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The Rat Homologue of the Bovine α_{1c}-Adrenergic Receptor Shows the Pharmacological Properties of the Classical α_{1A} Subtype

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SUMMARY

The cDNA for the rat α_{1c}-adrenergic receptor (AR) has been cloned using a probe derived from the bovine α_{1c}-AR sequence. Clone rB7a has a 2.6-kilobase insert with a 1390-base pair open reading frame and encodes a receptor of 466 amino acids. The cloned receptor has 91% amino acid identity with the bovine α_{1c}-AR. The rat α_{1c}-AR mRNA was detected in tissues known to be enriched for the α_{1A}-AR subtype, including vas deferens, heart, kidney, and hippocampus. Rat α_{1c}-AR mRNA was absent from liver and spleen when assayed by Northern blot analyses and RNase protection assays. In COS-7 cells transfected with cDNAs encoding the three rat α_1-ARs, WB-4101 and benoxathian had similar binding affinities for the $\alpha_{1a/d}$-AR and the α_{1c}-AR and 10-fold lower affinities for the α_{1b}-AR. The affinity of 5-methylurapidil was found to be 10- and 30-fold higher at the α_{1c}-AR than at the $\alpha_{1a/d}$- and α_{1b}-ARs, respectively. (*S*)-(+)-Niguldipine was found to have high affinity for the rat α_{1c}-AR, with 42- and 22-fold lower affinity at the $\alpha_{1a/d}$- and α_{1b}-ARs, respectively. Treatment of intact transfected COS-7 cells with chlorethylclonidine resulted in the inactivation of 19% of the α_{1c}-ARs, in contrast to 72% and 85% inactivation of the $\alpha_{1a/d}$- and α_{1b}-ARs, respectively. Similarly to the other two α_1-ARs, the rat α_{1c}-AR is coupled to the activation of phospholipase C. Our data suggest that the rat α_{1c}-AR cDNA encodes an α_1-AR with the pharmacological properties previously defined for the α_{1A} subtype found in tissues.

Although ARs bind the same endogenous catecholamines, epinephrine and norepinephrine, their physiological and pharmacological specificity is markedly diverse. This diversity is due primarily to the existence of at least nine different genes encoding three distinct AR types (α_1, α_2, and β) (1). These proteins belong to the superfamily of G protein-coupled receptors and are characterized by a single polypeptide chain that is predicted to span the plasma membrane seven times, with an extracellular amino terminus and a cytoplasmic carboxyl terminus. Pharmacological studies have demonstrated the existence of at least two α_1-AR subtypes. Binding studies of rat brain α_1-ARs provided strong evidence for the existence of receptor heterogeneity, based on the relative affinities for prazosin and WB-4101 (2). These observations were supported by the finding that CEC inactivated 50% of the ^{125}I-HEAT binding sites from rat cerebral cortex and 80% of the binding sites from liver or spleen (α_{1B})[1] but did not inactivate α_1-ARs from the hippocampus or vas deferens (α_{1A}) (2–4). Taken together, these results suggested a classification of the α_{1A} subtype as exhibiting high affinity for WB-4101 and resistance to alkylation by CEC and the α_{1B} subtype as having 10–20-fold lower affinity for WB-4101 and being sensitive to CEC inactivation.

The molecular cloning of three genes encoding α_1-ARs supports the existence of pharmacologically and anatomically distinct α_1-AR subtypes. The α_{1b}-AR gene was originally cloned from a hamster smooth muscle cell line cDNA library and encodes a 515-amino acid peptide. The transfection of this gene into COS-7 cells induced the expression of an α_1-AR with low affinity for WB-4101, 95% of which could be inactivated by CEC (5). The cloned rat α_{1b}-AR (6, 7) displays pharmacology similar to that of the classically defined α_{1B} subtype, and the localization of the rat α_{1b}-AR mRNA in rat liver, heart, cerebral cortex, and kidney agrees with the tissue distribution of the classically defined α_{1B} subtype (6). Recently the human α_{1b}-AR has been cloned, and it exhibits a similar pharmacological profile (8). A second α_1-AR gene was cloned from a bovine brain cDNA library and was found to encode a 466-residue polypeptide with 52% homology to the α_{1b}-AR (9). Upon expression it showed 16- and 30-fold lower affinity for WB-4101 and

[1] In this manuscript, the pharmacologically defined subtypes are designated using uppercase subscripts and the cloned receptors are designated using lowercase subscripts.

ABBREVIATIONS: AR, adrenergic receptor; CEC, chlorethylclonidine; PCR, polymerase chain reaction; bp, base pair(s); SDS, sodium dodecyl sulfate; kb, kilobase(s); SSC, standard saline citrate; RT, reverse transcription; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; HEAT, 2[(p-hydroxyphenyl)ethylaminomethyl]1,2,3,4-tetrahydro-1-keto-naphtalin.

phentolamine, respectively, than did the α_{1b}-AR and was partially inactivated (65%) by CEC (9). Although the pharmacological properties of this bovine α_1-AR were found to be similar to those of the pharmacologically defined α_{1A} subtype, this α_1-AR was designated the α_{1c}-AR, due in part to the failure to detect its mRNA in rat tissues where the α_{1A} subtype had been previously characterized (9). Two clones encoding a third α_1-AR subtype, which differ from each other by two amino acids, have been isolated from rat tissues (6, 10). The expression of the mRNA for this third α_1-AR in rat vas deferens, aorta, cerebral cortex, and hippocampus, together with a pharmacological profile showing 10-fold higher affinity for WB-4101, compared with the α_{1b}-AR, led to its designation as the α_{1a}-AR (6). However, the second clone of this rat α_1-AR (10) was found to be sensitive to CEC and, compared with the tissue α_{1A} subtype, it was found to have lower affinities for phentolamine, (S)-(+)-niguldipine, and 5-methylurapidil. These findings suggested that this α_1-AR gene did not encode the α_{1A} subtype, and it was designated the rat α_{1d}-AR (10).

Because the three cloned α_1-ARs (rat $\alpha_{1a/d}$, rat α_{1b}, and bovine α_{1c}) do not account for the classical α_{1A} subtype pharmacology as defined in rat tissues, a fourth α_1-AR was proposed to exist (11). However, we have recently found that the human homologue of the bovine α_{1c}-AR encodes an α_1-AR that meets the pharmacological criteria for the α_{1A} subtype (12). In an attempt to elucidate the relationship between the cloned and pharmacologically defined α_1-AR subtypes, we have cloned, sequenced, and expressed the rat homologue of the bovine α_{1c}-AR. Compared with the properties of the rat $\alpha_{1a/d}$-AR and rat α_{1b}-AR, the rat α_{1c}-AR shows the pharmacological properties of the classical α_{1A} subtype. Furthermore, the mRNA of the rat α_{1c}-AR is present in rat tissues that show the classical α_{1A} subtype pharmacology (hippocampus and vas deferens) and was not detectable, by RNase protection or Northern blot analyses, in tissues rich in the α_{1B} subtype (liver and spleen).

