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Cloning, Expression, and Tissue Distribution of the Rat Homolog of the Bovine α_{1C}-Adrenergic Receptor Provide Evidence for Its Classification as the α_{1A} Subtype

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SUMMARY

Three α_1-adrenergic receptors (ARs) have been cloned, i.e., the α_{1B}-, α_{1C}-, and α_{1D}-ARs. Compared with the α_{1B} subtype, the α_{1A} subtype in tissue is described as being insensitive to chloroethylclonidine and sensitive to SZL-49 and having a 10–100-fold higher affinity for a number of agonists and antagonists. The α_{1A} subtype is also expressed in a variety of rat tissues (as assessed by pharmacology), with greatest abundance in the cerebral cortex, hippocampus, vas deferens, and submaxillary gland. The cloned bovine α_{1C}-AR, though having an α_{1A}-AR pharmacology, was first reported as not being expressed in any rat tissue (as determined by Northern analysis) and was therefore designated as a new subtype. We report the cloning, expression, and characterization of the rat homolog of the bovine α_{1C}-AR. Using a human α_{1C}-AR probe obtained by polymerase chain reaction screening of a neuroblastoma cell line (SK-N-MC), both exon 1 and exon 2 of the rat α_{1C}-AR gene were cloned from a rat genomic library. These two exons were spliced together and cloned into the expression vector pMT2′. Transfection into COS-1 cells and analysis of the ligand-binding profile of the expressed protein receptor using ^{125}I-HEAT revealed a 10–100-fold higher affinity for the α_1-AR antagonists 5-methylurapidil, (+)-niguldipine, WB-4101, and phentolamine and the agonists oxymetazoline and methoxamine, compared with the α_{1B}-AR. This ligand-binding profile is similar to that for endogenously expressed tissue α_{1A}-ARs. In addition, the rat α_{1C}-AR was the least sensitive of the three cloned subtypes to the alkylating effects of chloroethylclonidine but was the most sensitive to the alkylating prazosin analog SZL-49, properties also observed for the tissue α_{1A} subtype. Furthermore, by three different techniques, i.e., RNase protection assays, reverse transcription-polymerase chain reaction Northern blotting, and *in situ* hybridization histochemistry, the rat α_{1C}-AR mRNA was localized to α_{1A}-AR-rich tissues, such as rat vas deferens, hippocampus, aorta, and submaxillary gland. Taken together, these data suggest that this receptor may actually represent the α_{1A} subtype.

α_1-ARs mediate a variety of sympathetic nervous system responses, such as vascular smooth muscle and cardiac contraction, and thus play a major role in circulatory homeostasis. These receptors are coupled to their effector molecules by various G proteins. Evidence for heterogeneity of α_1-ARs was initially based upon studies using various rat tissues (1). Two major subtypes (α_{1A} and α_{1B}) were identified based on pharmacological studies and on their sensitivity to certain alkylating agents. The α_{1A} subtype was described as having a higher affinity (10–100-fold) than the α_{1B} subtype for the antagonists WB-4101, 5-methylurapidil, (+)-niguldipine, and phentolamine and the agonists oxymetazoline and methoxamine (1–4). Sensitivity to irreversible inactivation by alkylating analogs of clonidine and prazosin, CEC and SZL-49, respectively, has been used as a criterion for differentiating between the α_{1A}- and α_{1B}-ARs in various tissues. CEC irreversibly inactivates the α_{1B}-AR but not the α_{1A}-AR (5). In contrast, SZL-49 has been shown to completely inactivate the α_{1A}-AR while causing only partial inactivation of the α_{1B}-AR (6, 7).

Based on molecular cloning studies, three distinct α_1-AR subtypes (α_{1B}, α_{1C}, and α_{1D}) have been identified (8–10). An additional clone, which is identical to the α_{1D} clone, has also been described, and it has been suggested that the receptor encoded by this cDNA is the α_{1A}-AR (11). However, as a result of more detailed evaluations of the pharmacological properties of this receptor, it is now agreed that the α_{1D}-AR (or $\alpha_{1A/D}$-AR)

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ABBREVIATIONS: AR, adrenergic receptor; CEC, chloroethylclonidine; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pair(s); SSC, standard saline citrate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; [^{125}I]HEAT, 2-[β-(4-hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl]tetralone.

is a novel subtype (12). The α_{1C} subtype, as initially cloned from bovine tissue, was categorized as a novel subtype, although its pharmacological properties closely matched those of the α_{1A} subtype. The basis for this classification was its lack of expression in rat tissues, where the α_{1A} subtype had been clearly demonstrated in pharmacological studies, and its sensitivity to CEC. We now report the cloning of the gene and cDNA for the rat homolog of the bovine α_{1C}-AR and the characterization of the pharmacological properties of the expressed encoded protein. These studies reveal a ligand-binding profile that is similar to that of the rat α_{1A} subtype. The mRNA for the α_{1C}-AR is expressed in α_{1A}-AR-rich tissues, such as rat vas deferens, submaxillary gland, and hippocampus. In addition, the rat α_{1C}-AR is the least sensitive of the three cloned α_1-AR subtypes to the alkylating effects of CEC, and it is the most sensitive to SZL-49. Taken together, the data suggest that the α_{1C}-AR was misclassified and actually represents the "classical" α_{1A} subtype described originally in rat tissues.

Materials and Methods

PCR cloning of the human α_{1C}-AR. Highly degenerate primers [primer A, 5′-GAATTCGAATTCTT(T/C)TG(T/C)(A/G)A(T/C)(G/A)T(A/T)TGGG-3′; primer B, 5′-GAATTCGAATTC(A/G)AA(A/G)AA(T/C)GG(T/C)A(G/A)CCA(A/G)CC-3′] corresponding to the conserved third and sixth transmembrane domains of α_1-ARs were synthesized on a Milligen 8750 DNA synthesizer. The primers were designed with two *Eco*RI restriction sites at the 5′ ends to facilitate subcloning. Single-stranded cDNA was prepared from 1 μg of poly(A)$^+$ mRNA from the human cell line SK-N-MC, as described previously (10). PCR was then performed on one half of the sample, with 40 pmol of each primer (primer A plus primer B), 10 mM tricine, pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, deoxynucleotide triphosphates (200 μM concentrations of each base), and 2.5 units of *Thermus aquaticus* DNA polymerase. The amplification profile, run for 45 cycles, consisted of 1 min at 95°, 3 min at 45°, and 5 min at 73°, followed by a 15-min extension at 73°. The resulting DNA (564-bp fragment) was separated, subcloned into pBluescript, and screened for α_1 subtypes by dideoxy sequencing. To obtain a probe that would allow the cloning of the rat α_{1C}-AR, another set of degenerate primers were designed to isolate the 3′ end of the human α_{1C}-AR cDNA. These primers [primer C, 5′-GAATTCGAATTCATGGCTCTGCTCTGCGTCTGGGCACTCTCCCTGGTC-3′; primer D, 5′-GCGGCCGCGCGGCCGCGAC(A/G)TC(A/G)TCCCC(A/G)TT(C/T)TCACTGAGGGAGATGGT(A/G)TG-3′], corresponding to the fourth transmembrane region of the human α_{1C}-AR and the carboxyl-tail region of the bovine α_{1C}-AR, respectively, were used for PCR after RT of mRNA prepared from the human SK-N-MC cell line, exactly as described above. These primers contained two *Eco*RI and *Not*I restriction sites for subcloning. The DNA product (980 bp) obtained after 45 cycles of PCR was subcloned and sequenced.

