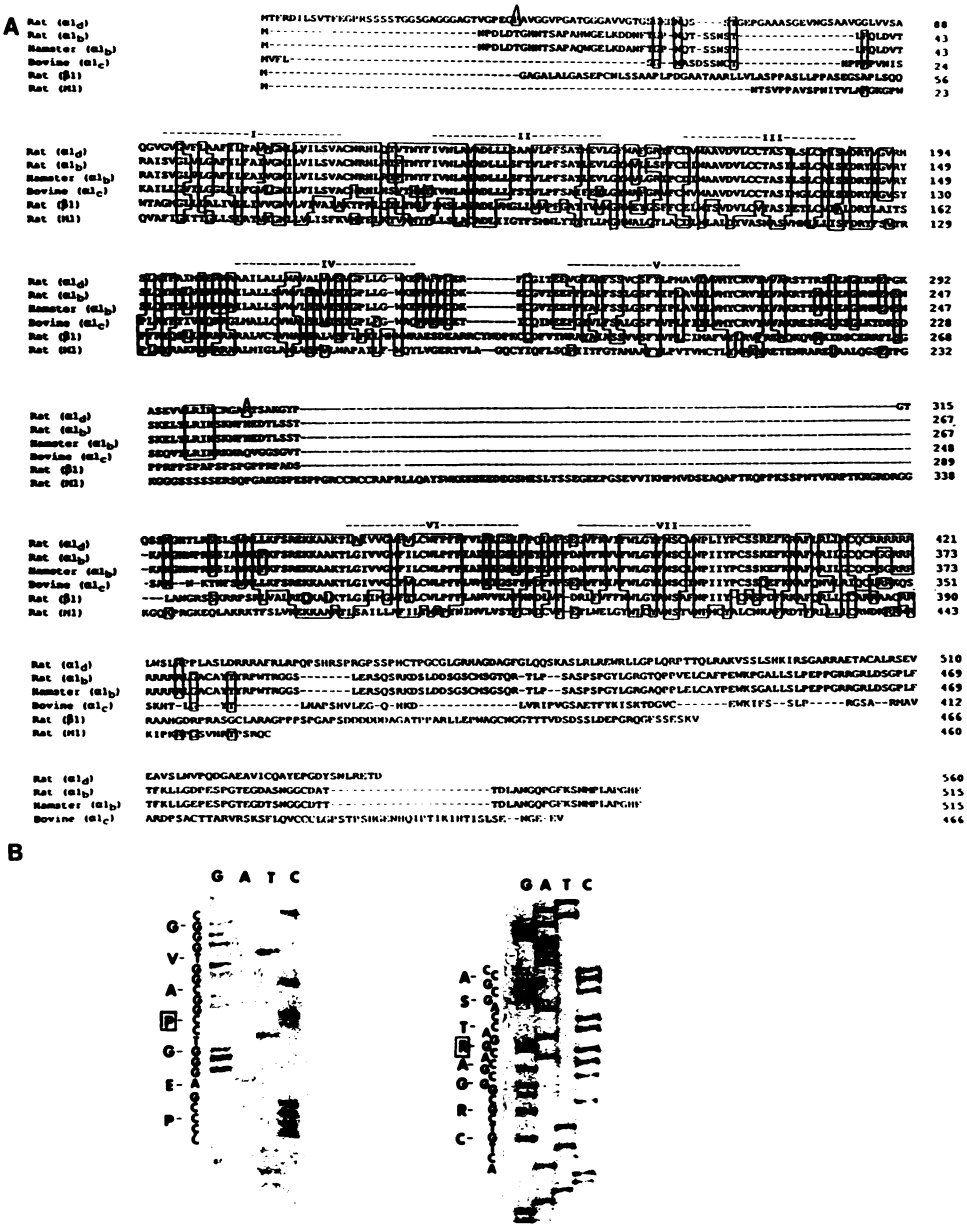

Fig. 2. A, Comparison of the rat α_{1D}-AR primary structure with other members of the G protein-coupled receptor family. B, Nucleotide sequences of the codons for the amino acids that differ between the α_{1D}-AR and the receptor published recently by Lomasney *et al.* (27). A, The amino acid sequence of the rat α_{1D}-AR is compared with that of the rat α_{1B}-AR (15), hamster α_{1B}-AR (14), bovine α_{1C}-AR (16), rat β_1-AR (28), and rat M_1 muscarinic receptor (29). Aligned residues identical in at least three different receptors are *boxed*. The concensus sites for *N*-linked glycosylation are indicated by *wavy lines*. Amino acid differences between the α_{1D}-AR and the α_{1A}-AR recently reported by Lomasney *et al.* (27) are indicated by *triangles*. B, Nucleotide sequence of the codon differences between the α_{1D}-AR and the α_{1A}-AR reported by Lomasney *et al.* (27). In the amino terminus of the α_{1D}-AR, residue 37 is a proline (*boxed*), indicated by the codon CCG, rather than a glycine, and, in the 5–6 loop, residue 306 is an arginine (*boxed*), indicated by the codon CGG, rather than the alanine noted by Lomasney *et al.* (27). The entire α_{1D}-AR was sequenced in both directions to verify the primary sequence.

Table 3). Alternatively, the α_{1X}-AR may differ from the endogenously expressed α_{1A}-AR that has been extensively characterized previously (Table 3) and may be more akin to the atypical CEC-sensitive α_1-AR characterized in rat aorta (30, 31).

Data from our studies with CEC also argue against the fact that the receptor we have cloned is the α_{1A}-AR. One of the criteria used to define the α_{1A} subtype is the fact that, either in tissue experiments or with solubilization and partial purification (32), it is insensitive to CEC. We show in two different incubation protocols (10 min at 37° or 24 hr at 4°) (Table 2) that the α_{1X}-AR is extensively inactivated by CEC, although it is less sensitive to inactivation than the hamster α_{1B}-AR. One caveat to these studies is the fact that the α_{1B}-AR is expressed more abundantly in the COS-7 membranes than is the α_{1X}-AR. Thus, the membrane protein concentration in the CEC experiments was greater for the studies with the α_{1X}-AR than the α_{1B}-AR, which may have reduced the free