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Cloning and Pharmacological Characterization of Human *Alpha*-1 Adrenergic Receptors: Sequence Corrections and Direct Comparison with Other Species Homologues[1]

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ABSTRACT

We have cloned cDNAs encoding three human *alpha*-1 adrenergic receptor (AR) subtypes and characterized pharmacological properties of the expressed receptor protein. A number of significant sequence corrections have been identified and compared with previously published data, at both nucleotide and amino acid levels; the most major differences occur for the human *alpha*-$1_{a/d}$AR. Pharmacological characterization was performed simultaneously using six cloned *alpha*-1AR subtypes (human and rat *alpha*-$1_{a/d}$, human and hamster *alpha*-1_b, human and bovine *alpha*-1_c) stably expressed in rat-1 fibroblasts at approximately equal receptor concentrations (1–2 pmol/mg of total protein). In general, human *alpha*-1AR subtypes have similar pharmacology compared to their rat, hamster and bovine homologs, although a few minor species differences important for *alpha*-1AR classification are noted. In addition, much lower inactivation (≈20%) by the alkylating agent chloroethylclonidine is noted in this study compared to previous reports for both human and bovine *alpha*-1_cAR membrane preparations. All six *alpha*-1AR subtypes couple to phosphoinositide hydrolysis in a pertussis toxin-insensitive manner, including the cloned human *alpha*-$1_{a/d}$AR which had not been expressed previously. In spite of significant sequence differences between human *alpha*-1ARs and their other species counterparts, previously established ligand selectivity remains fairly comparable. In summary, these data represent the first side-by-side comparison of pharmacological properties between species homologs of *alpha*-1AR subtypes and should facilitate the development of *alpha*-1AR subtype selective drugs for clinical use.

Alpha-1-ARs are members of the G-protein-coupled receptor family, coupling *via* Gq to the hydrolysis of membrane phospholipids and ultimately to smooth muscle contraction (Minneman, 1988a; McGrath *et al.*, 1989; Ruffolo *et al.*, 1991; Lomasney *et al.*, 1991a). *Alpha*-1ARs have been implicated in many human diseases such as benign prostatic hypertrophy, hypertension, myocardial hypertrophy and myocardial arrhythmias (Caine *et al.*, 1975; Nichols and Ruffolo, 1991; Terzic *et al.*, 1993). Two subtypes of *alpha*-1ARs (*alpha*-1_A and *alpha*-1_B) have been defined pharmacologically (Morrow and Creese, 1986; Han *et al.*, 1987; Minneman *et al.*, 1988b; Gross *et al.*, 1988), whereas cDNAs encoding three *alpha*-1ARs [cloned rat *alpha*-$1_{a/d}$ (also referred to as *alpha*-1_a or *alpha*-1_d), hamster *alpha*-1_b and bovine *alpha*-1_c] have been identified by using molecular techniques (Cotecchia *et al.*, 1988; Schwinn *et al.*, 1990; Lomasney *et al.*, 1991b, Perez *et al.*, 1991, Schwinn and Lomasney, 1992). In this manuscript we refer to pharmacologically defined *alpha*-1AR subtypes by upper case subscripts (*e.g.* *alpha*-1_B) and cloned *alpha*-1AR subtypes by lower case subscripts (*e.g.* *alpha*-1_b), in analogy to current muscarinic nomenclature.

The role of *alpha*-1ARs in many human diseases, together with the development of *alpha*-1AR subtype-selective agonists and antagonists, make it important to define the exact pharmacological properties of cloned human *alpha*-1AR subtypes. This is especially true for the human *alpha*-$1_{a/d}$AR,

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ABBREVIATIONS: AR, adrenergic receptor; CEC, chloroethylclonidine; bp, base pair; SSC(1X) (150 mM NaCl and 15 mM sodium citrate; pH 7); SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; TE solution/buffer, (5 mM Tris HCl and 5 mM EDTA, pH 7.4); HEAT, 2-{β-(4-hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl}-tetralone; PBS, phosphate-buffered saline; IP, inositol phosphate; NE, norepinephrine; PI, phosphoinositide.

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because this cDNA has never been expressed and characterized pharmacologically (Bruno *et al.*, 1991). In addition, it is important to compare expressed human *alpha*-1ARs with those of other species to evaluate species differences in pharmacological properties of the receptor subtypes (as has been reported for human and mouse *alpha*-2AR homologs) (Link *et al.*, 1992). We have therefore cloned three human *alpha*-1AR cDNAs, expressed them in rat-1 fibroblasts in approximately equal concentrations (1–2 pmol/mg of total protein) and compared their pharmacological profile to their rat, hamster and bovine counterparts. Significantly, our human *alpha*-1AR cDNAs demonstrate substantial deviation in nucleotide sequence compared to recently published reports, particularly for the human *alpha*-$1_{a/d}$. These sequence differences result in significant changes at the nucleotide and amino acid levels in regions potentially involved in receptor regulation (amino terminus and carboxyl terminus) as well as isolated amino acid changes which could impact ligand binding and interpretation of structure-function relationships in these receptors. In addition to examining ligand binding characteristics by competition experiments and saturation binding isotherms, we report CEC sensitivity and second messenger coupling assays (phosphoinositide hydrolysis with and without pertussis toxin) obtained simultaneously for all six cloned *alpha*-1AR receptors. These results represent the first demonstration of detailed pharmacology of human *alpha*-1AR subtypes as well as direct comparison with previously cloned *alpha*-1ARs from other species.

Methods

Isolation of *alpha*-1AR cDNA clones. General cloning methods utilized in isolating human *alpha*-1AR cDNA clones are listed first, followed by the exact cloning strategy used for each individual *alpha*-1AR subtype. Human heart and prostate cDNA libraries (Stratagene [La Jolla, CA] and Clontech [Palo Alto, CA], respectively) and a human genomic library (Clontech) were used to clone three human *alpha*-1AR cDNAs. Portions of previously published *alpha*-1AR cDNAs were used as initial probes: rat *alpha*-$1_{a/d}$AR probe (686 bp PvuII fragment, nucleotide no. 433-1119), hamster *alpha*-1_bAR probe (905 bp PvuII fragment, nucleotide no. 131-1036) and bovine *alpha*-1_cAR probe (694 bp BglII/PvuII fragment, nucleotide no. 356-1050). DNA probes were labeled with ^{32}P by using random-priming and were added to prehybridization solution (50% formamide, 5× Denhardt's and 5× SSC, 0.1% SDS, 100 μg/ml of denatured fish sperm DNA) to give a specific activity for each probe of 5×10^5 cpm/ml. After an overnight incubation at 42°C, the filters were washed to 55°C in 0.2 × SSPE 1(×) (150 mM NaCl, 20 mM EDTA and 10 mM NaH_2PO_4), pH 7.4 and 0.1% SDS, and were subjected to autoradiography. Positive clones were subjected to two further rounds of plaque purification. DNA inserts were released from the phage DNA by EcoRI digestion and ligated into pSP72 (Promega Biotec, Madison, WI). Further cDNA library and genomic library screenings to obtain missing portions of clones were performed by initially screening amplified library subpools by polymerase chain reaction, then plating out positive pools at 10,000 plaque-forming units per 230-mm^2 plate. Sequencing of final full-length cDNAs encoding each human *alpha*-1AR subtype was performed manually by using standard ^{35}S-dideoxy sequencing techniques as well as by using automated fluorescent methods.