Cloning of the rat α_{1C}-AR gene. To obtain the gene for the rat α_{1C}-AR, a rat genomic library (Stratagene) was screened separately with exon 1 and exon 2 probes, which were made from the human α_{1C}-AR cDNA generated as described above. A 368-bp exon 1 probe was obtained by *Eco*RI/*Xma*I digestion of the human α_{1C}-AR cDNA, whereas a 410-bp exon 2 probe was generated by *Not*I/*Eco*RV digestion of the human α_{1C}-AR cDNA. The exon 1 probe corresponded to the fourth through sixth transmembrane regions of the human α_{1C}-AR, whereas the exon 2 probe corresponded to the carboxyl-terminal region. These probes were used to isolate exon 1 and exon 2 of the gene for the putative rat α_{1C}-AR by plaque hybridization, as described (13).

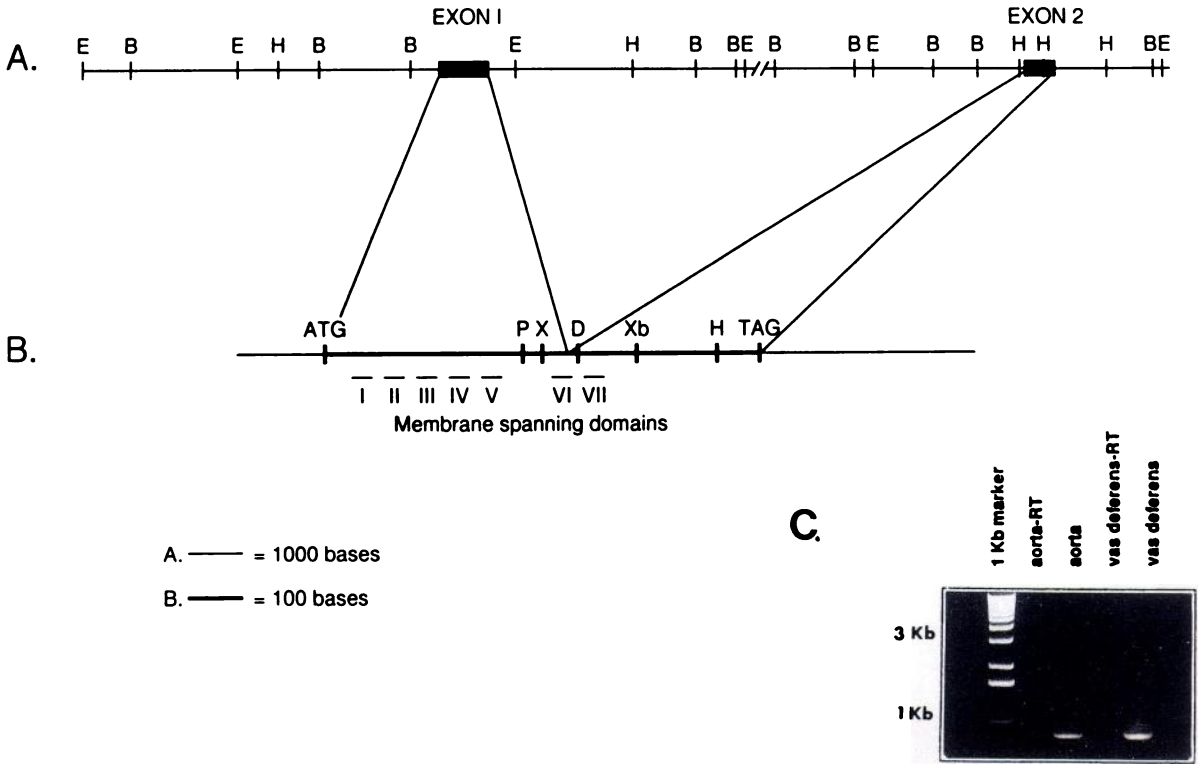

Fig. 1. Genomic organization of the rat α_{1C}-AR. A, Restriction map of the gene. The *line* representing the gene is interrupted at the location corresponding to the region in the intron that was not isolated with either the exon 1 or exon 2 clone. *Solid boxes*, exons. *Vertical lines*, restriction sites. B, Fusion construct of the rat α_{1C}-AR cDNA and detailed restriction map of the coding region. C, RT-PCR analysis of the rat α_{1C}-AR mRNA. PCR was performed as described in the text, with exon 1- and exon 2-specific primers that spanned the intron boundaries. PCR was performed on 3 μg of mRNA from either rat aorta or vas deferens, to confirm that exon 1 and exon 2 were spliced to form a single message *in vivo*. The 840-bp product is the size predicted if the mRNA species exists. *–RT lanes*, controls in which no reverse transcriptase was added to the mRNA in generating first-strand cDNA. *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hind*III; *P*, *Pst*I; *X*, *Xho*I; *D*, *Dra*I; *Xb*, *Xba*I; *ATG*, translation start site; *TAG*, termination codon.

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RAT      MVLLSENASEGSNCTHPPAPVNISKAILLGVILGGLIIFGVLGNILVILSVACHR    55
HUMAN    MVFLSGNASDSSNCTQPPAPVNISKAILLGVILGGLILFGVLGNILVILSVACHR    55
BOVINE   MVFLSGNASDSSNCTHPPPPVNISKAILLGVILGGLILFGVLGNILVILSVACHR    55
         **.**.***..****.**.******************.*****************

RAT      HLHSVTHYYIVNLAVADLLLTSTVLPFSAIFEILGYWAFGRVFCNIWAAVDVLCC    110
HUMAN    HLHSVTHYYIVNLAVADLLLTSTVLPFSAIFEVLGYWAFGRVFCNIWAAVDVLCC    110
BOVINE   HLHSVTHYYIVNLAVADLLLTSTVLPFSAIFEILGYWAFGRVFCNVWAAVDVLCC    110
         ********************************.************.*********

RAT      TASIMGLCIISIDRYIGVSYPLRYPTIVTQRRGVRALLCVWVLSLVISIGPLFGW    165
HUMAN    TASIMGLCIISIDRYIGVSYPLRYPTIVTQRRGLMALLCVWALSLVISIGPLFGW    165
BOVINE   TASIMGLCIISIDRYIGVSYPLRYPTIVTQKRGLMALLCVWALSLVISIGPLFGW    165
         ********************************.**..*******.************