concentration of CEC available to irreversibly inactivate the α_{1X}-AR. To control for this possibility, additional studies were performed with CEC, in which mock-transfected COS-7 membranes were added to α_{1B}-AR preparations to equal the protein concentration in the CEC studies with the α_{1X}-AR. However, in these studies the sensitivity of α_{1B}-AR to CEC was unchanged (data not shown).

Based on these properties, as shown in Table 3, in comparison with previously characterized α-AR subtypes it is apparent that the α_1-AR encoded by the cDNA clone that we have isolated is not the α_{1A}-AR but a novel (α_{1D}) subtype.

A controversy obviously exists between our work and conclusions and those of Lomasney *et al.* (27). First, there is the issue of the minor difference in sequence. We have sequenced the entire clone twice and the regions containing the codons in question an additional time. Indeed, we have included our sequencing gels of this region in Fig. 2B. Therefore, we feel confident that we have correctly sequenced our cDNA. The fact that the cDNA are 99.8% identical in nucleotide sequence indicates that they are the same clone. The possible sequencing errors could have easily been made by overlooking a compressed

TABLE 1

Pharmacological characterization of expressed rat α_{1D}-AR and hamster α_{1B}-AR

COS-7 cell membranes transfected with the pMT2′ expression vector containing either the rat α_{1D} or hamster α_{1B} cDNA were incubated with the α_1-AR antagonist [^{3}H]prazosin, in the absence or presence of increasing concentrations of various agonists or antagonists. Each point represents the mean of at least two individual experiments, in duplicate. Ten concentrations of each ligand were tested, and the points were chosen to be on the linear portion of the displacement curve. K_i values were generated using the iterative curve-fitting program LIGAND.

	K_i	
	Rat α_{1D}	Hamster α_{1B}
	nM	
Agonists		
(−)-Norepinephrine	320	1,210
(+)-Norepinephrine	4,300	>10,000
(−)-Epinephrine	239	230
Antagonists		
CEC[a]	35,000	40,000
Prazosin	0.32	0.25
Phentolamine	138	82
WB 4101	1.9	5.9
5-Methylurapidil	15	41
(+)-Niguldipine[b]	46	8
SZL-49[a]	3	1.5
Rauwolsine	1,060	1,190
Propranolol	3,400	>10,000

[a] Values for these ligands represent the concentration necessary to inhibit the binding of [^{3}H]prazosin by 50%.