Materials and Methods

cDNA library construction. Total RNA from rat whole brain was isolated by the guanidinium/cesium chloride method (13). Poly(A)$^+$ RNA was prepared from the rat whole-brain total RNA with the FastTrack mRNA isolation kit (Invitrogen). The cDNA was synthesized and ligated to *Eco*RI adaptors (Promega). Size selection of the cDNA was performed on a Sepharose CL-4B column (Pharmacia). The cDNA was then separated on a SeaPlaque GTG low-temperature agarose gel (FMC), and the cDNAs between 1.5 kb and 6.1 kb were recovered with β-agarase (New England Biolabs). The cDNA was ligated into the λZAP II vector (Stratagene) and packaged with the Gigapack II packaging extract (Stratagene). The resulting cDNA library contained 4.4×10^6 primary clones, with a mean insert size of 2.3 kb. The phage were aliquotted into 87 pools consisting of 50,000 primary clones each.

Library screening and DNA sequencing. The homology screening of the cDNA library was done with a ^{32}P-labeled probe derived from bovine α_{1c}-AR cDNA (9) nucleotides 1–70. The hybridization was performed at reduced stringency (40°, 37.5% formamide), and the membranes were washed at 45° with 0.1× SSC (0.15 M sodium chloride, 0.015 M sodium citrate)/0.1% SDS. A partial cDNA clone isolated from this screening of the rat cDNA library was designated rB5a. Primers were designed, using the rB5a sequence, for PCR screening of the rat brain cDNA library to identify a phage pool that contained a cDNA clone with the entire coding region. The primers were derived from the second cytoplasmic loop region at nucleotides 402–424 (sense), the third cytoplasmic loop region at nucleotides 775–800 (antisense), and the carboxyl-terminal region at nucleotides 1146–1171 (sense). The primers for the pBluescript SK(−) vector sequence (Stratagene) were derived from nucleotides 729–755 [(+)-strand] and nucleotides 669–691 [(−)-strand]. The primers for the second and third cytoplasmic loops were used to screen four pools of phage that represented 4.4×10^6 clones of the rat brain cDNA library. A positive pool of 1×10^6 phage clones was divided into 23 pools, and the pools were screened by PCR to determine which pool contained the cDNA. A positive pool was rescreened with a PCR primer set that contained one rat primer (nucleotides 775–800 or nucleotides 1146–1171) and a pBluescript primer, to confirm that the pool contained the entire coding region. The PCRs were done in a buffer containing 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each deoxynucleoside triphosphate, 1 μM each primer, 2.5 units/100-ml volume *Thermus aquaticus* polymerase (Perkin-Elmer), and 2 μl of the phage pool. The reaction mixture was heated at 95° for 5 min and then subjected to 94° for 2 min and 68° for 4 min for 40 cycles, followed by 5 min at 72°. The positive phage pool was plated and 1.8×10^5 phage were screened with a probe for rat α_{1c}-AR nucleotides 1227–1296, at high stringency (50% formamide at 40°; washed with 0.1× SSC/0.1% SDS at 50°). The cDNA clone isolated from this screening of the rat cDNA library was designated rB7a. Positive phage clones were converted into phagemids by *in vivo* excision, with coinfection of the R408 helper phage (Stratagene). The nucleotide sequences of the double-stranded cDNAs were analyzed by the Sanger dideoxy method (14) using Sequenase (United States Biochemical Corp.) and by cycle sequencing using Vent DNA polymerase (New England Biolabs).

Northern blot analysis. Rat poly(A)$^+$ RNA (10 μg; Clontech) was separated by electrophoresis on a 1% agarose/16% formaldehyde gel and transferred to a nylon membrane (Genescreen Plus; New England Nuclear) by overnight capillary blotting in 10× SSC. A PCR fragment from nucleotide 402 to nucleotide 1280 of the cloned rat α_{1c}-AR was randomly primed and used to probe the Northern blot, as described previously (15).

RT-PCR localization. The first-strand cDNA synthesis was performed with 1.5 μg of total RNA in a 30-μl reaction volume. The reaction buffer contained 10 mM Tris, pH 8.3, 75 mM KCl, 3.0 mM $MgCl_2$, 20 mM dithiothreitol, 0.5 mM levels of each deoxynucleoside triphosphate, 1.5 μg of random hexamer, and 600 units of Superscript II reverse transcriptase (GIBCO-BRL). The reaction mixture was incubated at 42° for 1 hr, and the reaction was terminated by heating of the tube at 90° for 10 min. The RNA was removed by addition of 1.5 unit of RNase H (Boehringer Mannheim) to the cDNA synthesis reaction tube and incubation at 37° for 30 min. A 1-μl aliquot of the first-strand cDNA was used as a template for PCR amplification with rB5a primers derived from nucleotides 799–823 (sense) and nucleotides 1255–1280 (antisense). The reaction mixture was heated at 95° for 5 min and subjected to 94° for 2 min and 68° for 4 min for 30 cycles, followed by 5 min at 72°. A carboxyl-terminal 45-mer derived from nucleotides 1166–1209 (sense) was used as the probe for the RT-PCR experiment. Rat α_{1b}-AR mRNA was detected in rat liver and spleen with primers that span the intron between transmembrane domains VI and VII (data not shown), confirming the quality of the RNA.