RAT      RQPAPEDETICQINEEPGYVLFSALGSFYVPLAIILVMYCRVYVVAKRESRGLKS    220
HUMAN    RQPAPEDETICQINEEPGYVLFSALGSFYLPLAIILVMYCRVYVVAKRESRGLKS    220
BOVINE   RQPAPEDETICQINEEPGYVLFSALGSFYVPLTIILVMYCRVYVVAKRESRGLKS    220
         ********************************.**.**********************

RAT      GLKTDKSDSEQVTLRIHRKNVPAEGGGVSSAKNKTHFSVRLLKFSREKKAAKTLG    275
HUMAN    GLKTDKSDSEQVTLRIHRKNAPAGGSGMASAKTKTHFSVRLLKFSREKKAAKTLG    275
BOVINE   GLKTDKSDSEQVTLRIHRKNAQVGGSGVTSAKNKTHFSVRLLKFSREKKAAKTLG    275
         ********************....*.*..***.**********************

RAT      IVVGCFVLCWLPFFLVMPIGSFFPDFKPSETVFKIVFWLGYLNSCINPIIYPCSS    330
HUMAN    IVVGCFVLCWLPFFLVMPIGSFFPDFKPSETVFKIVFWLGYLNSCINPIIYPCSS    330
BOVINE   IVVGCFVLCWLPFFLVMPIGSFFPDFRPSETVFKIAFWLGYLNSCINPIIYPCSS    330
         ****************************.********.******************

RAT      QEFKKAFQ:IVLRIQCLRRRQSSKHALGYTLHPPSQALEGQHRDMVRIPVGSGETF    385
HUMAN    QEFKKAFQNVLRIQCLRRKQSSKHALGYTLHPPSQAVEGQHKDMVRIPVGSRETF    385
BOVINE   QEFKKAFQNVLRIQCLRRKQSSKHTLGYTLHAPSHVLEGQHKDLVRIPVGSAETF    385
         ******************.******.*******.**...****.*.********.***

RAT      YKISKTDGVCEWKFFSSMPQGSARITVPKDQSACTTARVRSKSFLQVCCCVGSSA    440
HUMAN    YRISKTDGVCEWKFFSSMPRGSARITVSKDQSSCTTARVRSKSFLEVCCCVGPST    440
BOVINE   YKISKTDGVCEWKIFSSLPRGSARMAVARDPSACTTARVRSKSFLQVCCCLGPST    440
         *.************.***.*.****..*..*.*.************.****.*.*.

RAT      PRPEENHQVPTIKIHTISLGENGEEV    466
HUMAN    PSLDKNHQVPTIKVHTISLSENGEEV    466
BOVINE   PSHGENHQIPTIKIHTISLSENGEEV    466
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Fig. 2. Amino acid sequences of the rat, human, and bovine α_{1C}-ARs. *, identical amino acids; ·, conserved amino acid changes; *blanks*, unconserved amino acid changes.

TABLE 1

Pharmacological characterization of the expressed rat α_{1c}-AR, Hamster α_{1B}-AR, and rat α_{1D}-AR

COS-1 cell membranes transfected with the pMT2′ expression vector containing either the rat α_{1C}-AR, hamster α_{1B}-AR, or rat α_{1D}-AR cDNA were incubated with the α_1-AR antagonist ^{125}I-HEAT, in the absence or presence of increasing concentrations of various agonists or antagonists. Each point represents the mean of at least two individual experiments performed in duplicate. Ten concentrations of each ligand were used, and the points were chosen to be on the linear portion of the displacement curve. K_i values were generated using the iterative, nonlinear, curve-fitting program LIGAND.

Ligand	K_i		
	Rat α_{1C}-AR	Hamster α_{1B}-AR	Rat α_{1D}-AR
		μM	
Agonists			
(−)-Norepinephrine	11.9 ± 0.7	13.5 ± 1.1	1.4 ± 0.1
(+)-Norepinephrine	168 ± 22	396 ± 40	26.6 ± 2.9
(−)-Epinephrine	4.4 ± 0.4	7.1 ± 0.7	0.13 ± 0.01
Phenylephrine	35.5 ± 2.5	27.7 ± 1.9	24.6 ± 3.7
Methoxamine	80.0 ± 0.8	2119 ± 124	661.0 ± 79.0
Oxymetazoline	0.20 ± 0.04	4.4 ± 0.3	36.9 ± 3.0
		nM	
Antagonists			
Phentolamine	11 ± 2	70 ± 20	90.5 ± 11
WB-4101	0.21 ± 0.003	6.2 ± 0.8	14.1 ± 1.2
5-Methylurapidil	3.35 ± 0.5	430 ± 51	282 ± 42
(+)-Niguldipine	0.06 ± 0.03	86.6 ± 11	230 ± 25
Abanoquil	1.13 ± 0.09	0.07 ± 0.01	1.58 ± 0.13
YM617	6.4 ± 0.7	8.5 ± 0.6	5.8 ± 0.5

Construction of the exon 1/exon 2 gene fusion construct. Exon 1 and exon 2 of the rat α_{1C}-AR gene were ligated to create a cDNA, by using a synthetic DNA linker that spanned the *Xho*I (at base position 858) (exon 1) to *Dra*I (at base position 988) (exon 2) restriction sites. This 130-bp linker encoded the rat α_{1C}-AR-coding sequence between these two restriction sites but lacked the single intron separating the two exons. The generated cDNA was cloned into the mammalian expression plasmid pMT2′, to yield pMT2′α_{1C}, and the resulting plasmid was sequenced to verify the nucleotide sequences at the 5′ and 3′ cloning sites and at the exon 1/exon 2 splice site.

Transfection of COS-1 cells and ligand binding. Plasmid DNA from the gene fusion construct, purified by CsCl gradient centrifugation and Biogel A-50m (Bio-Rad) column chromatography, was used to transfect COS-1 cells (American Type Culture Collection, Bethesda, MD) by the DEAE-dextran method, as described (14). Membranes from COS-1 cells transfected with pMT2′α_{1C} or plasmids containing the hamster α_{1B}-AR cDNA (pMT2′α_{1B}) or rat α_{1D}-AR cDNA (pMT2′α_{1D}) were prepared as described (10) and tested with various α_1-AR subtype-selective and nonselective agonists/antagonists in competition binding assays using the α_1-selective radioligand ^{125}I-HEAT. Nonspecific binding was determined in the presence of 100 μM phentolamine. Competition reactions (total volume, 0.25 ml) contained HEM buffer (20 mM HEPES, pH 7.5, 1.4 mM EGTA, 12.5 mM $MgCl_2$), 200 pM ^{125}I-HEAT, COS-1 membranes, and increasing amounts of unlabeled ligand. To avoid interassay variability, the ability of each ligand to compete for ^{125}I-HEAT binding by each of the three α_1-AR subtypes was examined in a single assay. 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 using a Packard Auto-gamma 500 counter. Binding data were analyzed by the iterative curve-fitting program LIGAND.