Cloning of the human *alpha*-$1_{a/d}$ cDNA was accomplished first by screening a human prostate cDNA library (Clontech), from which a portion of the coding region just before the NotI site (nucleotide no. 638) through the 3′ untranslated sequence was obtained. This DNA fragment was then used to screen a human leukocyte genomic library (Clontech, one donor) and the rest of the coding sequence (as well as some 5′ sequences) was obtained. Once the sequence was known from these two cDNA libraries, the region between the initial ATG and the NotI site was amplified by using PCR to optimize the Kozak consensus sequence, and the two fragments were spliced together. The entire coding sequence was fully sequenced including matching PCR sequence with original genomic coding sequence, and ensuring splice sites were joined correctly. In the case of the *alpha*-1_bAR, while studies were in progress to complete cloning of this cDNA from a human heart cDNA library (Stratagene), the sequence of the human *alpha*-1_b gene was published (Ramarao *et al.*, 1992). Therefore, the missing portion of this gene (the carboxyl terminal third of the clone) was amplified from human heart mRNA (Clontech) by using PCR (1 min at 94°C, 1 min at 55°C and 2.5 min at 72°C for 30 cycles in the presence of 10% formamide) with primers derived from the published sequence. PCR products were subcloned into the pCRII vector (InVitrogen) and subjected to sequence analysis. Sequencing of several distinct PCR products ($n \geq 3$) was performed to confirm the sequence in regions in which PCR had been used; differences with previously published sequences were noted only when at least three identical PCR products differed from published results. The human *alpha*-1_cAR was cloned from a human prostate cDNA library (Clontech, 65-year-old patient), and a large fragment encoding the initial two-thirds of the coding sequence was obtained. The carboxyl one-half of the coding sequence was obtained from another human prostate cDNA library (Clontech, 25-year-old patient) and the overlapping sequence was validated. The entire coding block was then amplified by using PCR and the sequence again was validated against original cDNA sequences.

Stable expression of human *alpha*-1AR subtypes in rat-1 fibroblasts. Transfection constructs consisted of the appropriate human, rat, hamster or bovine *alpha*-1AR coding sequence inserted in either pZipNeo (Cepko *et al.*, 1984) or pcDNA3 (InVitrogen) vectors. Transfection of each cDNA into rat-1 fibroblasts was accomplished by using the calcium phosphate precipitation method as described previously (Cullen, 1987). Individual colonies were isolated, subcloned and screened for high expression (1–2 pmol/mg of total protein). Rat-1 fibroblasts and transfected rat-1 fibroblasts stably expressing either rat *alpha*-$1_{a/d}$, human *alpha*-$1_{a/d}$, hamster *alpha*-1_b, human *alpha*-1_b, bovine *alpha*-1_c or human *alpha*-1_cARs were grown in monolayers in DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) in 5% CO_2 at 37°C. Selection was maintained in cells expressing *alpha*-1AR subtypes by adding the antibiotic G418 (0.8 mg/ml) to the media.

Radioligand binding. Rat-1 cells stably expressing individual *alpha*-1AR subtypes from culture flasks (150 cm^2) were scraped into 10 ml of TE. A lysate was prepared with a Brinkman Polytron (model PT3000, setting 8 for 10 sec); after pelleting, membranes were resuspended in 150 mM NaCl, 50 mM Tris HCl and 5 mM EDTA, pH 7.4, quickly frozen and stored at −70°C. Binding of the *alpha*-1-adrenergic antagonist [^{125}I]HEAT (DuPont-New England Nuclear, Boston, MA) was measured using a total volume of 0.25 ml including diluted membranes (5–10 μg of total protein) in 150 mM NaCl, 50 mM Tris HCl and 5 mM EDTA, pH 7.4, with protease inhibitors (5 μg/ml leupeptin, 10 μg/ml of benzamidine and 10 μg/ml of soybean trypsin inhibitor). For saturation binding isotherms, [^{125}I]HEAT concentrations ranged from 10 to 960 pM. Competition curves were performed in triplicate, with a final [^{125}I]HEAT concentration of 100 pM; agonist competition was performed in the presence of 1 μM ascorbic acid to prevent agonist oxidation. Incubations were at 25°C for 45 min; the reaction was terminated with ice-cold 50 mM Tris HCl (pH 7.4) and the entire reaction mixture was filtered rapidly over GF/C filters by using a Brandel harvester. Nonspecific binding was measured in the presence of 1 μM prazosin with nonspecific binding 5 to 10% of total counts. Curves were fit by using noniterative regression analysis with InPlot (Graphpad, San Diego, CA).

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CEC inactivation. For CEC experiments, 100 μM CEC was incubated with transfected rat-1 fibroblast cell membrane lysates (50–100 μg of protein) in hypotonic TE at 37°C for 10 and 20 min. The reaction was stopped by addition of 4 ml of ice-cold TE, followed by immediate centrifugation at 40,000 × *g* at 4°C. Membranes were then washed 2 to 3 more times with 5 ml of ice-cold PBS before ligand binding. A saturating concentration of [^{125}I]HEAT (300 pM final) was used to determine *alpha*-1AR receptor number before and after CEC treatment in triplicate.

IP determination in intact cells. Rat-1 fibroblasts stably expressing individual *alpha*-1AR subtypes, grown in 30-mm dishes (1 × 10^6 cells/dish), were labeled with [^{3}H]inositol (DuPont-New England Nuclear) for 18 hr at 2.5 μCi/ml in DMEM supplemented with 5% fetal bovine serum (GIBCO). After labeling, cells were washed and incubated in PBS (no added calcium) for 30 min, followed by a 30-min incubation in PBS with 20 mM LiCl. IPs were extracted as described by Martin (1983) and were separated on AG 1-X8 columns (0.8 ml packed, 100–200 mesh) in the formate phase (Berridge *et al.*, 1983). Total IPs were eluted with 1 M ammonium formate-0.1 M formic acid. For experiments with pertussis toxin, incubation with 100 ng/ml of pertussis toxin was performed for 18 hr before initiation of the PI experiment.

Materials. Drugs and reagents were obtained from the following sources: EDTA, epinephrine, methoxamine, NE, oxymetazoline, phentolamine, phenylephrine, prazosin and propranolol (Sigma Chemical Co., St. Louis, MO); 5-methylurapidil, (+)-niguldipine, spiperone and WB4101 (Research Biochemical Inc., Wayland, MA); and pertussis toxin (List Biological Laboratories, Inc. (Campbell, CA). Terazosin was a gift from Dr. Kenneth Minneman (Emory University). For drugs that exist as racemic mixtures, the (−)-isomer was used unless indicated otherwise.