RNase protection assays. PCR was used to generate DNA templates for rat α_{1c}-AR cDNA and rat *G3PDH* genes for use in generating RNA probes, as described previously (16). Primers directed to the carboxyl terminus of the rat α_{1c}-AR gene contained T3 and T7 RNA polymerase promoter sequences at the 5′ ends of the sense (nucleotides 1014–1037) (see Fig. 1) and antisense (nucleotides 1213–1235) (see Fig. 1) primers, respectively. Similarly, primers directed to exon 8 of the rat *G3PDH* gene contained T3 and T7 RNA polymerase promoter sequences at the 5′ ends of the sense (nucleotides 577–599; GenBank accession number M17701) and antisense (nucleotides 796–819; GenBank accession number M17701) primers, respectively. The cloned rat α_{1c}-AR recombinant plasmid (5 ng) and rat brain total cDNA (250 ng of cDNA converted from RNA; Clontech) served as templates for amplification of rat α_{1c}-AR (generating a 286-bp PCR product) and rat

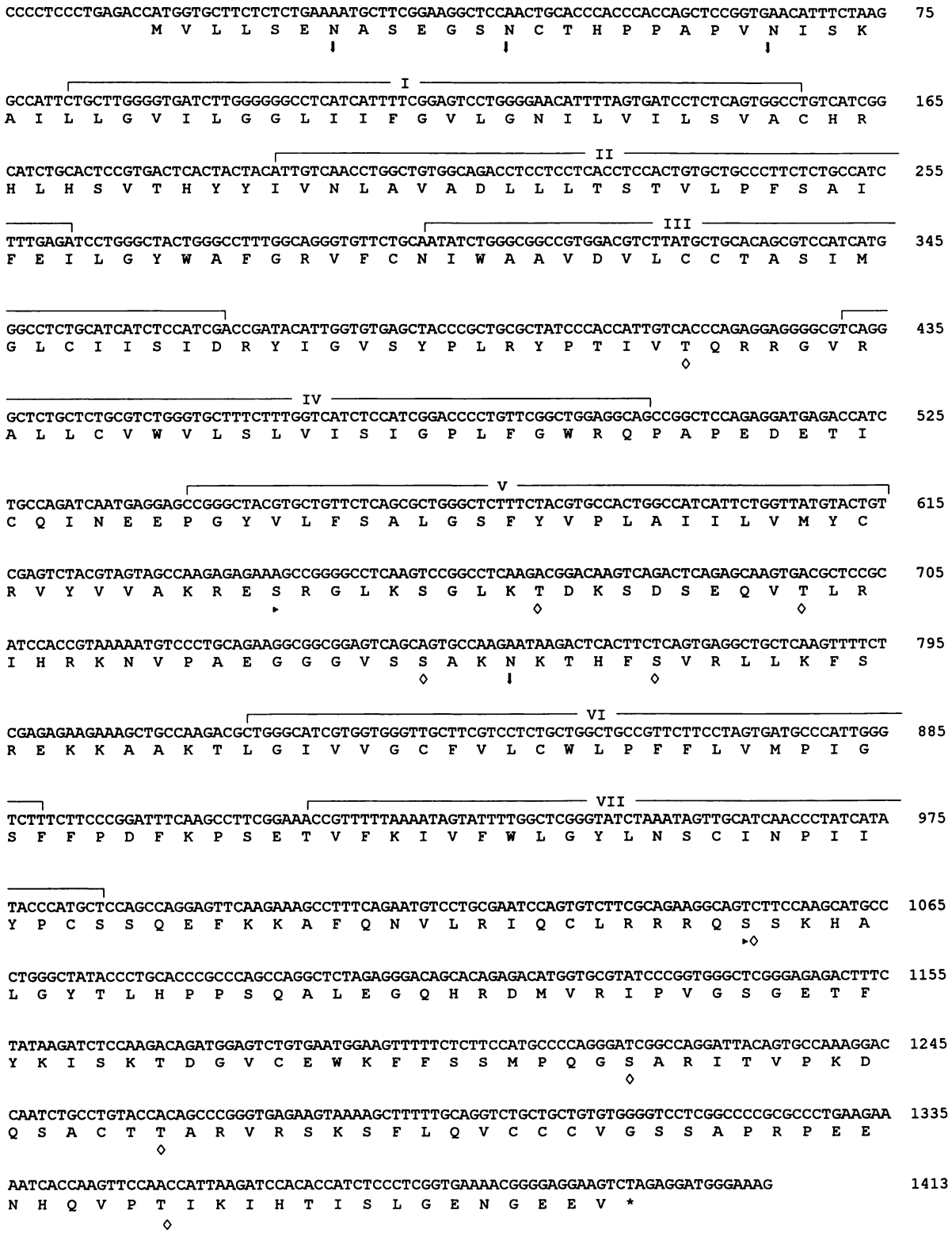

Fig. 1. Nucleotide sequence of the rB7a clone and the deduced amino acid sequence of the rat α_{1c}-AR. The nucleotide numbering begins with 1 at the translation initiation codon. The single-letter amino acid code is used. *Lines over the amino acids*, predicted transmembrane domains. *Arrows*, potential *N*-linked glycosylation sites. ◇ and ▶, consensus sequences for phosphorylation by protein kinase C and cAMP-dependent protein kinase, respectively. *, termination codon.

G3PDH (generating a 307-bp PCR product) genes in a PCR mixture containing 1× PCR buffer (described above), sense and antisense primers at a final concentration of 1 μM each, 1.5 mM $MgCl_2$, and 2.5 units of *T. aquaticus* DNA polymerase (AmpliTaq kit). The tubes were placed in a Perkin-Elmer DNA thermal cycler with the following program: initial denaturing step for 5 min at 95°; five cycles of 1 min at 94°, 2 min at 68°, and 3 min at 72°; 30 cycles of 1 min at 94°, 2 min at 72°, and 3 min at 72°; and an extension period of 10 min at 72°. The reaction mixtures were separated on a 1.0% low-melting point agarose gel (SeaPlaque; FMC), and the single bands were excised, extracted, and quantitated.

In vitro transcription was performed using the MAXIscript T7 *in vitro* transcription kit (Ambion), as described in detail in the instruction manual provided by the manufacturer, with the following modifications: 50 μCi of [α-^{32}P]UTP with a 5 μM final concentration of nonradiolabeled UTP were added to the reaction containing 50 ng of the PCR-generated minigene template. The probes (260 nucleotides for rat α_{1c}-AR and 281 nucleotides for rat *G3PDH*) were gel purified, as described by the manufacturer. Probes consistently possessed specific activities of 5–8 × 10^8 cpm/μg. The RNase protection assay was performed using the Ambion RPA II kit (Ambion), as described in detail in the instruction manual provided by the manufacturer. Hybridization of radiolabeled probe (2 × 10^5 cpm) with 10 μg of poly(A)$^+$ RNA (or 55 μg of total RNA) from different rat tissues was carried out at 45° for both the α_{1c}-AR and *G3PDH* probes. Samples were separated on a denaturing 8 M urea/5% acrylamide gel, followed by drying and exposure to XAR-5 (Kodak) film for 14–96 hr. Autoradiographs were scanned using an ENV6100 scanner (Envisions Solutions Tech., Inc.) and quantitated using Photoshop version 2.5 for Windows, set to 256 grayscale at 600 dpi (Adobe Systems, Inc.). The relative abundance of the α_{1c}-AR subtype mRNAs in each tissue was normalized by using *G3PDH* as the control for RNA levels. A longer exposure time (7-fold) was used to compensate for the smaller amount of poly(A)$^+$ RNA (7-fold) assayed for vas deferens, assuming ≈2–3% mRNA in total RNA [or 1.4 μg of poly(A)$^+$ RNA in 55 μg of total RNA from vas deferens, compared with 10 μg of poly(A)$^+$ RNA for the other tissues]. Yeast RNA served as a negative control. Undigested probe migrated at a higher molecular weight (i.e., 260 nucleotides) than the protected fragment (i.e., 222 nucleotides) (see Fig. 5) because it contains the T3 RNA polymerase promoter sequence at the 5′ end of the sense PCR primer used to generate the DNA template for the RNA probe.