Inactivation by chemically reactive receptor probes. After initial characterization, the ability of CEC and SZL-49 to inactivate the expressed α_{1C}-, α_{1B}-, and α_{1D}-ARs was assessed. Membrane aliquots were incubated with various concentrations of CEC or SZL-49, in HEM buffer, for 10 min at 37°. After incubation, the reactions were diluted by the addition of 1.5 ml of ice-cold HEM buffer, and the membranes were centrifuged in a desktop microfuge. Washing and resuspension

TABLE 2

α_1-AR subtype pharmacology

Ligand[a]	K_i				
	"Classical" α_{1A}-AR	Rat α_{1C}-AR	Bovine α_{1C}-AR	Hamster α_{1B}-AR	Rat α_{1D}-AR
			nM		
WB-4101	0.2 ± 0.1	0.21 ± 0.03	0.6 ± 0.04	14 ± 5	8 ± 5
5-Methylurapidil	0.6 ± 0.1	3.3 ± 0.5	7.0	40 ± 10	148 ± 80
Phentolamine	1.0 ± 0.4	11 ± 2	10.0 ± 3.0	146 ± 48	138 ± 20
(+)-Niguldipine	0.06 ± 0.03	0.06 ± 0.03	80.0	47 ± 30	138 ± 46

[a] Values are based on those reported from competition binding studies for the α_{1A}-AR (based on three or four values for each ligand reported in Refs. 1–4), α_{1B}-AR (three to eight values) (1–4, 8–10, 12), α_{1D}-AR (two values) (Ref. 10 and this study), bovine α_{1C}-AR (two values) (9, 12), and rat α_{1C}-AR (this study).

TABLE 3

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

COS-1 cell membranes transfected with either the rat α_{1C}-AR, hamster α_{1B}-AR, or rat α_{1D}-AR were incubated with the indicated concentrations of CEC or SZL-49, for 10 min at 37°. The membranes were washed extensively, and then the level of receptor inactivation was quantitated as described in the text. Results are the means of three independent experiments for each subtype.

Agent	Inactivation		
	α_{1B}-AR	α_{1D}-AR	α_{1C}-AR
		%	
CEC			
10 μM	53.5	27.0	7.5
100 μM	83.5	56.0	39.0
SZL-49			
1 nM	5.5	12.6	43.5
10 nM	17.0	21.0	57.0
100 nM	24.0	33.0	74.0

were repeated three additional times. To control for noncovalently bound CEC and SZL-49 that may not have been completely removed from the membranes, timed-control reactions were run in which the ligand was added to the native membranes after the incubation period but immediately before the washing steps.

RNase protection assays. A PCR-generated fragment, corresponding to a region of the rat α_{1C}-AR gene encoding from the second transmembrane domain to approximately the end of the third intracellular loop, was subcloned into the *Eco*RI site of pBluescript KS. *Hind*III-linearized plasmid DNA was used to generate radiolabeled cRNA (~700 nucleotides) using T7 RNA polymerase, in the presence of ^{35}S-ribonucleotide ATP, using an Ambion Maxiscript kit. Total RNA from various rat tissues was prepared by the guanidinium isothiocyanate method (15) and was pelleted through a cesium chloride cushion by ultracentrifugation. Total RNA (30 μg) was hybridized with the cRNA (0.5×10^6 cpm total) at 42° for 18 hr, using an Ambion RPA kit. Unhybridized probe was digested by the addition of RNase A and T_1 (1/500 dilution), according to the manufacturer's directions. After ethanol precipitation, the sample was resuspended in formamide loading buffer and electrophoresed on a 6% sequencing gel.

RT-PCR Northern blots. PCR primers (primer E, 5′-GGGCCTTTGGCAGGGTGTTCTGCAATATC-3′; primer F, 5′-ACTTGTCCGTCTTGAGGCCGGACTTC-3′) corresponding to the first extracellular loop and the fifth/sixth loop region, respectively, of the rat α_{1C}-AR were used to amplify first-strand cDNA generated from the various rat tissue mRNAs. Using the same conditions for PCR as described for the isolation of the human α_{1C}-AR fragment, the PCR product generated after 45 cycles was separated on a 1.2% agarose gel, transferred onto nitrocellulose, and probed with an end-labeled oligonucleotide. This oligonucleotide was made to a region of the rat α_{1C}-AR sequence that was internal to the region of the cDNA that hybridized with the oligonucleotides used in the initial PCR. The 5′-end-labeled probe (5′-GGGGCGTCAGGGCTCTGCTC-3′) corresponded

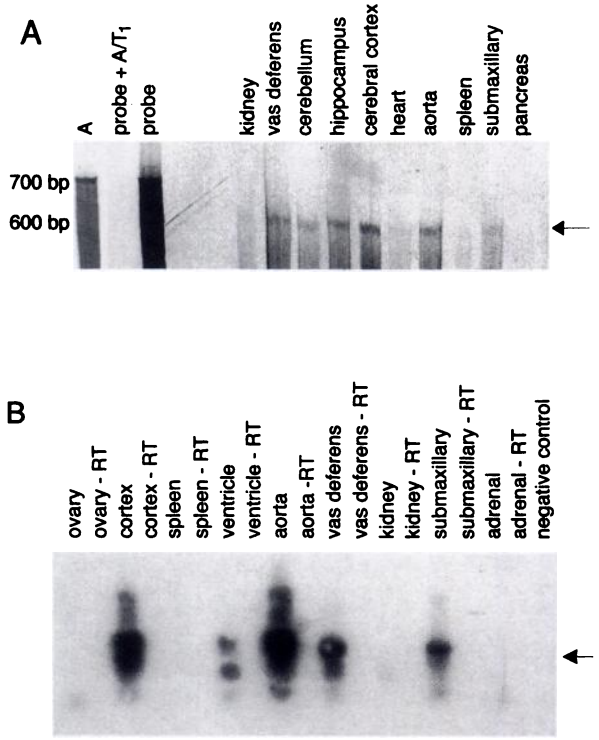

Fig. 3. RNase protection assays (A) and RT-PCR Northern analysis (B) of various mRNAs isolated from rat tissues. A, Total RNA (30 μg) from the indicated rat tissues was hybridized overnight at 42° with a 700-nucleotide cRNA probe, as described in the text. The mixture was then subjected to RNase A and T digestion and electrophoresis as described, which resulted in the identification of a protected 600-bp fragment ([larrow]). *Lane A*, 700-bp probe, which was deliberately underexposed to allow visualization. All other lanes were subjected to 14 days of autoradiography. B, mRNA (3 μg) from the indicated tissues was subjected to first-strand cDNA synthesis and then 45 cycles of PCR as described in the text. The DNA products were electrophoresed on a 1.2% agarose gel, transferred to nitrocellulose, and probed with a ^{32}P-labeled oligonucleotide specific for the rat α_{1C}-AR sequence. *Arrow* indicates predicted product. *–RT lanes*, control mRNA samples subjected to first-strand cDNA synthesis without reverse transcriptase.

to the second intracellular loop of the rat α_{1C}-AR. After hybridization overnight at 42° in 6× SSC, 5× Denhardt's solution, 0.1% SDS, the filters were washed at 60° in 6× SSC (1[times]SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% SDS, and subjected to autoradiography.