Results

Nucleotide sequence differences with previously cloned human *alpha*-1AR cDNAs. Comparisons of the translated amino acid sequences for all three cloned human *alpha*-1AR cDNAs and their mammalian counterparts (rat, hamster and bovine) are shown in figure 1; differences between our sequences and those published previously (Bruno *et al.*, 1991; Ramarao *et al.*, 1992; Hirasawa *et al.*, 1993) are highlighted in boxes. The largest variation between our cloned human *alpha*-1AR cDNAs and other previously published sequences occurs for the human *alpha*-$1_{a/d}$AR subtype. The translated sequence of the human *alpha*-$1_{a/d}$AR contains 572 amino acids; comparison with the human *alpha*-$1_{a/d}$ clone published by Bruno *et al.* (1991) reveals that our clone does not match bases 1 to 178 of this human sequence in spite of the fact that this region of our clone is homologous with the rat *alpha*-$1_{a/d}$AR (Lomasney *et al.*, 1991b; Perez *et al.*, 1991). When bases 1 to 172 are reverse-complemented and compared to the entire human *alpha*-$1_{a/d}$AR coding sequence, this fragment matches bases 454 to 626, suggesting the presence of a cloning artifact at the 5′ end of the previously published human *alpha*-$1_{a/d}$AR cDNA (Bruno *et al.*, 1991). Another discrepancy between our human *alpha*-$1_{a/d}$AR cDNA and that published by Bruno *et al.* (1991) occurs in the carboxyl terminus where again significant deviations in amino acid sequence are noted. Of note, the human *alpha*-$1_{a/d}$AR clone published by Bruno *et al.* (1991) also differs in this region from previously reported rat *alpha*-$1_{a/d}$AR sequences (Lomasney *et al.*, 1991b; Perez *et al.*, 1991). In order to clarify these sequencing differences, in addition to cloning the human *alpha*-$1_{a/d}$AR cDNA, we also resequenced the identical rat *alpha*-$1_{a/d}$AR clone published by Lomasney *et al.* (1991b). Resequencing the rat *alpha*-$1_{a/d}$ cDNA in this region indicates two errors, a two bp insertion (GC) at base 1749 followed 177 bp later by a single base insertion (C), leading to a 59 amino acid frame shift in the carboxyl terminus; this mistake is present in both published rat *alpha*-$1_{a/d}$ cDNAs (Lomasney *et al.*, 1991b; Perez *et al.*, 1991) and may have resulted from the GC rich nature of this region which makes sequencing more difficult. When these corrections are inserted into the rat *alpha*-$1_{a/d}$AR sequence, the reading frame shows greater alignment to the human sequence and homology increases significantly from 83 to 87%. Close to the area in which this marked divergence is noted between our human and rat *alpha*-$1_{a/d}$ cDNAs, we found a single base missing in the published human sequence (Bruno *et al.*, 1991) (at bases 1393–1394 there are two adenines in the published sequence instead of three in our sequence). This results in the translated sequence moving into another reading frame, and accounts for differences between the published human *alpha*-$1_{a/d}$ clone (Bruno *et al.*, 1991) and rat *alpha*-$1_{a/d}$AR cDNAs (Lomasney *et al.*, 1991b; Perez *et al.*, 1991). Other minor differences between the published human *alpha*-$1_{a/d}$AR sequence and our sequence include a silent base change at 1358 (C to A) and a GC inversion at base 1492 to 1493. Due to the complexity of these corrections in the human *alpha*-$1_{a/d}$AR nucleotide and translated amino acid sequences, the open-reading frame of the human *alpha*-$1_{a/d}$AR is given in figure 2.

Fewer differences between our cloned sequences and previously published sequences were found for human *alpha*-1_b and *alpha*-1_c subtype cDNAs. The human *alpha*-1_bAR and *alpha*-1_cAR are 95 and 92% identical to the rat *alpha*-1_b and bovine *alpha*-1_c, respectively. Comparison of our human *alpha*-1_bAR sequence to the previously isolated human *alpha*-1_bAR gene sequence (Ramarao *et al.*, 1992) showed identity, except for a three bp insertion (CGC) at base 1114 which adds another arginine residue, three single guanine insertions after bases 1486, 1490 and 1492 and a base substitution at base 1489 (A to G). These latter changes alter three amino acids (P-R-H) to encode four amino acids (A-A-A-D) in the human *alpha*-1_bAR. These changes close a gap in the published comparison between hamster and human *alpha*-1_BAR with the new human sequence being now more homologous to the hamster (A-T-T-D). In addition, there is a sequence difference between the publication and the Genbank entry for the human *alpha*-1_b (Ramarao *et al.*, 1992); our sequence at amino acid 33 to 35 (S-N-S) and at amino acid 486 (D) is identical to the sequence described in the manuscript (Ramarao *et al.*, 1992), but differs from the R-G-G and E present in Genbank, respectively. Comparison of our human *alpha*-1_cAR nucleotide sequence to the recently published human *alpha*-1_cAR sequence shows identity except for base 1727, which is a C in our sequence instead of a G. This changes the glutamic acid at position 431 (GAG) to a glutamine (CAG), which is the same residue that is found in the bovine *alpha*-1_cAR. It is important to note at this point that single bp differences could be the result of ethnic or other naturally occurring polymorphisms. However, inasmuch as changes noted above result in closer alignment between human and nonhuman homologs, the possibility of polymorphisms becomes less likely. Corrected nucleotide sequences for all three human *alpha*-1AR cDNAs, as well as for the rat *alpha*-$1_{a/d}$, have been entered into Genbank.

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              1
α1a/d human   MTFRDLLSVS  FEGPRPDSSA  GGSSAGGGGG  SAGGAAPSEG  PAVGGVPGGA
α1a/d rat     MTFRDILSVT  FEGPRSSSST  GGSGAGGGAG  TVG....PEG  TAVGGVP GA
α1b human     ..........  ..........  ..........  ..........  ..........
α1b hamster   ..........  ..........  ..........  ..........  ..........
α1c human     ..........  ..........  ..........  ..........  ..........
α1c bovine    ..........  ..........  ..........  ..........  ..........

              51                                                    100
α1a/d human   GGGGGVVGAG  SGEDNRSSAG  EPGSAGAGGD  VNGTAAVGGL  VVSAQGVGVG
α1a/d rat     TGGGAVVGTG  SGEDNQSSTG  EPG.AAASGE  VNGSAAVGGL  VVSAQGVGVG
α1b human     .MNPDLDTGH  NTSAPAHWGE  LKNANFTGPN  QTSSNSTLPQ  LDITRAISVG
α1b hamster   .MNPDLDTGH  NTSAPAQWGE  LKDANFTGPN  QTSSNSTLPQ  LDVTRAISVG
α1c human     ..........  ..........  MVFLSGNASD  SSNCTQPPAP  VNISKAILLG
α1c bovine    ..........  ..........  MVFLSGNASD  SSNCTHPPPP  VNISKAILLG