Expression. A 2.6-kb *ClaI-BamHI* fragment containing the entire cDNA clone (rB7a) was subcloned from pBluescript into the *ClaI* and *BamHI* sites of the eukaryotic expression vector EXJ.RH (provided by Dr. J. Bard, Synaptic Pharmaceutical Corp.), which was derived from pcEXV-3 (17). COS cells were transfected as described previously (15). The rat $\alpha_{1a/d}$-AR cDNA was obtained from Dr. J. Lomasney (Department of Pathology, Northwestern University) (6). The rat α_{1b}-AR cDNA was obtained from Dr. H. Chin (Laboratory of Neurochemistry, National Institutes of Health) (7) and was subcloned into the EXJ expression vector.

Radioligand binding assays. Transfected cells from culture flasks were scraped into 5 ml of 5 mM Tris·HCl, 5 mM EDTA, pH 7.5, and lysed by sonication (Branson Sonic Power Co., Danbury, CT). The cell lysates were centrifuged at 1000 rpm for 5 min at 4°, and the supernatant was centrifuged at 30,000 × *g* for 20 min at 4°. The pellet was suspended in 50 mM Tris·HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, at pH 7.5. Membrane preparations of transfected COS-7 cells were incubated at 37° for 30 min with 0.5 nM [^{3}H]prazosin (specific activity, 76.2 Ci/mmol; New England Nuclear, Boston, MA), in a final volume of 0.25 ml. The amount of protein added to the binding reaction was adjusted for each receptor subtype, in each experiment, so that the total bound [^{3}H]prazosin did not exceed 10% of the radioactivity added to the reaction mixture. The reaction was stopped by filtration through GF/B filters using a cell harvester (Tomtec, Orange, CT), and tritium was determined by liquid scintillation counting. Data were analyzed by a computerized nonlinear regression program (Inplot; GraphPad Software, San Diego, CA). Protein concentration was determined by a colorimetric assay using a commercial kit, with bovine serum albumin as the standard (Bio-Rad Laboratories, Hercules, CA).

CEC treatment. COS-7 cells were harvested 24 hr after transfection and plated again into culture dishes, to obtain homogeneous distribution of the transfected cells. After another 24 hr in culture, the medium was removed and substituted with Dulbecco's PBS. CEC was dissolved in 5 mM phosphate buffer and added to the cultures, and cells were incubated at 37°. After 20 min the medium was removed by aspiration and both control and CEC-treated cells were washed three times with 20 ml of fresh PBS. Cells were harvested and membrane protein was obtained as described above. Receptor numbers and K_d values were obtained by Scatchard analysis of saturation binding isotherms using [^{3}H]prazosin (0.03–6 nM). Nonspecific binding was determined in the presence of 10 μM phentolamine, and it accounted for <6% of the total bound radioactivity. The reaction was stopped as described above, and the data were analyzed using the LIGAND computer program.

Formation of [^{3}H]inositol phosphates. COS-7 cells transfected with the rat α_{1c}-AR cDNA were grown to confluency in 24-well plates. The culture medium was removed and 0.5 ml of PBS containing 5 μCi/ml *myo*-[^{3}H]inositol was added. After 60 min at 37° the unincorporated radioactivity was removed, 0.4 ml of PBS containing 10 mM LiCl was added, and cells were incubated for 20 min. The reaction was started by addition of either PBS (basal) or the agonist and was incubated for 60 min at 37°. The reaction was stopped by addition of 0.03 ml of 50% trichloroacetic acid, followed by neutralization with 1 M Tris base. Total [^{3}H]inositol phosphates were separated by ion exchange chromatography as described (18).

Results and Discussion

An oligonucleotide probe derived from the amino terminus of the bovine α_{1c}-AR cDNA (9) was used to screen 2 × 10^6 plaques from a rat brain cDNA library in λZAP II at reduced stringency. One positive plaque was identified (rB5a) and the cDNA was analyzed. A comparison of the deduced amino acid sequence of the rat clone with the bovine α_{1c}-AR sequence (9) suggested that the rB5a clone was truncated in the carboxyl terminus (data not shown).[2] The cDNA library was rescreened by PCR and a cDNA clone containing the entire coding region was isolated. Clone rB7a contained a 2.6-kb cDNA with a 1398-bp open reading frame encoding a protein of 466 amino acids. The nucleotide and deduced amino acid sequences of the open reading frame are shown in Fig. 1. The coding sequence contains the seven putative transmembrane domains characteristic of the G protein-coupled receptors. There are four potential glycosylation sites in the receptor, three in the amino terminus (positions 7, 13, and 22) and one in the third cytoplasmic loop (position 253). There are two potential cAMP-dependent kinase phosphorylation sites, one in the third cytoplasmic loop (position 212) and one at the transmembrane domain VII-carboxyl terminus boundary (position 348). Finally, there are nine potential protein kinase C phosphorylation sites in the intracellular domains, one site in the second cytoplasmic loop (position 139), four sites in the third cytoplasmic loop (positions 224, 233, 250, and 258), and three sites in the carboxyl terminus (positions 351, 407, 421, and 451). An amino acid alignment of the rat, bovine, and human α_{1c}-ARs is shown in Fig. 2. The rat α_{1c}-AR has 91% amino acid identity to the bovine α_{1c}-AR and 93% identity to the human α_{1c}-AR, with only 49% identity to the rat α_{1a}-AR and 53% identity to the rat α_{1b}-AR. As a comparison, the human (12) and rat (6, 10) $\alpha_{1a/d}$-ARs have 84%

[2] The sequence of the rat α_{1c}-AR has been deposited in GenBank (accession number U07126).