Riboprobe design for *in situ* hybridizations. The cDNA for the α_{1C}-AR was cloned into the *Eco*RI-*Not*I sites of pBluescript KSII. A sense probe for the α_{1C}-AR mRNA was synthesized by first cutting the plasmid with *Xma*III. Transcription with T7 RNA polymerase yielded a 372-nucleotide probe. The α_{1C}-AR antisense probe was constructed

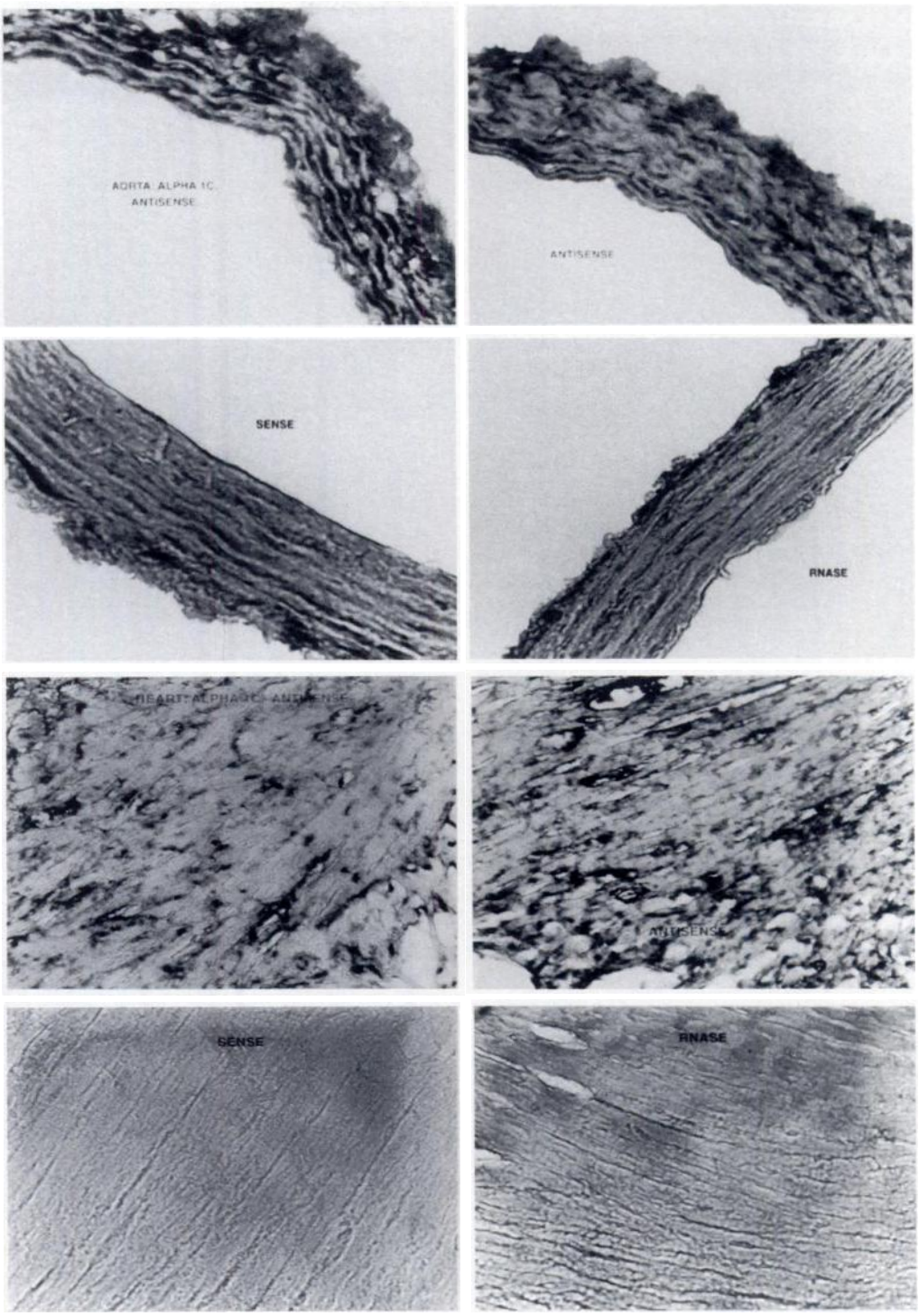

Fig. 4. Photomicrographs showing the α_{1C}-AR mRNA in rat aorta (A) and rat heart (B), detected using *in situ* hybridization histochemistry, as detailed in Materials and Methods. A and B, *Upper*, the tissues were probed using a biotinylated 477-nucleotide α_{1C}-AR cRNA. The hybridizing mRNA species are seen as diffuse black dotted regions, particularly over the nuclear zones. A and B, *Lower left*, hybridizations were performed with a α_{1C}-AR sense probe; *lower right*, the tissues were digested with RNase before hybridization with the cRNA probe.

by first cutting the plasmid containing the cDNA with *Dra*I. Transcription of this linearized plasmid with T3 RNA polymerase yielded a 477-nucleotide probe. The antisense probe was made to the carboxyl-terminal tail of the receptor. There is virtually no sequence homology between the α_{1C}-AR and either the α_{1D}- or α_{1B}-ARs in this region (10).

Preparation of biotinylated probes for the α_{1C}-AR. Biotinylated riboprobes for the α_{1C} mRNA were synthesized using an RNA-labeling system from GIBCO/BRL (catalog no. 8093 SA). Reactions contained transcription buffer, 5 mM dithiothreitol, 1 mM unlabeled ribonucleotides, 2 mM biotin-C_{14}-CTP, human placental RNase inhibitor, T3 or T7 RNA polymerase, and linearized DNA template. Transcription was allowed to proceed for 2.5 hr before the reaction was stopped by the addition of EDTA. Biotinylated riboprobes were separated from unincorporated nucleotides by centrifugation through Sephadex G-50 columns and were precipitated with ethanol. The final pellet was solubilized with 2× *in situ* hybridization buffer (4× SSC, 0.2 M sodium phosphate, 2× Denhardt's solution, 0.1 mg/ml sodium azide).