              101                                                   150
α1a/d human   VFLAAFILMA  VAGNLLVILS  VACNRHLQTV  TNYFIVNLAV  ADLLLSATVL
α1a/d rat     VFLAAFILTA  VAGNLLVILS  VACNRHLQTV  TNYFIVNLAV  ADLLLSAAVL
α1b human     LVLGAFILFA  IVGNILVILS  VACNRHLRTP  TNYFIVNLAM  ADLLLSFTVL
α1b hamster   LVLGAFILFA  IVGNILVILS  VACNRHLRTP  TNYFIVNLAI  ADLLLSFTVL
α1c human     VILGGLILFG  VLGNILVILS  VACHRHLHSV  THYYIVNLAV  ADLLLTSTVL
α1c bovine    VILGGLILFG  VLGNILVILS  VACHRHLHSV  THYYIVNLAV  ADLLLTSTVL

              151                                                   200
α1a/d human   PFSATMEVLG  FWAFGRAFCD  VWAAVDVLCC  TASILSLCTI  SVDRYVGVRH
α1a/d rat     PFSATMEVLG  FWAFGRTFCD  VWAAVDVLCC  TASILSLCTI  SVDRYVGVRH
α1b human     PFSAALEVLG  YWVLGRIFCD  IWAAVDVLCC  TASILSLCAI  SIDRYIGVRY
α1b hamster   PFSATLEVLG  YWVLGRIFCD  IWAAVDVLCC  TASILSLCAI  SIDRYIGVRY
α1c human     PFSAIFEVLG  YWAFGRVFCN  IWAAVDVLCC  TASIMGLCII  SIDRYIGVSY
α1c bovine    PFSAIFEILG  YWAFGRVFCN  VWAAVDVLCC  TASIMGLCII  SIDRYIGVSY

              201                                                   250
α1a/d human   SLKYPAIMTE  RKAAAILALL  WVVALVVSVG  PLLGWKEPVP  PDERFCGITE
α1a/d rat     SLKYPAIMTE  RKAAAILALL  WAVALVVSVG  PLLGWKEPVP  PDERFCGITE
α1b human     SLQYPTLVTR  RKAILALLSV  WVLSTVISIG  PLLGWKEPAP  NDDKECGVTE
α1b hamster   SLQYPTLVTR  RKAILALLSV  WVLSTVISIG  PLLGWKEPAP  NDDKECGVTE
α1c human     PLRYPTIVTQ  RRGLMALLCV  WALSLVISIG  PLFGWRQPAP  EDETICQINE
α1c bovine    PLRYPTIVTQ  KRGLMALLCV  WALSLVISIG  PLFGWRQPAP  EDETICQINE

              251                                                   300
α1a/d human   EAGYAVFSSV  CSFYLPMAVI  VVMYCRVYVV  ARSTTRSLEA  GVKRERGKAS
α1a/d rat     EVGYAIFSSV  CSFYLPMAVI  VVMYCRVYVV  ARSTTRSLEA  GIKREPGKAS
α1b human     EPFYALFSSL  GSFYIPLAVI  LVMYCRVYIV  AKRTTKNLEA  GVMKEMSNSK
α1b hamster   EPFYALFSSL  GSFYIPLAVI  LVMYCRVYIV  AKRTTKNLEA  GVMKEMSNSK
α1c human     EPGYVLFSAL  GSFYLPLAII  LVMYCRVYVV  AKRESRGLKS  GLKTDKSDSE
α1c bovine    EPGYVLFSAL  GSFYVPLTII  LVMYCRVYVV  AKRESRGLKS  GLKTDKSDSE

              301                                                   350
α1a/d human   EVVLRIHCRG  AATGADGAHG  MRSAKGHTFR  SSLSVRLLKF  SREKKAAKTL
α1a/d rat     EVVLRIHCRG  AATSAKGYPG  TQSSKGHTLR  SSLSVRLLKF  SREKKAAKTL
α1b human     ELTLRIHSKN  FHEDT...LS  STKAKGHNPR  SSIAVKLFKF  SREKKAAKTL
α1b hamster   ELTLRIHSKN  FHEDT...LS  STKAKGHNPR  SSIAVKLFKF  SREKKAAKTL
α1c human     QVTLRIHRKN  APAGG...SG  MASAKT...K  THFSVRLLKF  SREKKAAKTL
α1c bovine    QVTLRIHRKN  AQVGG...SG  VTSAKN...K  THFSVRLLKF  SREKKAAKTL
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Fig. 1. Translated amino acid sequences for cDNAs encoding human and rat *alpha*-1 (α_1) $_{a/d}$ARs, human and hamster α_{1b}ARs, human and bovine α_{1c}ARs. Differences with previously published sequences are highlighted in boxes.

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Pharmacological characterization of *alpha*-1AR subtypes. In order to assess the pharmacological characteristics of human *alpha*-1ARs, each cDNA was stably transfected into rat-1 fibroblasts, and the resulting receptors were compared to other expressed *alpha*-1AR subtypes (rat *alpha*-$1_{a/d}$, hamster *alpha*-1_b and bovine *alpha*-1_c) in rat-1 fibroblasts. Expression levels were roughly equivalent between all six clonal lines tested (1–2 pmol/mg of total protein). Membranes prepared from these stable cell lines expressing *alpha*-1AR subtypes were both used in saturation binding isotherms to determine K_d for [^{125}I]HEAT and, in competition analysis with various *alpha*-1-subtype selective drugs, both agonists and antagonists (table 1). Of note, all *alpha*-1AR subtypes demonstrate high and approximately equal affinity for [^{125}I]HEAT. Although it is evident that human *alpha*-1ARs have approximately the same affinities for various agonists and antagonists compared to their rat *alpha*-$1_{a/d}$, hamster *alpha*-1_b and bovine *alpha*-1_c homologs, some minor

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               351                                                         400
α1a/dhuman     AIVVGVFVLC  WFPFFFVLPL  GSLFPQLKPS  EGVFKVIFWL  GYFNSCVNPL
α1a/drat       AIVVGVFVLC  WFPFFFVLPL  GSLFPQLKPS  EGVFKVIFWL  GYFNSCVNPL
α1bhuman       GIVVGMFILC  WLPFFIALPL  GSLFSTLKPP  DAVFKVVFWL  GYFNSCLNPI
α1bhamster     GIVVGMFILC  WLPFFIALPL  GSLFSTLKPP  DAVFKVVFWL  GYFNSCLNPI
α1chuman       GIVVGCFVLC  WLPFFLVMPI  GSFFPDFKPS  ETVFKIVFWL  GYLNSCINPI
α1cbovine      GIVVGCFVLC  WLPFFLVMPI  GSFFPDFRPS  ETVFKIAFWL  GYLNSCINPI

               401                                                         450
α1a/dhuman     IYPCSSREFK  RAFLRLLRCQ  C...RRRRRR  RPLWRVYGHH  WRASTSGLRQ
α1a/drat       IYPCSSREFK  RAFLRLLRCQ  C...RRRRRR  ..LWAVYGHH  WRASTGDARS
α1bhuman       IYPCSSKEFK  RAFVRILGCQ  CRGRRRRRRR  RRRLGGCAYT  YRPWTRGGSL
α1bhamster     IYPCSSKEFK  RAFMRILGCQ  CRSGRRRRRR  RR.LGACAYT  YRPWTRFFSL
α1chuman       IYPCSSQEFK  KAFQNVLRIQ  C..LRRKQSS  KHALG...YT  LHPPSQAVEG
α1cbovine      IYPCSSQEFK  KAFQNVLRIQ  C..LRRKQSS  KHTLG...YT  LHAPSHVLEG