Fig. 2. Amino acid alignment of the cloned rat, bovine, and human α_{1c}-ARs. *Brackets*, seven putative transmembrane domains (*I–VII*). *Shading*, residues that differ between the receptors. The rat α_{1c}-AR was encoded by rB7a; the bovine α_{1c}-AR (9) and human α_{1c}-AR (33) sequences have been published.

amino acid identity, whereas the human (8) and rat (6, 7) α_{1b}-ARs have 95% amino acid identity. The nucleotide identity between the coding regions for the rat α_{1c}-AR and the bovine α_{1c}-AR was 86% and the nucleotide identity for the rat α_{1c}-AR and the human α_{1c}-AR was 88%. The human and bovine cloned α_{1c}-ARs had the highest nucleotide identity, 90%.

Localization of the rat α_{1c}-AR mRNA in rat tissues was done by converting total RNA into cDNA, and this first-strand cDNA was used as a template for PCR. The PCR primers (third cytoplasmic loop and carboxyl terminus) span an intron that is located between transmembrane domains VI and VII (8). As shown in Fig. 3A, the primers amplified the cDNA but did not amplify genomic DNA. Furthermore, the PCR primers and probes showed no cross-reactivity when tested against plasmids containing the rat $\alpha_{1a/d}$-AR (6) and α_{1b}-AR (7) cDNAs (Fig. 3B). A second set of primers within the carboxyl terminus, which did not span the intron, amplified the genomic DNA template and the rB7a cDNA (Fig. 3A). The rat α_{1c}-AR mRNA was found in hippocampus, vas deferens, aorta, and cerebral cortex tissues (Fig. 3B), where the pharmacologically defined rat α_{1A} subtype has been shown to be present. Rat liver and spleen, which contain predominantly the α_{1B} subtype, showed undetectable levels of the rat α_{1c}-AR mRNA, compared with the levels found in other tissues (Fig. 3A); a faint hybridization signal was detectable in liver and spleen only after overnight exposure of the blot (data not shown).

Further localization studies of rat α_{1c}-AR mRNA were performed by RNase protection assays using radiolabeled antisense RNA probes. Specificity of the rat α_{1c}-AR probe was verified by hybridization to *in vitro* transcribed sense RNA for

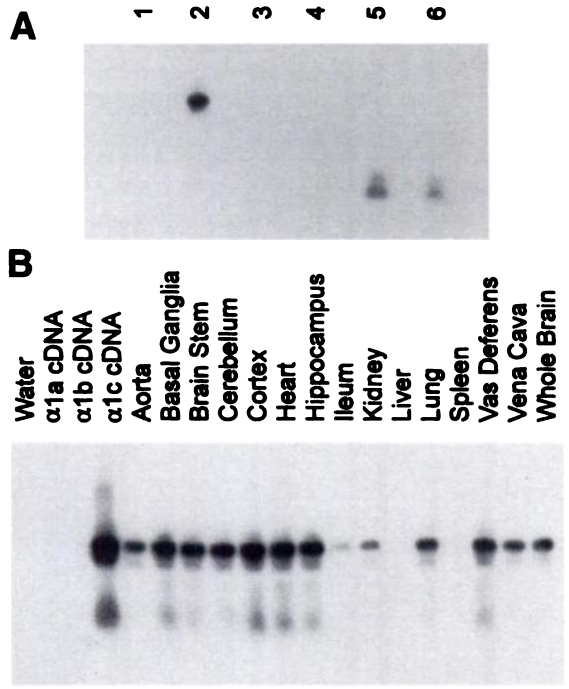

Fig. 3. Expression of the rat α_{1c}-AR mRNA in rat tissues, as determined by RT-PCR. A, PCR of the rat α_{1c}-AR DNA from genomic DNA and plasmid templates. The templates in *lanes 1* (water), *2* (rB7a), and *3* (genomic DNA) were amplified with primers (nucleotides 799–823 and nucleotides 1255–1280) spanning the intron region. The templates in *lanes 4* (water), *5* (genomic DNA), and *6* (rB7a) were amplified with primers (nucleotides 1146–1171 and nucleotides 1255–1280) spanning the carboxyl-terminal region. The PCR mixture was separated on an agarose gel, blotted to nylon membrane, and hybridized at high stringency with a rat α_{1c}-AR oligonucleotide, derived from nucleotides 1166–1209, as the probe. A 1-hr exposure of the Southern blot autoradiograph is shown. B, Tissue distribution of the rat α_{1c}-AR mRNA determined by RT-PCR. Single-stranded cDNA converted from total RNA was used as the template for PCR. The amplification products were blotted and probed with a rat α_{1c}-AR oligonucleotide. Plasmids containing the rat α_{1a}-, α_{1b}-, and α_{1c}-ARs were amplified under the same conditions. A 30-min exposure of the Southern blot autoradiograph is shown.

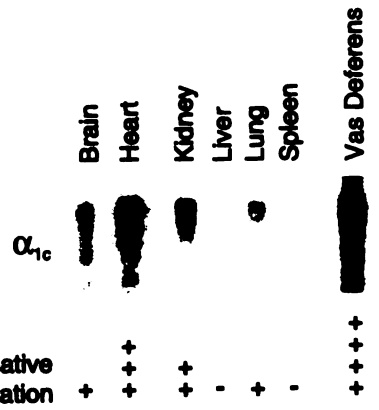

Fig. 4. RNase protection assay and relative quantitation of α_{1c}-AR mRNA expression in rat tissues. This autoradiograph demonstrates the results of a representative RNase protection assay, in which 10 μg of poly(A)$^+$ RNA (for all tissues except vas deferens, where 55 μg of total RNA were used) from several rat tissues were hybridized with radiolabeled antisense α_{1c}-AR RNA probe. A protected fragment of 222 nucleotides representing the α_{1c}-AR mRNA is shown. Quantitation ranges from a minimum of – (not present) to a maximum of ++++. These values were derived from autoradiographs of protected fragments that were scanned and quantitated (normalization of RNA levels was achieved using a constitutively expressed gene, *G3PDH*). Autoradiographic exposure times were 14 hr for all tissues except vas deferens, which represents a 96-hr exposure to compensate for the differences between total and poly(A)$^+$ RNA (see Materials and Methods).