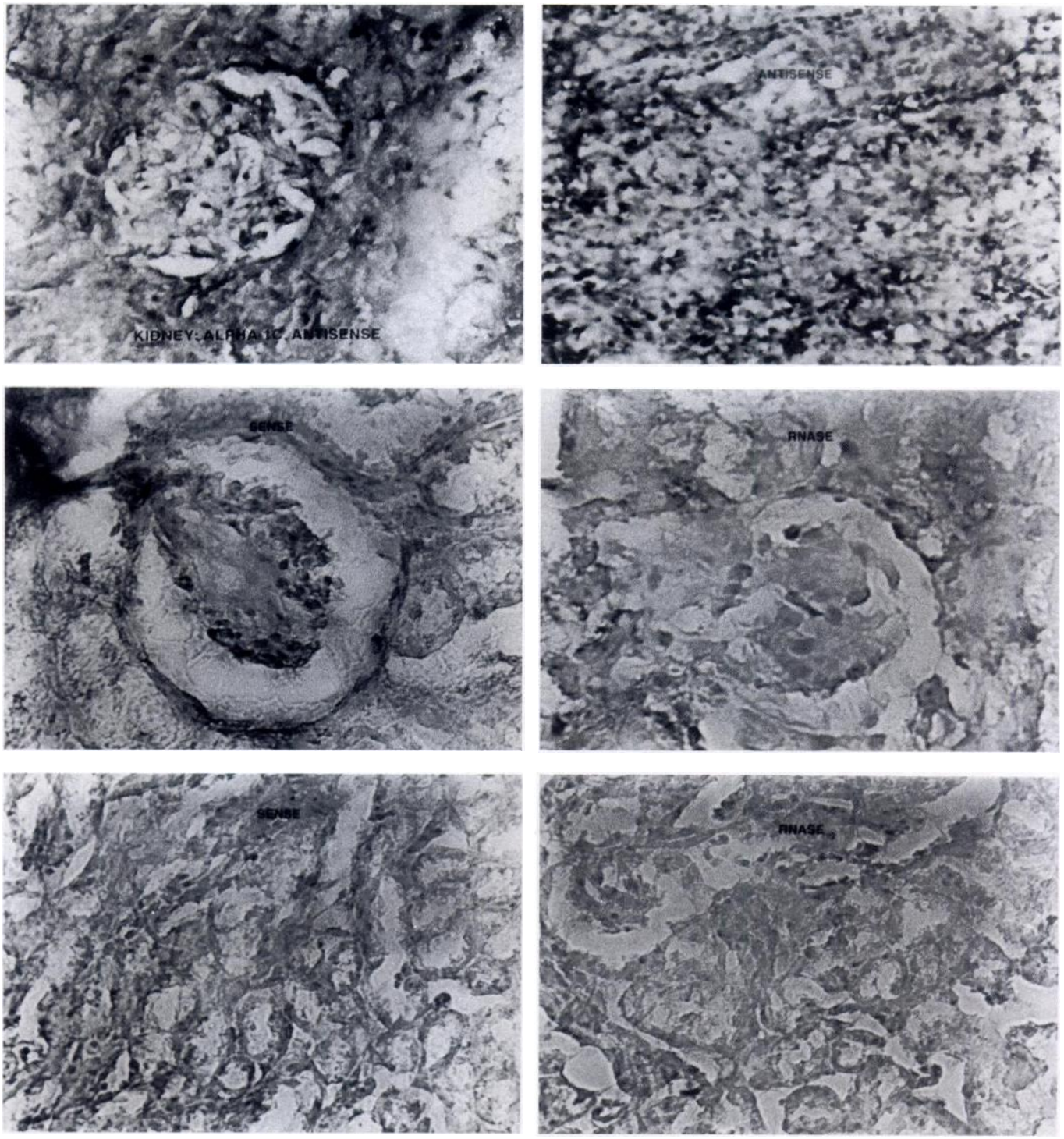

Fig. 5. Photomicrograph showing the α_{1C}-AR mRNA in rat kidney. *Upper*, sections probed with biotinylated 477-nucleotide α_{1C}-AR cRNA, as in Fig. 4 (*left*, cortex; *right*, medulla); *middle*, sense (*left*) and RNase (*right*) controls for the cortex; *lower*, sense (*left*) and RNase (*right*) controls for the medulla.

The final probe concentration was determined by quantitating absorbance at a wavelength of 260 nm.

***In situ* hybridization histochemistry.** Frozen 12-μm sections of aorta, heart, and kidney were prepared and stored at −70°. On the day of experimentation, sections were first fixed with 4% paraformaldehyde, rinsed with 2× SSC, and then treated with proteinase K (15 μg/ml) for 5 min at 25°. In certain experiments, sections were incubated with 100 μg/ml ribonuclease A for 1 hr at 37°, followed by two rinses in 2× SSC. The tissue was then acetylated by reaction for 10 min at 25° with a mixture of 0.25% acetic anhydride and 0.1 M triethanolamine, pH 8. After acetylation, the tissue was successively dehydrated using increasing concentrations (50, 70, 95, and 100%) of ethanol, with final delipidation by a 5-min incubation with chloroform. The sections were allowed to air-dry for 2 hr before hybridization. The hybridization solution contained 2× SSC, 0.1 M sodium phosphate, 1× Denhardt's solution, 10% dextran, 50% formamide, placental RNase inhibitor, and the appropriate riboprobe (80–150 ng/μl). This solution was applied directly to each slide, followed by careful placement of a coverslip. Rubber cement was placed around the coverslip to retain hydration. Hybridization was allowed to proceed for 18–20 hr. After that time the

coverslips were carefully removed and the slides were washed five times in 0.2× SSC. Detection of an cRNA-mRNA hybrid was accomplished by using a commercially available *in situ* hybridization and detection system (GIBCO/BRL catalog no. 8250SA). This system uses alkaline phosphatase bound to strepavidin to deposit a colored insoluble reaction product at the site of a cRNA-mRNA hybrid. After the slides were washed and treated with a blocking solution, the tissue sections were exposed to the strepavidin-alkaline phosphatase conjugate for 15 min at 25°. After being rinsed with Tris-buffered saline, pH 7.5, the slides were incubated with the alkaline phosphatase substrates nitroblue tetrazolium (0.3 mg/ml; GIBCO/BRL) and 4-bromo-5-chloro-3-indolylphosphate (0.166 mg/ml; GIBCO/BRL). The incubation time varied according to the tissue being probed. The reaction was stopped by rinsing in water and then dehydration with increasing concentrations of alcohol. The slides were then coverslipped and used for microscope examination.

Materials. Drugs were obtained from the following manufacturers: abanoquil, Pfizer, Inc.; WB-4101, 5-methylurapidil, and CEC, Research Biochemicals (Natick, MA); (−)-epinephrine, (−)-norepinephrine, phenylephrine, phentolamine, oxymetazoline, methoxamine, and prazosin, Sigma. (+)-Niguldipine was generously provided by Byk Gulden (Germany). SZL-49 was synthesized by the method of Pitha *et al.* (16). ^{125}I-HEAT and ^{35}S-ribonucleotide ATP were obtained from New England Nuclear-DuPont (Boston, MA). The human SK-N-MC cell line was a gift from Dr. Kenneth Minneman (Emory University). YM617 was a gift from the Yamaguchi Corp. (Japan).

Results and Discussion

The SK-N-MC human cell line was initially used to screen for the classical α_{1A} subtype because it had been reported to express a high percentage of the α_{1A}-AR, relative to the α_{1B}- or α_{1D}-ARs, as assessed by both ligand binding and functional assays (17). Using degenerate primers corresponding to highly conserved regions of the cDNAs of the α_1-AR family and RT-PCR, an appropriately sized PCR product was obtained. This provided evidence for the expression of mRNA for the human α_{1C}-AR in this cell line. Moreover, the amino acid sequence of the PCR-generated fragment, deduced from the nucleotide sequence in the transmembrane regions, was identical to that reported previously for the bovine α_{1C}-AR, with the exception of changes in the third base position of the codons. Such codon changes are expected for a species homolog. This verified that the isolated PCR fragment encoded the human α_{1C}-AR and not another α_1-AR subtype. The sequence of the human α_{1C}-AR was subsequently published (18), which also confirmed our assessment that the PCR product encoded the human α_{1C}-AR.