               451                                                         500
α1a/dhuman     DCAPSSGDAP  PGAPLALTAL  PDPDPEPPGT  PEMQAPVASR  RKPPSAFREW
α1a/drat       DCAPSPRIAP  PGAPLALTA.  .HPGAGSADT  PETQDSVSSS  RKPASALREW
α1bhuman       ERSQ......  SRK.......  .DSLDDSGSC  LSGSQRTLPS  ASPSPGYLGR
α1bhamster     ERSQ......  SRK.......  .DSLDDSGSC  MSGSQRTLPS  ASPSPGYLGR
α1chuman       QHKDMVRIPV  GSR.......  .ETFYRISKT  DGVCEWKFFS  SMPRGS..AR
α1cbovine      QHKDLVRIPV  GSA.......  .ETFYKISKT  DGVCEWKIFS  SLPRGS..AR

               501                                                         550
α1a/dhuman     RLLGPFRRPT  TQLRAKVSS.  LSHKIRAGGA  QRAEAACAQR  SEVEAVSLGV
α1a/drat       RLLGPLQRPT  TQLRAKVSS.  LSHKIR.SGA  RRAETACALR  SEVEAVSLNV
αlbhuman       GAPPPVELCA  FPEWKAPGAL  LSLPAPEPPG  RRGRHDSGPL  FTFKLLTEPE
α1bhamster     GAQPPLELCA  YPEWK.SGAL  LSL..PEPPG  RRGRLDSGPL  FTFKLLFEPE
α1chuman       ITVSKDQSSC  TTARVRSKSF  LQVCCCVGPS  TPSLDKNHQV  PTIKVHTISL
α1cbovine      MAVARDPSAC  TTARVRSKSF  LQVCCCLGPS  TOSHGENHQI  PTIKIHTISL