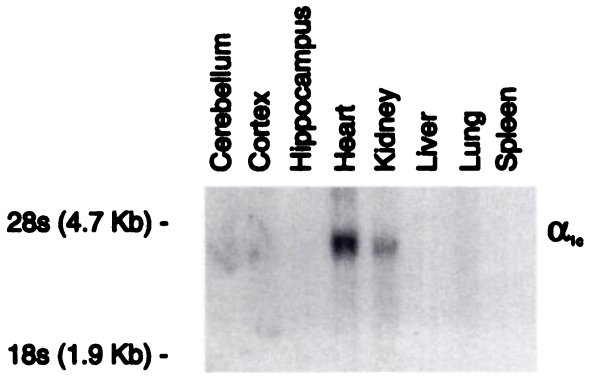

Fig. 5. Northern blot analysis of the rat α_{1c}-AR mRNA. Poly(A)$^+$ RNA (10 μg) from rat tissues was separated on a formaldehyde-agarose gel, blotted to a nylon membrane, and hybridiidized at high stringency with a rat α_{1c}-AR PCR fragment (nucleotides 402–1280). An overnight exposure of the autoradiograph is shown. The locations of the ribosomal bands are indicated. The α_{1c}-AR transcript is approximately 4.0 kb.

all three α_1-ARs, and no cross-hybridization was detected (data not shown). Fig. 4 shows an autoradiograph demonstrating the distribution and relative abundance of the α_{1c}-AR mRNA in several rat tissues. The mRNA is present most abundantly in the vas deferens, with heart being the next most abundant source among the tissues tested. Lower levels of expression of the α_{1c}-AR mRNA were observed in the kidney, followed by the brain and lung in descending rank order of abundance. No signals were detected in the spleen or liver, consistent with results obtained by RT-PCR and the sensitivity differences between the assays. The presence of multiple bands for the rat α_{1c}-AR mRNA in the RNase protection assay is consistent with those observed for the human α_{1c}-AR mRNA in the same assay (19).

Northern blots made with poly(A)$^+$ RNA from a variety of tissues showed a 4.0-kb transcript in heart and kidney that hybridized at high stringency with the rat α_{1c}-AR cDNA (Fig. 5). No transcripts were detected in cerebellum, cortex, hippocampus, liver, lung, or spleen. Given the decreased sensitivity of Northern analysis, relative to RNase protection assays, we were not suprised to observe a signal in lung in RNase protection assays (Fig. 4), whereas one was absent in Northern blot analyses (Fig. 5). Similarly, Price *et al.* (20) observed a positive signal for rat α_{1c}-AR mRNA in cortex and hippocampus (a tissue rich in the α_{1A} subtype) in RNase protection assays; however, we did not observe a signal in either cortex or hippocampus in Northern blot analyses.

Membrane preparations of COS-7 cells transiently transfected with the rat α_{1c}-AR cDNA showed saturable, high affinity binding of [^{3}H]prazosin (K_d = 0.4 ± 0.02 nM). The potency of a series of α_1-AR antagonists to displace [^{3}H]prazosin in these membrane preparations was compared with that in COS-7 cells transfected with rat $\alpha_{1a/d}$- and α_{1b}-AR cDNAs (Table 1). The endogenous catecholamines norepinephrine and epinephrine were found to have 20- and 7-fold higher affinity for the $\alpha_{1a/d}$-AR than for the other two AR subtypes, respectively. Conversely, the synthetic agonist oxymetazoline was found to have 180- and 30-fold higher affinity at the α_{1c}-AR than at the $\alpha_{1a/d}$-AR and α_{1b}-AR, respectively. In agreement with findings

TABLE 1

Binding affinity of agonists and antagonists at the three cloned rat α_1-AR subtypes expressed in COS-7 cells

Membrane protein from COS-7 cells transfected with each of the three cDNAs encoding the rat $\alpha_{1a/d}$-AR (6), α_{1b}-AR (7), and α_{1c}-AR were incubated with 0.5 nM [^{3}H]prazosin and 12 concentrations of the test compounds (from 0.1 nM to 100 μM), as described in Materials and Methods. Data are presented as the mean ± standard error from three independent experiments. Statistical significance was assessed by one-way analysis of variance.

Compounds	K_i		
	α_{1a}	α_{1b}	α_{1c}
	nM		
Agonists			
(−)-Norepinephrine	254 ± 49[a,b]	5918 ± 662	4637 ± 898
Epinephrine	393 ± 74[a,b]	3080 ± 436	2769 ± 482
Methoxamine	5422 ± 675	5787 ± 678	4634 ± 700
Oxymetazoline	1099 ± 75	181 ± 26[c]	6 ± 1.6[b,c]
Phenylephrine	1808 ± 242	6840 ± 943	2314 ± 310
Antagonists			
Benoxathian	4 ± 1.1	21 ± 3	2 ± 0.7[b]
HEAT	3 ± 0.3	1 ± 0.2	0.7 ± 0.1
5-Methylurapidil	40 ± 3	118 ± 15	4 ± 1.9[b,c]
(S)-(+)-Niguldipine	209 ± 20	109 ± 26	5 ± 1.1[b,c]
Prazosin	0.3 ± 0.06	0.2 ± 0.02	0.3 ± 0.02
Phentolamine	43 ± 4	67 ± 3	9 ± 1.5[b,c]
Spiperone	14 ± 2	9 ± 2[a]	30 ± 4
WB-4101	2 ± 0.4[b]	22 ± 3	0.9 ± 0.2[b]

[a] $p < 0.05$ versus α_{1c}.
[b] $p < 0.05$ versus α_{1b}.
[c] $p < 0.05$ versus α_{1a}.

TABLE 2

Inactivation of the three cloned rat α_1-AR subtypes by CEC

Intact COS-7 cells transiently transfected with the cDNA encoding the $\alpha_{1a/d}$-, α_{1b}-, or α_{1c}-AR were incubated with (CEC) or without (Control) 100 μM CEC in Dulbecco's PBS. After 20 min at 37°, the medium was aspirated and the cells were washed three times with 2 volumes of fresh PBS. Membrane protein was prepared and assayed for [^{3}H]prazosin binding as described in Materials and Methods. Data are shown as the mean ± standard error from three independent experiments performed in triplicate.

Subtype	Control		CEC		Inactivation
	K_d	B_{max}	K_d	B_{max}	
	nM	pmol/mg of protein	nM	pmol/mg of protein	%
α_{1a}	0.18 ± 0.03	0.64 ± 0.03	0.29 ± 0.06	0.18 ± 0.03[a]	72
α_{1b}	0.36 ± 0.08	19 ± 1.7	0.11 ± 0.01	2.9 ± 0.2[a]	85
α_{1c}	0.44 ± 0.02	10 ± 0.7	0.36 ± 0.02	8.4 ± 1.2	19

[a] $p < 0.05$ versus control.