A region of the human α_{1C}-AR cDNA extending from the fourth transmembrane region to the carboxyl terminus was then generated by RT-PCR of the SK-N-MC cell line, for use in the generation of probes for screening of a rat genomic library. This approach was used because the α_{1C}-AR was reported not to be detectable by Northern analysis of any rat tissue and, therefore, was unlikely to be present in a cDNA library or was likely to be difficult to isolate by PCR without specific primers for the rat sequence. Based on the assumption that the genomic organization of the rat α_{1C}-AR would be similar to that of the human α_{1B}-AR (13), the rat genomic library was screened with an exon 1/exon 2 probe made from the human α_{1C}-AR cDNA. One exon 1 clone and two exon 2 clones were identified. The exon 1 clone contained a 2.5-kb *Bam*HI/*Eco*RI restriction fragment that encoded a sequence that was highly homologous to that of the bovine or human α_{1C}-AR but was interrupted in the coding region at the end of the putative sixth transmembrane region by an intron (Fig. 1A). The exon 2 clone contained a 6-kb *Bam*HI restriction fragment that encoded the rest of the coding region. The exon/intron boundaries, at the 3′ end of the exon 1 clone and the 5′ end of the exon 2 clone, contain the consensus splice sequence AG/GT at a type I splice phase, which is identical to that we observed for the human α_{1B}-AR gene (13). In additional studies we also isolated an exon 1 clone from a human genomic library by using the human α_{1C}-AR cDNA probe, and this clone also contains a similarly located intron at its 3′ end. This suggests that all α_1-ARs have an identical genomic organization. The intron for the rat α_{1C}-AR contains at least 14 kb and, thus, is comparable to the ~20-kb intron that we found in the human α_{1B}-AR gene (13).

To obtain a functional receptor, we elected to splice together the two exons and express the product in COS-1 cells to determine whether a functional α_{1C}-AR is encoded by the gene fusion construct (Fig. 1B). Fusion was simplified by the location of *Xho*I and *Dra*I restriction sites, which spanned the exon/intron boundaries and were only 130 bp apart. A synthetic oligonucleotide duplex corresponding to this region of the gene was synthesized and ligated in-frame with the exon 1 and exon 2 fragments. To confirm that, indeed, exon 1 and exon 2 are spliced together in the α_{1C}-AR mRNA, PCR primers were made to the rat α_{1C}-AR sequence spanning the intron boundaries and were used to amplify single-stranded cDNA prepared from rat vas deferens and aorta mRNA (Fig. 1C). The finding of an expected 840-bp product confirms that the two exons form a single message. The rat α_{1C}-AR cDNA contains a 1398-bp open reading frame encoding a protein of 466 amino acids, which is the same length reported for the bovine and human α_{1C}-ARs (Fig. 2). The amino acid identity between the rat α_{1C}-AR and the bovine α_{1C}-AR is 89% and that between the rat α_{1C}-AR and the human α_{1C}-AR is 93%.

To examine the pharmacological and biochemical characteristics of the rat α_{1C}-AR, the fusion construct was inserted into the mammalian expression vector pMT2′. The resulting construct, pMT2′α_{1C}, and the constructs for the hamster α_{1B}-AR (pMT2′α_{1B}) and rat α_{1D}-AR (pMT2′α_{1D}) were used to transiently transfect COS-1 cells. The level of expression of the rat α_{1C}-AR was approximately 0.8 pmol/mg of membrane protein, whereas those for the α_{1B}- and α_{1D}-ARs were 1.0 and 0.7 pmol/mg, respectively. Nontransfected COS-1 cells showed no specific ^{125}I-HEAT binding. Nonspecific binding was <10% of total binding for the α_{1B}- and α_{1C}-ARs and approximately 20% of total binding for the α_{1D} subtype.

Early classification of α_1 subtypes, based on pharmacological studies of tissues and cells, suggested the existence of two α_1-ARs (α_{1A} and α_{1B}). Those studies indicated differences in affinities for the antagonists WB-4101, phentolamine, 5-methylurapidil, and (+)-niguldipine and for the agonists oxymetazoline and methoxamine, with the α_{1A} subtype having at least a 10-fold higher affinity than the α_{1B}-AR for these ligands. Consistent with this α_{1A}-AR pharmacology, the affinity of the rat α_{1C}-AR for the agonists oxymetazoline and methoxamine was 10–100-fold higher than that of the hamster α_{1B}-AR (Table 1). In addition, the rat α_{1C}-AR had a 10–100-fold higher affinity for the antagonists (+)-niguldipine, 5-methylurapidil, WB-4101, and phentolamine, compared with those of the other two subtypes (Table 1). The affinity of the expressed rat α_{1C}-AR homolog for these compounds was also analogous to those

observed with the expressed bovine (9) and human (18) α_{1C}-ARs. The only significant exception in this regard was the K_i of 0.06 nM for (+)-niguldipine at the rat α_{1C}-AR, compared with a K_i value of 80 nM reported for the bovine α_{1C}-AR (9) (Table 2). This may be due to a true species difference in the ligand-binding properties of this receptor, because there is a preliminary report of a similar species difference being observed with this ligand (19).

Additional pharmacological studies involving the use of new, putatively α_{1A}-selective drugs revealed interesting results. In our studies, abanoquil (UK-52,046, a quinoline derivative) (20) was found to be "α_{1B}-selective," having at least a 10-fold higher affinity at α_{1B}-ARs than at the other two subtypes (Table 1). In addition, YM617 (a benzenesulfonamide) (21), although it had a high affinity, was not recognized selectively by any of the three subtypes. Currently, there are no α_1 subtype-selective drugs (i.e., drugs that show at least a 100–1000-fold increased affinity for one subtype, compared with the others) available, although there has been a preliminary report of a compound (SNAP-5150) that apparently displays a 100–500-fold higher affinity at the cloned human α_{1C}-AR than at the cloned α_{1D}- or α_{1B}-ARs (22). (+)-Niguldipine may be a candidate α_{1C} subtype-selective compound, showing a 1443-fold increased affinity at the α_{1C}-AR, compared with the α_{1B}-AR (Table 1). However, this ligand is very hydrophobic, light sensitive, and poorly soluble. This may result in a spuriously low affinity if the ligand is not used correctly, because nonspecific binding, precipitation, and inactivation may markedly reduce the concentration of free ligand available for interaction with the receptor. Distinguishing the α_{1B} and α_{1D} subtypes in tissues is also difficult, because both are sensitive to CEC and their affinities for the available agonists and antagonists are not markedly different. However, the use of (+)-norepinephrine might provide a differentiating factor, because the α_{1D}-AR has a 150-fold higher affinity for this agent.