               551                                 587
α1a/dhuman     PHEVAEGATC  QAYELADYSN  LRETDI....  .......
α1a/drat       PQDGAEAVIC  QAYEPGDYSN  LRETDI....  .......
α1bhuman       SPGTDGGASN  GGCEAAADVA  NGQPGFKSNM  PLAPGQF
α1bhamster     SPGTEGDASN  GGCDATTDLA  NGQPGFKSNM  PLAPGHF
α1chuman       SENGEEV...  ..........  ..........  .......
α1cbovine      SENGEEV...  ..........  ..........  .......
```

Fig. 1. *Continued*

differences occur which impact on *alpha*-1AR classification. These include slightly higher affinity for spiperone by the bovine *alpha*-1_c compared to the human *alpha*-1_c, as well as larger differences in affinity for methoxamine between human *alpha*-1_b and *alpha*-1_c than seen between hamster and bovine homologs.

Further pharmacological characterization included inactivation of *alpha*-1AR subtypes by the alkylating agent CEC. Conditions were chosen such that the *alpha*-1_bAR subtype would be completely inactivated (100 μM CEC, 37°C, 20 min); for comparison, a slightly shorter incubation time (10 min) also was performed. Human *alpha*-1AR subtypes were inactivated by CEC to the same extent as their rat, hamster and bovine counterparts. For 20-min incubations, *alpha*-1_bARs were inactivated by CEC, 89 to 98%, followed by *alpha*-$1_{a/d}$ARs at 75 to 86% and *alpha*-1_cARs at 11 to 18% (table 1). Of note, CEC inactivation for the *alpha*-1_cAR subtype is significantly less than that reported previously for membrane assays (Schwinn *et al.*, 1990, 1991). In order to test coupling to phospholipase C, PI hydrolysis experiments were performed. All six cloned *alpha*-1AR subtypes stably expressed in rat-1 fibroblasts produced IP with agonist stimulation with the following NE efficacy: *alpha*-1_cAR (human and bovine) > *alpha*-1_bAR (human and hamster) > *alpha*-$1_{a/d}$AR (human and rat) (table 1). Of note, the human *alpha*-1_cAR couples to PI hydrolysis with almost twice the NE efficacy as the bovine *alpha*-1_cAR. Activation of PI hydrolysis by the six cloned *alpha*-1AR subtypes is pertussis toxin-insensitive (100 and 500 ng/ml).

Discussion

In this study we report the cloning and characterization of three human *alpha*-1AR cDNAs. *Alpha*-1ARs are defined at both a pharmacological (*alpha*-1_A and *alpha*-1_B) and molecular (cloned rat *alpha*-$1_{a/d}$, hamster *alpha*-1_b and bovine *alpha*-1_c) level, and merging of the two classification schemes has proven difficult. The best correlation between pharmacological and molecular classification schemes for *alpha*-1AR subtypes occurs for the *alpha*-1_BAR subtype, in which both cloned (*alpha*-1_b) and pharmacological (*alpha*-1_B) subtypes have low affinity for several *alpha*-1AR subtype-selective compounds [(+)-niguldipine, WB4101 and 5-methylurapidil], are sensitive to inactivation by CEC and are present in the same rat tissues (Morrow and Creese, 1986; Han *et al.*, 1987; Minneman *et al.*, 1988b; Gross *et al.*, 1988; Cotecchia *et al.*, 1988; Schwinn *et al.*, 1991). In contrast, alignment of the pharmacologically defined *alpha*-1_A subtype with cloned *al*-

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   1  ATGACTTTCCGCGATCTCCTGAGCGTCAGTTTCGAGGGACCCCGCCCGGACAGCAGCGCA
      M  T  F  R  D  L  L  S  V  S  F  E  G  P  R  P  D  S  S  A

  61  GGGGGCTCCAGCGCGGGCGGCGGCGGGGGCAGCGCGGGCGGCGCGGCCCCCTCGGAGGGC
      G  G  S  S  A  G  G  G  G  G  S  A  G  G  A  A  P  S  E  G

 121  CCGGCGGTGGGCGGCGTGCCGGGGGGCGCGGGCGGCGGCGGCGGCGTGGTGGGCGCAGGC
      P  A  V  G  G  V  P  G  G  A  G  G  G  G  G  V  V  G  A  G

 181  AGCGGCGAGGACAACCGGAGCTCCGCGGGGGAGCCGGGGAGCGCGGGCGCGGGCGGCGAC
      S  G  E  D  N  R  S  S  A  G  E  P  G  S  A  G  A  G  G  D

 241  GTGAATGGCACGGCGGCCGTCGGGGGACTGGTGGTGAGCGCGCAGGGCGTGGGCGTGGGC
      V  N  G  T  A  A  V  G  G  L  V  V  S  A  Q  G  V  G  V  G

 301  GTCTTCCTGGCAGCCTTCATCCTTATGGCCGTGGCAGGTAACCTGCTTGTCATCCTCTCA
      V  F  L  A  A  F  I  L  M  A  V  A  G  N  L  L  V  I  L  S

 361  GTGGCCTGCAACCGCCACCTGCAGACCGTCACCAACTATTTCATCGTGAACCTGGCCGTG
      V  A  C  N  R  H  L  Q  T  V  T  N  Y  F  I  V  N  L  A  V

 421  GCCGACCTGCTGCTGAGCGCCACCGTACTGCCCTTCTCGGCCACCATGGAGGTTCTGGGC
      A  D  L  L  L  S  A  T  V  L  P  F  S  A  T  M  E  V  L  G

 481  TTCTGGGCCTTTGGCCGCGCCTTCTGCGACGTATGGGCCGCCGTGGACGTGCTGTGCTGC
      F  W  A  F  G  R  A  F  C  D  V  W  A  A  V  D  V  L  C  C

 541  ACGGCCTCCATCCTCAGCCTCTGCACCATCTCCGTGGACCGGTACGTGGGCGTGCGCCAC
      T  A  S  I  L  S  L  C  T  I  S  V  D  R  Y  V  G  V  R  H

 601  TCACTCAAGTACCCAGCCATCATGACCGAGCGCAAGGCGGCCGCCATCCTGGCCCTGCTC
      S  L  K  Y  P  A  I  M  T  E  R  K  A  A  A  I  L  A  L  L

 661  TGGGTCGTAGCCCTGGTGGTGTCCGTAGGGCCCCTGCTGGGCTGGAAGGAGCCCGTGCCC
      W  V  V  A  L  V  V  S  V  G  P  L  L  G  W  K  E  P  V  P

 721  CCTGACGAGCGCTTCTGCGGTATCACCGAGGAGGCGGGCTACGCTGTCTTCTCCTCCGTG
      P  D  E  R  F  C  G  I  T  E  E  A  G  Y  A  V  F  S  S  V

 781  TGCTCCTTCTACCTGCCCATGGCGGTCATCGTGGTCATGTACTGCCGCGTGTACGTGGTC
      C  S  F  Y  L  P  M  A  V  I  V  V  M  Y  C  R  V  Y  V  V

 841  GCGCGCAGCACCACGCGCAGCCTCGAGGCAGGCGTCAAGCGCGAGCGAGGCAAGGCCTCC
      A  R  S  T  T  R  S  L  E  A  G  V  K  R  E  R  G  K  A  S

 901  GAGGTGGTGCTGCGCATCCACTGTCGCGGCGCGGCCACGGGCGCCGACGGGGCGCACGGC
      E  V  V  L  R  I  H  C  R  G  A  A  T  G  A  D  G  A  H  G

 961  ATGCGCAGCGCCAAGGGCCACACCTTCCGCAGCTCGCTCTCCGTGCGCCTGCTCAAGTTC
      M  R  S  A  K  G  H  T  F  R  S  S  L  S  V  R  L  L  K  F

1021  TCCCGTGAGAAGAAAGCGGCCAAGACTCTGGCCATCGTCGTGGGTGTCTTCGTGCTCTGC
      S  R  E  K  K  A  A  K  T  L  A  I  V  V  G  V  F  V  L  C

1081  TGGTTCCCTTTCTTCTTTGTCCTGCCGCTCGGCTCCTTGTTCCCGCAGCTGAAGCCATCG
      W  F  P  F  F  F  V  L  P  L  G  S  L  F  P  Q  L  K  P  S

1141  GAGGGCGTCTTCAAGGTCATCTTCTGGCTCGGCTACTTCAACAGCTGCGTGAACCCGCTC
      E  G  V  F  K  V  I  F  W  L  G  Y  F  N  S  C  V  N  P  L

1201  ATCTACCCCTGTTCCAGCCGCGAGTTCAAGCGCGCCTTCCTCCGTCTCCTGCGCTGCCAG
      I  Y  P  C  S  S  R  E  F  K  R  A  F  L  R  L  L  R  C  Q

1261  TGCCGTCGTCGCCGGCGCCGCCGCCCTCTCTGGCGTGTCTACGGCCACCACTGGCGGGCC
      C  R  R  R  R  R  R  R  P  L  W  R  V  Y  G  H  H  W  R  A

1321  TCCACCAGCGGCCTGCGCCAGGACTGCGCCCCGAGTTCGGGCGACGCGCCCCCCGGAGCG
      S  T  S  G  L  R  Q  D  C  A  P  S  S  G  D  A  P  P  G  A

1381  CCGCTGGCCCTCACCGCGCTCCCCGACCCCGACCCCGAACCCCCAGGAACGCCCGAGATG
      P  L  A  L  T  A  L  P  D  P  D  P  E  P  P  G  T  P  E  M

1441  CAGGCTCCGGTCGCCAGCCGTCGAAAGCCACCCAGCGCCTTCCGCGAGTGGAGGCTGCTG
      Q  A  P  V  A  S  R  R  K  P  P  S  A  F  R  E  W  R  L  L

1501  GGGCCGTTCCGGAGACCCACGACCCAGCTGCGCGCCAAAGTCTCCAGCCTGTCGCACAAG
      G  P  F  R  R  P  T  T  Q  L  R  A  K  V  S  S  L  S  H  K

1561  ATCCGCGCCGGGGGCGCGCAGCGCGCAGAGGCAGCGTGCGCCCAGCGCTCAGAGGTGGAG
      I  R  A  G  G  A  Q  R  A  E  A  A  C  A  Q  R  S  E  V  E

1621  GCTGTGTCCCTAGGCGTCCCACACGAGGTGGCCGAGGGCGCCACCTGCCAGGCCTACGAA
      A  V  S  L  G  V  P  H  E  V  A  E  G  A  T  C  Q  A  Y  E

1681  TTGGCCGACTACAGCAACCTACGGGAGACCGATATTTAAGGACCCCAGAGCTAGGCCGCG
      L  A  D  Y  S  N  L  R  E  T  D  I

1741  GAGTGTGCTGGGCTTGGGGGTAAGGGGGACCAGAGAGGCGGGCTGGTGTTCTAAGAGCCC
1801  CCGTGCAAATCGGAGACCCGGAAACTGATC
```

Fig. 2. The nucleotide sequence and translated amino acid sequence of the human *alpha*-1$_{a/d}$AR open-reading frame.