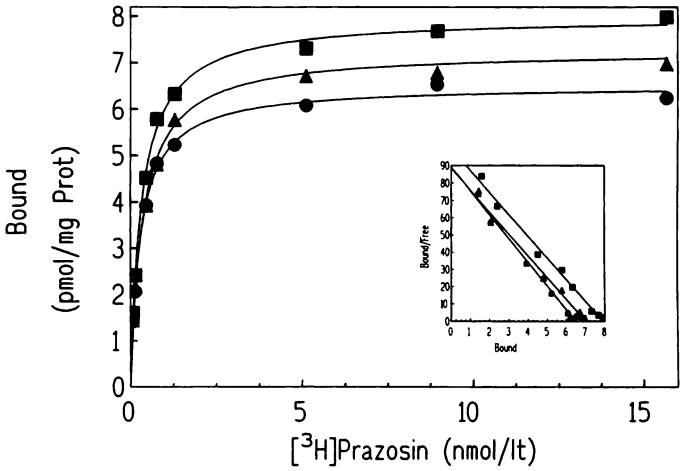

Fig. 6. Effects of CEC on membrane preparations of COS-7 cells transfected with the rat α_{1c}-AR cDNA. Membrane protein preparations from transfected COS-7 cells were obtained as described in Materials and Methods, suspended in either hypotonic buffer (50 mM Tris·HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.5) or isotonic buffer (50 mM Tris·HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.5), and incubated with 0.1 mM CEC for 20 min at 37°, in a final volume of 1 ml. The reaction was stopped by dilution of the reaction mixture in 30 ml of isotonic buffer and centrifugation at 30,000 × *g* for 20 min at 4°. The supernatant was discarded and the pellet was washed three times with 30 ml of isotonic buffer. The number of binding sites was determined by saturation binding experiments with [^{3}H]prazosin, as described in Materials and Methods. Data from a representative experiment are shown. ■, Control; ▲, CEC in isotonic buffer; ●, CEC in hypotonic buffer. The means ± standard errors from three independent experiments were as follows: control, K_d = 0.24 ± 0.08 nM, B_{max} = 8.3 pmol/mg of protein; CEC in isotonic buffer, K_d = 0.23 ± 0.09 nM, B_{max} = 7.2 ± 0.15 pmol/mg of protein; CEC in hypotonic buffer, K_d = 0.25 ± 0.05 nM, B_{max} = 6.7 ± 0.15 pmol/mg of protein. Inset: Plot of data from the same experiments transformed according to scatchard.[34]

from binding studies with rat tissues, prazosin and HEAT were found to be potent but nonselective ligands at the three cloned receptor subtypes. From the comparison of the binding properties of the three rat α_1-ARs it is apparent that the α_{1c}-AR has the highest affinity for antagonists that were characterized in binding studies in rat tissues as being selective for the α_{1A} subtype (21–23). Benoxathian, 5-methylurapidil, (*S*)-(+)-niguldipine, phentolamine, and WB-4101 were found to have their highest affinity at the α_{1c}-AR. However, only 5-methylurapidil, (*S*)-(+)-niguldipine, and phentolamine were selective for the cloned α_{1c}-AR. Of these three selective antagonists, 5-methylurapidil and phentolamine were 30- and 7-fold less potent at the cloned α_{1b}-AR and only 10- and 5-fold less potent at the $\alpha_{1a/d}$-AR, respectively. The calcium channel antagonist (*S*)-(+)-niguldipine showed the greatest separation between the affinities at the rat cloned $\alpha_{1a/d}$- and α_{1c}-ARs (42-fold). Spiperone was found to have similar affinities for binding to the $\alpha_{1a/d}$- and α_{1b}-ARs and 3-fold lower affinity at the rat α_{1c}-AR, suggesting that it could hardly differentiate between the cloned subtypes. When the binding data from cloned receptors are compared with those obtained in rat tissues in which mixtures of α_1 subtypes are expressed, an interesting pattern emerges depending on which antagonist is used. For example, WB-4101 or benoxathian could distinguish the α_{1b}-AR from the other two cloned receptors but could not differentiate between the $\alpha_{1a/d}$- and α_{1c}-ARs. Moreover, 5-methylurapidil or (*S*)-(+)-niguldipine could differentiate the α_{1c}-AR from $\alpha_{1a/d}$- or α_{1b}-ARs but could not differentiate between the latter two cloned subtypes. Our data indicate that the available antagonists were not sufficiently selective to pharmacologically identify three distinct receptor subtypes in rat tissues.

In addition to antagonist profiles, an important criterion for the differentiation of α_1-AR subtypes is their sensitivity to irreversible inactivation by CEC (2, 3). As shown in Table 2, the three rat receptor subtypes displayed different sensitivities to inactivation by CEC. Treatment of intact COS-7 cells transfected with the cDNA for the rat α_{1c}-AR with 100 μM CEC inactivated 19% of the receptors. Similar treatment of COS-7 cells transfected with the $\alpha_{1a/d}$-AR or the α_{1b}-AR resulted in 72 and 85% reduction, respectively, of the number of [^{3}H]prazosin binding sites. These data are in agreement with the sensitivity to CEC reported for the hamster α_{1b}-AR and the rat $\alpha_{1a/d}$-AR (5, 10). As shown in Fig. 6, CEC treatment of membrane preparations of COS-7 cells transfected with the rat α_{1c}-AR cDNA resulted in the inactivation of similar proportions of binding sites whether the incubation was performed in isotonic

(14%) or hypotonic (20%) medium, in agreement with earlier observations in rat tissues (24). In assays performed simultaneously with those for the rat α_1-ARs, CEC treatment of intact COS-7 cells transfected with the cloned bovine α_{1c}-AR resulted in 55 ± 3% (mean ± standard error, three experiments) reduction of the [^{3}H]prazosin binding sites. These data are in agreement with a previous report in which CEC inactivated 68% of the ^{125}I-HEAT binding sites of COS cell membranes transfected with the bovine α_{1c}-AR (9). In view of the high level of sequence identity between the rat and bovine α_{1c}-ARs, the difference in CEC sensitivity is probably due to a relatively small difference in amino acid sequence between these species homologues. In support of this contention is the evidence that species differences noted in pharmacological profiles for many G protein-coupled receptors can often be attributed to a relatively small number of amino acid changes in the primary amino acid sequences of the receptors (25, 26). Finally, in agreement with the evidence from experiments with rat tissues indicating that both α_{1A}- and α_{1B}-ARs activate the formation of [^{3}H]inositol phosphates (27), we found that the α_{1c}-AR expressed in COS-7 cells also activates this signaling pathway (Fig. 7).