[b] In separate studies with this hydrophobic ligand, the affinity of the hamster α_{1B}-AR remained unchanged when the protein concentration in the binding assay was increased to that used for the α_{1D} binding studies.

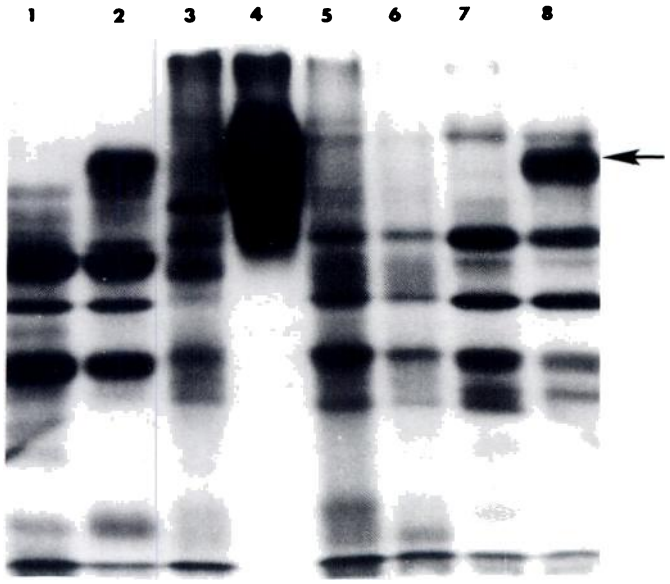

Fig. 3. Photoaffinity labeling of membranes prepared from rat liver, rat brain, or transfected COS-7. Rat liver or brain membranes (120 μg of protein) and COS-7 cell membranes transfected with either the hamster α_{1B}-AR or the rat α_{1D}-AR (40 fmol) were incubated with [^{125}I]azidoprazosin, in the absence or presence of 100 μM phentolamine, for 1 hr at 25° in the dark. Photolysis, SDS-polyacrylamide gel electrophoresis, and autoradiography were done as described in the text. A labeled M_r ~80,000 protein (*arrow*) is seen in rat liver (*lane 2*), brain (*lane 8*), and COS-7 cells (*lane 4*) transfected with the α_{1B}-AR. This labeling is blocked by phentolamine (*lanes 1, 3,* and *7*). An M_r of 80,000 for the α_{1B}-AR, which is higher than predicted for the protein backbone, indicates that the overexpressed COS-7 receptor, as well as the receptor in rat liver or brain, is most likely glycosylated, as noted previously (37). No specific labeling of the α_{1D}-AR (*lane 5*, buffer; *lane 6*, phentolamine) with [^{125}I] azidoprazosin is apparent.

TABLE 2

Inactivation of the hamster α_{1B}-AR and the rat α_{1D}-AR by the alkylating agents CEC and SZL-49

COS-7 cell membranes transfected with either the hamster α_{1B} or rat α_{1D} cDNA were incubated with the indicated concentrations of CEC or SZL-49, for 10 min at 37° or 24 hr at 4°. The membranes were washed extensively, and then the level of receptor inactivation was quantitated as described in the text.

Agent/protocol	Inactivation	
	α_{1B}	α_{1D}
	%	
CEC		
10 min, 37°		
3 μM	45	22
100 μM	98	72
24 hr, 4°		
3 μM	90	45
100 μM	95	74
SZL-49		
10 min, 37°		
1 nM	0	57
10 nM	0	72
100 nM	34	70
24 hr, 4°		
1 nM	0	58
10 nM	45	62
100 nM	70	83