A criterion that has been widely used to discriminate α_1 subtypes is their sensitivity to irreversible inactivation by CEC. α_{1B}- and α_{1A}-ARs have been described as being sensitive and insensitive to CEC, respectively. This classification was based on experiments in various tissue preparation where CEC inactivation correlated with the loss of low affinity sites for WB-4101 (i.e., α_{1B}-ARs), leaving only receptors with a high affinity for WB-4101 (i.e., α_{1A}-ARs). Previously described CEC inactivation experiments (9–11) with the three cloned α_1 subtypes (α_{1B}, bovine α_{1C}, and α_{1D}) were performed with differing concentrations of CEC and for varying times, making it difficult to correlate results. The original CEC inactivation experiments of Minneman and co-workers (4, 5), in which the α_{1A} subtype was first described in tissues, were performed using CEC concentrations of either 10 μM or 100 μM for only 10 min at 37°. Using exactly these conditions, we found that the rat α_{1C}-AR was the least sensitive of the three subtypes (Table 3) and, in fact, at 10 μM CEC the α_{1C}-AR was almost totally insensitive (7.5% inactivation). Previous studies reporting the bovine α_{1C}-AR as being partially inactivated (68%) were performed with 100 μM CEC for 20 min at 37° (9). This raises the following question: if the cloned rat α_{1C}-AR corresponds to the classical tissue-expressed α_{1A}-ARs, then why is it not insensitive to either 10 μM or 100 μM CEC? It is difficult to conclude from the original CEC experiments by Minneman and co-workers (4, 5), which were based on ligand binding studies with membranes prepared from tissue homogenates containing mixtures of α_1 subtypes, whether the α_{1A}-AR was completely insensitive to CEC or simply less sensitive than the α_{1B}-AR. It is quite possible that some fraction of the total population of the α_{1A}-AR in these tissue preparations was inactivated. In addition, additional evidence for the hypothesis that the rat α_{1C}-AR is indeed the α_{1A} subtype comes from our inactivation studies using the prazosin analog SZL-49 (Table 3). These studies indicate that the rat α_{1C}-AR is the most sensitive of the three receptors to alkylation by SZL-49, consistent with the finding that the α_{1A}-AR has the greatest sensitivity to this alkylating agent, both *in vitro* and *in vivo* (6, 7).

A finding with the bovine α_{1C}-AR that suggested that it represented a novel subtype was its inability to be detected in any rat tissue by Northern blot analysis. Using the rat α_{1C}-AR as a probe, we also were unable to detect transcripts for this receptor by Northern blot analysis. However, with the more sensitive RNase protection assays, we could detect appropriately sized protected products in rat vas deferens, cerebellum, hippocampus, cortex, heart, aorta, and submaxillary gland (Fig. 3A). No product could be detected in the pancreas. We speculate, therefore, that the rat α_{1C}-AR, though expressed from a transcript of low abundance, can be readily detected at the protein level in a variety of tissues, by radioligand binding and functional studies. Thus, for example, Esbenshade *et al.* (17) could demonstrate the predominant expression of α_{1A}-ARs in SK-N-MC cells, based on carefully performed functional and pharmacological studies, but could not identify a transcript for the α_{1C}-ARs by Northern blot analysis. Nevertheless, as detailed above, we were able to isolate a cDNA clone for the human α_{1C}-AR from the same batch of SK-N-MC cells (kindly provided by K. Minneman) used by Esbenshade *et al.* (17) for their pharmacological studies. Similarly, Simpson *et al.* (23) and Knowlton *et al.* (24) have clearly identified α_1-ARs of the α_{1A} subtype that mediate a hypertrophic response in rat cardiac myocytes. In these cells, Simpson and co-workers (25) have detected α_{1C}-AR mRNA using a probe prepared from a rat α_{1C}-AR RT-PCR product. Finally, α_{1A}-ARs have been identified in human prostate based on functional studies (26), and this tissue expresses mRNA for the human α_{1C}-AR (27).

Expression of the rat α_{1C}-AR mRNA was also confirmed by RT-PCR (Fig. 3B). Controls in which no reverse transcriptase was added were negative by PCR. In addition, the rat α_{1C}-AR could be localized to three rat tissues (heart, kidney, and aorta) by *in situ* hybridization histochemistry (Figs. 4 and 5). Controls in which the tissue was initially digested by RNase or in which "sense" probes to the rat α_{1C}-AR were used showed no specific hybridization. Taken together, these data indicate that the rat α_{1C}-AR mRNA, though low in abundance, can be detected in rat tissues and is observed in highest relative abundance in tissues where the classical α_{1A}-AR subtype has been described previously, such as rat vas deferens, hippocampus, and submaxillary gland.

The present study provides evidence to suggest that the rat α_{1C}-AR was misclassified and actually represents the α_{1A}-AR subtype. The rat α_{1C}-AR has 10–100-fold higher affinity for the agonists oxymetazoline and methoxamine and 10–100-fold higher affinity for the α_{1A}-selective antagonists WB-4101, 5-methylurapidil, (+)-niguldipine, and phentolamine. The rat α_{1C}-AR is less sensitive to inactivation by the alkylating agent CEC but has the greatest sensitivity to the alkylating prazosin

analog SZL-49. The unique pharmacology of this receptor, coupled with expression in rat tissues such as aorta, vas deferens, hippocampus, cerebral cortex, and submaxillary gland, indicates that this receptor most likely is of the α_{1A} subtype. Proof of this postulate will ultimately require additional studies. For example, the inability to demonstrate tissue α_{1A}-ARs, by radioligand binding or functional studies, in the tissues of rats in which the α_{1C}-AR gene had been totally inactivated would provide compelling evidence that the α_{1C}-AR is the α_{1A} subtype. Such studies are presently not possible, because gene disruption generally involves an embryonic stem cell/homologous recombination approach, which thus far has been applied successfully only in mice (28). Also, total inactivation of the α_{1C}-AR may be lethal. Alternative approaches to receptor inactivation using antisense technologies would only partially inactivate the receptors and, thus, may not provide a definitive result. More importantly, if one accepts that α_1-AR subtype analysis with alkylating agents is problematic, then the only reason for invoking the α_{1C}-AR as a novel subtype is the inability to demonstrate its expression in the predicted rat tissues. Because expression has now been demonstrated by the findings of the present study, we believe that a reclassification of α_1-ARs is justified. In this reclassification the classical α_{1A}-AR and the cloned α_{1C}-AR are now designated as either the α_{1A} or $\alpha_{1A/C}$ subtype, the α_{1B} subtype remains unchanged, and the α_{1D} (or $\alpha_{1A/D}$) subtype is now designated only as the α_{1D} subtype.

While this manuscript was being revised, an original article by Simpson and co-workers (29) and a review by Ford *et al.* (30) were published; both papers support our contention that the receptor encoded by the cDNA for the rat homolog of the bovine α_{1C}-AR is equivalent to the classical α_{1A} subtype, as described in functional studies of rat tissues (1–4).

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