The relative insensitivity to CEC of the α_1-AR encoded by the rat α_{1c}-AR gene, together with its high affinity for α_{1A} subtype-selective antagonists, presents the profile of the classically defined α_{1A} subtype. By comparing the pharmacological properties of the three cloned rat α_1-ARs, it is clear that the rat $\alpha_{1a/d}$-AR gene does not encode a receptor with the properties described for the α_{1A} subtype (6). This is in agreement with the findings of Perez *et al.* (10) that led to the designation of the rat α_{1a}-AR as the $\alpha_{1a/d}$-AR (11). However, our data indicate that the rat α_{1c}-AR gene suffices to account for α_{1A} subtype pharmacology, in terms of 1) selective antagonist potency, 2) CEC sensitivity, and 3) tissue localization. The contention that the properties of the classical α_{1A} subtype are encoded by a fourth α_1-AR gene arose in part because of the initial finding that the species homologue of the bovine α_{1c}-AR could not be detected in rat tissues (6, 9). By using exact rat α_{1c}-AR cDNA probes, we and others (20, 28) have detected this mRNA in rat tissues rich in the α_{1A} subtype (including vas deferens and hippocampus) by Northern blot analysis, RNase protection assay, or RT-PCR. The abundant expression of the α_{1c}-AR gene in vas deferens, a tissue rich in the α_{1A} subtype (4), represents a good correlation between mRNA levels and α_{1A} binding sites. In certain cases, however, the relative abundance of the α_{1c}-AR mRNA determined in Northern blot analyses and RNase protection assays does not exactly match the distribution of high affinity (S)-(+)-niguldipine binding sites described by Han and Minneman (4). For example, our results indicate that there are higher mRNA levels in heart than in kidney, whereas the binding data indicate that heart and kidney have similar receptor densities. In addition, α_{1A} binding sites in hippocampus are expected to be more abundant than those in heart (4). Although RNase protection studies revealed a signal for α_{1c}-AR mRNA in hippocampus (20), both we and Perez *et al.* (29) were unable to detect a signal by Northern blot in hippocampus, whereas we did detect a signal in heart, suggesting a greater abundance of α_{1c}-AR mRNA in heart than in hippocampus. The lack of correlation between message and receptor protein levels could be taken to suggest that these tissues might express a fourth gene encoding an α_1-AR. Alternatively, it is also possible that the pharmacologically defined α_{1A} subtype actually corresponds to a combination of existing subtypes. Previous data on the expression of the rat α_{1a}-AR mRNA indicated that it was found in rat hippocampus and vas deferens (6). Coexpression of the α_{1a}- and α_{1c}-AR genes in hippocampus and vas deferens probably accounts for the findings of heterogeneity of the α_1-ARs in these two tissues after treatment with CEC (4, 24). Finally, the level of receptor mRNA expression may not directly correlate with the levels of receptor protein.

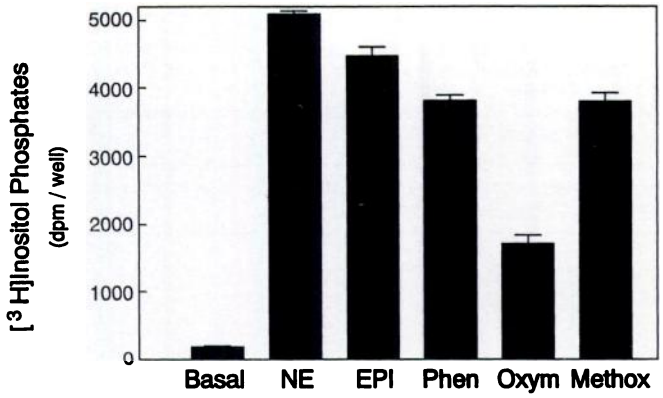

Fig. 7. Receptor-mediated activation of phospholipase C in COS-7 cells transfected with the cDNA encoding the rat α_{1c}-AR. The formation of [^{3}H]inositol phosphates was measured as described in Materials and Methods. The phosphoinositide response was stimulated with PBS (*Basal*), 100 μM norepinephrine (*NE*), 100 μM epinephrine (*EPI*), 100 μM phenylephrine (*Phen*), 100 μM oxymetazoline (*Oxym*), or 1 mM methoxamine (*Methox*). Data are shown as the mean ± standard error of three independent experiments.

The findings of this study are consistent with those of a recent study in which the binding affinities of selective compounds were studied with the three cloned human α_1-ARs (12). Thus, when human and rat α_1-ARs are compared side by side, the properties of the classical α_{1A} subtype are encoded by the α_{1c}-AR gene in both species. Our results do not support the conclusions of studies in guinea pig liver, which expresses an α_1-AR with the pharmacological properties of the α_{1A} subtype, in which the cognate of the rat $\alpha_{1a/d}$-AR was identified (30, 31). Although the latter findings can be interpreted as meaning that the $\alpha_{1a/d}$-AR gene encodes a receptor with α_{1A} pharmacology, there are several arguments against this contention. 1) In two species, rat and human, the $\alpha_{1a/d}$-AR shows an antagonist binding profile and CEC sensitivity that are inconsistent with its identity as an α_{1A} subtype (10, 12). Based on the known structural homology within α_1-AR subtypes, it seems unlikely that two receptor proteins that are 40% identical could still show virtually identical pharmacological properties. 2) The previous difficulties in detecting α_{1c}-AR mRNA in rat tissues using a bovine probe suggest that a negative result from a Northern analysis for this receptor subtype using the bovine α_{1c}-AR probe is not conclusive evidence for the absence of receptor mRNA and should be interpreted with caution. However, a more definitive resolution of this apparent controversy will have to wait until the genes encoding the α_1-ARs from guinea pig are cloned.

The relationship of the cloned α_1-AR subtypes to their pharmacologically defined counterparts in tissue preparations has recently been questioned (20, 29, 32). To address this we have cloned the rat homologue of the bovine α_{1c}-AR from a rat brain cDNA library. When expressed in COS-7 cells this cDNA

encodes an α_1-AR that has high affinity for α_{1A} subtype-selective antagonists and is relatively insensitive to inactivation by CEC. These pharmacological properties, as well as its mRNA expression in tissues known to be enriched in the α_{1A}-AR subtype, suggest that the rat α_{1c}-AR gene encodes the pharmacologically defined α_{1A} subtype. Further characterization awaits the development of more selective reagents to assay for receptor protein levels (subtype-selective ligands or antibodies) and will ultimately be needed to precisely define the physiological role of α_1-AR subtypes.

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