pha-1AR subtypes has been more difficult. The *alpha*-1$_{A}$ is defined as having high affinity for *alpha*-1AR subtype-selective drugs, resistance to inactivation by CEC and is the predominant *alpha*-1AR subtype present in the rat vas deferens and submaxillary gland (Morrow and Creese, 1986; Minneman *et al.*, 1988b; Michel *et al.*, 1989). Although the cloned bovine *alpha*-1$_{c}$, as described originally, has high affinity for all *alpha*-1$_{A}$AR selective drugs, the encoded receptor protein deviates in its partial sensitivity to CEC, absence from pharmacologically predicted rat tissues as determined by Northern analysis (Schwinn *et al.*, 1990, 1991) and lower than expected (+)-niguldipine affinity (Schwinn and Lomasney, 1992). However, recent studies from our laboratory (Price *et al.*, 1994a) and by others have suggested that the cloned *alpha*-1$_{c}$AR might encode the pharmacological *alpha*-1$_{A}$AR after all. First, although CEC sensitivity is known in general to be lower in whole cells than in membrane preparations (Schwinn *et al.*, 1991), a recent study suggests even less CEC sensitivity than described originally for human *alpha*-1$_{c}$ARs in whole cells (Forray *et al.*, 1994a); our current study suggests for the first time that lower CEC sensitivity exists (~20% inactivation) for human and bovine *alpha*-1$_{c}$ARs in membrane assays. Second, by using more sensitive techniques, the tissue distribution of the *alpha*-1$_{c}$AR has now been shown to be more extensive than originally thought. RNase protection assays and *in situ* hybridization studies of *alpha*-1AR mRNAs have demonstrated recently the presence of cloned *alpha*-1$_{c}$ARs in many human (Price *et al.*, 1994b) and rat (Price *et al.*, 1994a) tissues, particularly in the stroma of the human prostate (Price *et al.*, 1993) where the *alpha*-1$_{c}$AR appears to be involved in smooth muscle contraction (Smith *et al.*, 1993; Forray *et al.*, 1994b). Finally, recent studies have demonstrated higher affinity for (+)-niguldipine in the rat *alpha*-1$_{c}$AR compared with the bovine homolog (Forray *et al.*, 1994a). With the cloned *alpha*-1$_{c}$ now thought to encode the pharmacological *alpha*-1$_{A}$ subtype, the only remaining cloned *alpha*-1AR not encoding a pharmacologically defined *alpha*-1AR subtype is the *alpha*-1$_{a/d}$. Historically, the cloned rat *alpha*-1$_{a/d}$ was first called the *alpha*-1$_{a}$AR due to high affinity for phentolamine and WB4101, initial studies demonstrating resistance to inactivation by CEC and appropriate tissue distribution in the rat (Lomasney *et al.*, 1991b). However, more extensive evaluation of this receptor (Perez *et al.*, 1991) resulted in the name *alpha*-1$_{d}$AR due to lower affinity for oxymetazoline and (+)-niguldipine and some inactivation by CEC (Perez *et al.*, 1991); until this controversy is resolved completely, this receptor has been called the *alpha*-1$_{a/d}$ (Schwinn and Lomasney, 1992).

Because of the role of *alpha*-1ARs in many human diseases, and the possibility that species homologs with different pharmacologies may shed further light on *alpha*-1AR classification, it is important to define the exact pharmacologic properties of cloned human *alpha*-1AR subtypes. Therefore, we have cloned cDNAs encoding three human *alpha*-1AR subtypes and compared their pharmacological properties to the previously cloned rat, hamster and bovine *alpha*-1ARs. In addition to demonstrating sequence inaccuracies in human *alpha*-1ARs and the rat *alpha*-1$_{a/d}$ subtype, this study represents the first expression of human *alpha*-1$_{a/d}$AR protein and subsequent pharmacological characteriza-

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TABLE 1

Pharmacological characteristics of *Alpha*-1 AR subtypes

Mean ± S.E.M. All results are rounded to two significant digits.

Characteristic:	Human *Alpha*-$1_{a/d}$	Rat *Alpha*-$1_{a/d}$	Human *Alpha*-1_b	Hamster *Alpha*-1_b	Human *Alpha*-1_c	Bovine *Alpha*-1_c	Rank Order of Affinity CEC Inactivation/Efficacy
K_d [^{125}I]HEAT (pM)	130 ± 13	85 ± 12	120 ± 30	99 ± 6.0	131 ± 13	73 ± 4.0	a/d = b = c
Competition analysis:[a] pK_i							
Agonists:							
Epinephrine	6.6 ± 0.11	6.6 ± 0.0070	5.5 ± 0.11	5.8 ± 0.13	5.0 ± 0.036	5.1 ± 0.063	Human: a/d > b > c Other: a/d > b > c
Methoxamine	4.1 ± 0.13	4.2 ± 0.081	2.7 ± 0.11	3.3 ± 0.40	4.1 ± 0.11	3.6 ± 0.058	Human: a/d = c > b Other: a/d > c ≥ b
Norepinephrine	6.7 ± 0.058	6.9 ± 0.013	5.4 ± 0.12	5.7 ± 0.013	4.7 ± 0.086	4.7 ± 0.066	Human: a/d > b > c Other: a/d > b > c
Oxymetazoline	6.1 ± 0.050	6.1 ± 0.11	6.4 ± 0.071	6.8 ± 0.14	7.8 ± 0.12	7.6 ± 0.0010	Human: c > b ≥ a/d Other: c > b > a/d
Antagonists:							
5-Methylurapidil	7.2 ± 0.063	7.1 ± 0.015	6.6 ± 0.083	6.5 ± 0.11	8.5 ± 0.36	8.7 ± 0.067	Human: c > a/d > b Other: c > a/d > b
(+)Niguldipine	6.3 ± 0.15	6.3 ± 0.16	6.1 ± 0.19	6.3 ± 0.20	8.1 ± 0.15	8.0 ± 0.12	Human: c > a/d ≥ b Other: c > a/d = b
Phentolamine	7.5 ± 0.073	7.7 ± 0.24	6.7 ± 0.037	7.1 ± 0.039	7.7 ± 0.055	7.9 ± 0.10	Human: c ≥ a/d > b Other: c ≥ a/d > b
Prazosin	10.4 ± 0.095	10.1 ± 0.068	9.5 ± 0.15	9.9 ± 0.27	9.9 ± 0.16	9.3 ± 0.25	Human: a/d ≥ c ≥ b Other: a/d ≥ b > c
Spiperone	7.6 ± 0.042	7.4 ± 0.025	7.4 ± 0.031	7.5 ± 0.18	6.8 ± 0.016	7.5 ± 0.042	Human: a/d = b > c Other: a/d = b = c
Terazosin	8.4 ± 0.082	8.3 ± 0.062	8.4 ± 0.0011	8.5 ± 0.073	7.6 ± 0.056	7.5 ± 0.19	Human: a/d = b > c Other: a/d = b > c
WB4101	8.9 ± 0.16	8.9 ± 0.055	8.0 ± 0.041	8.1 ± 0.097	8.9 ± 0.061	9.2 ± 0.040	Human: c = a/d > b Other: c ≥ a/d > b
% Inactivation by CEC[b]							
100 μM, 37°C, 10 min	66 ± 4.7%	70 ± 6.4%	89 ± 4.0%	74 ± 9.0%	24 ± 8.2%	26 ± 3.2%	Human: b > a/d > c
100 μM, 37°C, 20 min	75 ± 14%	86 ± 6.6%	98 ± 3.4%	89 ± 4.8%	18 ± 9.4%	11 ± 2.4%	Other: b ≥ a/d > c
PI hydrolysis[c]							
NE, 100 μM	×1.5 ± 0.12	×1.8 ± 0.00	×4.6 ± 0.16	×6.5 ± 0.60	×15.5 ± 0.60	×7.7 ± 0.60	Human: c >> b >> a/d
NE, 100 μM + 100 ng/ml Pertussis toxin[d]	×1.5 ± 0.16	×1.9 ± 0.060	×5.4 ± 0.67	×4.8 ± 1.0	×18 ± 0.89	×7.8 ± 0.60	Other: c ≥ b >> a/d

[a] Competition assays were performed in triplicate, with all six subtypes tested simultaneously, n = 3–5 separate experiments.

[b] Ligand binding for CEC inactivation experiments was performed in triplicate, n = 3–5 separate experiments.