GG doublet and by transpositional errors. Second, there is the issue that we and Lomasney *et al.* (27) have virtually identical cDNAs and yet conclude that we have different receptors. We have already discussed our reasons for concluding that our receptor cannot be α_{1A}. This conclusion is based on sound pharmacological and biochemical principles. Indeed, if one closely compares our data with those reported by Lomasney *et al.* (27), it can be seen that our binding data agree quite well with theirs. Furthermore, they show that their receptor exhibits lower affinity for oxymetazoline than does the rat α_{1B}-AR. In pharmacological test systems, oxymetazoline exhibits higher affinity for the α_{1A}- than α_{1B}-AR. Therefore, their oxymetazoline data also do not agree with α_{1A} pharmacology. Finally, there is the issue of CEC (and SZL-49) sensitivity. Lomasney *et al.* (27) incubated their receptor with 10 μM CEC for 10 min at 37° and showed only 15% inactivation. It was concluded that the receptor encoded by their clone was insensitive to CEC. We show that CEC sensitivity of the α_{1D}-AR is a matter of concentration and time of exposure (see Table 2) and that α_{1D}-AR is highly sensitive to CEC. In summary, we and Lomasney *et al.* (27) have isolated virtually identical cDNAs. Our disagreement comes in the conclusions regarding the nature of the receptor encoded by the cDNA. We believe that our data clearly show that the receptor does not have the pharmacological properties of an α_{1A}-AR but rather represents a novel α_{1D}-AR.

The present study demonstrates the utility of solution-phase library screening to rapidly identify, amplify, and purify rare clones in a library. If only a single copy of a cDNA is present in a library, screening of large aliquots of the library should allow the identification of hybridizing inserts of the appropriate molecular weight. Dilution and amplification of the culture will then concentrate the cDNA until traditional replica plating can isolate the positive clone. This technique has been applied to the isolation of a rat α_{1D}-AR cDNA. Recent isolation of receptor genes and cDNAs whose expression could not be detected by Northern analysis or PCR (16, 35) indicated that additional AR subtypes are present, which have not been defined on the

TABLE 3

α_1-AR subtype characteristics

Characteristic	α_{1A}	α_{1B}	α_{1C}	α_{1D}
Tissue expression[a]				
Cerebral cortex	++	++	–	++
Hippocampus	++	–	–	++
Dentate gyrus	ND[b]	ND	++	ND
Aorta	?[c]	+	–	+
Spleen[d]	–	++/–	–	+
Liver	–	++	–	–
Vas deferens	++	–	–	++
Ligand binding (K_i, nM)[e]				
WB4101	0.19 ± 0.08	14.1 ± 5.2	0.68	1.9 ± 0.15
5-Methylurapidil	0.6 ± 0.08	40 ± 10	ND	15 ± 1.4
Prazosin	1.9 ± 1.8	1.0 ± 0.5	0.37	0.32 ± 0.005
Phentolamine	0.9 ± 0.15	146 ± 48	15.3	138 ± 10
(+)-Niguldipine	0.06 ± 0.04	8 ± 3	ND	46 ± 5.5
CEC sensitivity	–	+++	+++	++
SZL-49 sensitivity	+++	+	ND	+
M_r	ND	80,000	ND	ND
Amino acids	ND	515	466	560
[^{125}I]Azidoprazosin photolabeling	–	+++	ND	–
Glycosylation	++	++	ND	ND

[a] Based on identification by ligand binding for the α_{1A}-AR and on Northern blots for the other subtypes.
[b] ND, not determined.
[c] ?, the α_{1A}-AR characterized pharmacologically in rat aorta is atypical (31).
[d] Based on ligand binding studies, the α_1-AR subtype in spleen is almost exclusively α_{1B}, but no transcript for this subtype is observed by Northern blot analysis.
[e] Values (mean ± standard error) are based on those reported from competition binding studies for α_{1A} (based on three or four values for each ligand reported in Refs. 8, 10, 32, and 34); α_{1B} (three to eight values; Refs. 8, 10, 14, 16, 27, and 38), α_{1C} (one value; Ref. 16), and α_{1D} (this study).

basis of pharmacological and biochemical studies. Thus, molecular cloning and expression of isolated cDNAs provide the only direct tool for the classification of receptor subtypes and, indeed, this approach has already resulted in the identification of additional subtypes of muscarinic receptors (35) and α_2-ARs (36). Similarly, cloning and characterization of the AR described here indicate that this receptor is a novel α_1-AR (α_{1D}-AR), which is distinct from the α_{1A} and other subtypes reported previously. Although the physiological role of this receptor is unclear, it is of interest that transcripts for this protein are present in a variety of tissues. Further studies are necessary to define the functions of this novel α_{1D}-AR.

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