[c] PI hydrolysis results are expressed as fold increase over base line in the presence of 1 μM propranolol; NE, norepinephrine, n = 3–5 separate experiments each performed in triplicate.

[d] One additional set of PI hydrolysis experiments was performed with 500 ng/ml of pertussis toxin; identical results were obtained (*i.e.*, no pertussis toxin sensitivity).

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tion. Of importance, characterization of *alpha*-1AR subtypes was performed simultaneously for all six subtypes, by using receptors expressed in the same cell line (rat-1 fibroblasts) at approximately equal expression level (1–2 pmol/mg of total protein). Although human and rat *alpha*-$1_{a/d}$, human and hamster *alpha*-1_b and human and bovine *alpha*-1_cAR subtypes have similar pharmacological properties, some differences do exist.

In general, affinities for agonists and antagonists are similar between human, rat, hamster and bovine *alpha*-1ARs. However, subtle differences in ligand affinities noted in this study do impact *alpha*-1AR classification. One of these differences is spiperone affinity, where the pK_i value is 6.8 for the human *alpha*-1_c and 7.5 for the bovine *alpha*-1_c. Although not a large difference, spiperone is the only *alpha*-1_B-selective compound available to date, and original descriptions of spiperone in rat tissues demonstrated higher affinity for the pharmacological *alpha*-1_BAR compared to the *alpha*-1_AAR (Michel *et al.*, 1989). Studies to date with cloned (rat, hamster and bovine) *alpha*-1AR subtypes demonstrate spiperone affinities of *alpha*-1_c ≥ *alpha*-1_b ≥ *alpha*-$1_{a/d}$ (Schwinn and Lomasney, 1992). In this study, the human *alpha*-1_b has higher affinity for spiperone compared with the human *alpha*-1_cAR, matching the previously described pharmacological *alpha*-1_B > *alpha*-1_A (Michel *et al.*, 1989) and is consistent with the cloned *alpha*-1_c encoding the pharmacological *alpha*-1_A subtype. Another subtle difference between human and nonhuman homolog affinities occurs with the agonist methoxamine. Original descriptions of pharmacological *alpha*-1AR subtypes demonstrated higher methoxamine affinity for the *alpha*-1_A compared with the *alpha*-1_B (Minneman, 1988a). We demonstrate that whereas the cloned bovine *alpha*-1_c has approximately similar affinity for methoxamine compared with the hamster *alpha*-1_b (pK_i, 3.6 compared with 3.3, respectively), the human *alpha*-1_c has higher affinity for methoxamine (pK_i, 4.1) compared with the human *alpha*-1_b (pK_i, 2.7); this again is consistent with the cloned *alpha*-1_c encoding the pharmacological *alpha*-1_A subtype. Another difference between cloned bovine *alpha*-1AR pharmacology and the classically described *alpha*-1_AAR subtype is absolute (+)-niguldipine affinity, with the bovine *alpha*-1_cAR 10- to 100-fold lower compared to experiments performed in rat tissues (Boer *et al.*, 1989; Han and Minneman, 1991). Of note, whereas absolute (+)-niguldipine affinity for the rat *alpha*-1_cAR has been reported to be higher than the bovine *alpha*-1_cAR (Forray *et al.*, 1994a), we find no difference between human and bovine in (+)-niguldipine affinity in this study.

Compared with fairly subtle differences in ligand affinities, more striking differences from previously reported pharmacological characterization of cloned *alpha*-1ARs are apparent in CEC inactivation studies. In this study, we find that both human and bovine *alpha*-1_cARs are inactivated less by CEC compared with that reported previously in hypotonic membrane preparations (Schwinn *et al.*, 1990, 1991; Perez *et al.*, 1991). CEC conditions in this study were chosen carefully to inactivate completely the *alpha*-1_bAR (100 μM CEC, 37°C, 20 min). Under these conditions, *alpha*-1_bARs are 89 to 98% inactivated, *alpha*-$1_{a/d}$ARs 75 to 86% and *alpha*-1_cARs 11 to 18%. Hence although *alpha*-1_cARs are inactivated in part by CEC, they are clearly the most resistant of all of cloned *alpha*-1AR subtypes. With minimal CEC inactivation, this gives further support to the idea that the cloned *alpha*-1_c subtype encodes the pharmacological *alpha*-1_AAR.

All six *alpha*-1AR subtypes couple to the hydrolysis of membrane phospholipids *via* phospholipase C, as demonstrated by production of IP isomers upon stimulation with NE. As reported previously, the bovine *alpha*-1_cAR couples with greater NE efficacy to PI hydrolysis compared to either the hamster *alpha*-1_b or the rat *alpha*-$1_{a/d}$ (Schwinn *et al.*, 1991). We now report similar results with human *alpha*-1AR subtypes; in fact, the human *alpha*-1_cAR has the highest NE efficacy of coupling to PI hydrolysis of any of the *alpha*-1AR subtypes studied. Coupling of rat *alpha*-$1_{a/d}$, hamster *alpha*-1_b and bovine *alpha*-1_cARs to PI hydrolysis has been reported to occur *via* Gq (Wu *et al.*, 1992) and to be insensitive to pertussis toxin (Schwinn *et al.*, 1991; Perez *et al.*, 1993). Similar results are presented here for human *alpha*-1AR subtypes. Typically, pertussis toxin ADP-ribosylates G_i (Simon *et al.*, 1991). However, Gq associated with *alpha*-1ARs has been classically described as pertussis toxin-insensitive (Blank *et al.*, 1991; Simon *et al.*, 1991). Isolated reports of *alpha*-1AR-mediated inhibition of PI hydrolysis in the rat heart have occurred (Han *et al.*, 1989), but not confirmed, with cloned *alpha*-1AR subtypes. These results demonstrate that cloned *alpha*-1AR subtype-mediated PI hydrolysis is not pertussis toxin-sensitive in rat-1 fibroblasts.

In summary, we have cloned three human *alpha*-1AR subtype cDNAs, expressed them stably in rat-1 fibroblasts and characterized the expressed receptors pharmacologically; this is particularly important for the *alpha*-$1_{a/d}$ subtype which has not previously been characterized pharmacologically. In addition, these data represent the first side-by-side comparison of pharmacological properties between species homologs of *alpha*-1AR subtypes. Although pharmacological characterization of *alpha*-1AR subtypes is similar in many aspects between human and other species homologs, a few potentially important differences are apparent. The availability of clones for distinct *alpha*-1AR subtypes in the same species should now facilitate refinement not only of the structural basis of receptor ligand interactions, but also of signal transduction mechanisms ultimately utilized by different *alpha*-1AR subtypes. In addition, these data may help in the development of *alpha*-1AR subtype-selective drugs for use in clinical medicine.

Note in proof. While this manuscipt was in review, a paper describing the sequences of all three human *alpha*-1ARs was published (Forray *et al.*, 1994b). Comparison of our human *alpha*-$1_{a/d}$ sequence with results reported by Forray *et al.* (1994b) in GenBank reveals 100% sequence identity at the amino acid level, confirming our human *alpha*-$1_{a/d}$ corrections.

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