0026-895X/95/050977-09$3.00/0

MOLECULAR PHARMACOLOGY, 47:977-985 (1995).

Cloning of the Human α_{1d}-Adrenergic Receptor and Inducible Expression of Three Human Subtypes in SK-N-MC Cells

TIMOTHY A. ESBENSHADE, AKIRA HIRASAWA, GOZOH TSUJIMOTO, TERUO TANAKA, JUNICHI YANO, KENNETH P. MINNEMAN, and T. J. MURPHY

Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322 (T.A.E., K.P.M., T.J.M.), Department of Molecular and Cell Pharmacology, National Children's Medical Research Center, National Children's Hospital, Tokyo 154, Japan (A.H., G.T.), and Molecular Biology Department, Research Laboratories, Nippon Shinyaku Co., Kyoto 601, Japan (T.T., J.Y.)

Received August 8, 1994; Accepted February 10, 1995

SUMMARY

We have cloned the human α_{1d}-adrenergic receptor (AR) and compared the pharmacological properties of the three recombinant human α_1-AR subtypes in SK-N-MC cells. SK-N-MC cells natively express a mixture of α_1-AR subtypes, and the use of an inducible expression system allowed us to directly compare the recombinant and native subtypes without concern for cell-specific processing or microenvironment. The human α_{1d}-AR was expressed from a cDNA/gene fusion construct cloned from human SK-N-MC cell cDNA and human genomic libraries. This receptor is deduced to contain 572 amino acids with 98% identity to the rat α_{1d}-AR in the transmembrane domains and, when expressed in human embryonic kidney 293 cells, has α_1-AR binding properties similar to those of the rat α_{1d}-AR. Norepinephrine increased inositol phosphate formation and mobilized intracellular Ca^{2+} in transfected 293 cells. Reverse transcription-polymerase chain reaction analysis of the three cloned human subtypes (α_{1a}, α_{1b}, and α_{1d}) in mRNA from SK-N-MC cells, which natively express α_{1A}- and α_{1B}-like pharmacology, showed abundant α_{1a} and α_{1d} but fewer α_{1b} transcripts. The three human clones were expressed in SK-N-MC cells using isopropyl-β-D-thiogalactoside-inducible vectors. Upon induction, α_1-AR density was increased, with the recombinant subtype comprising 67–80% of total α_1-ARs. Inhibition curves for (+)-niguldipine and 5-methylurapidil fit best to a two-site model in uninduced cells, indicating significant receptor heterogeneity. Isopropyl-β-D-thiogalactoside induction altered the potencies of both compounds, causing most inhibition curves to fit best to a one-site model. (+)-Niguldipine was 100-fold more potent at the α_{1a}-AR than at α_{1b}- or α_{1d}-ARs, whereas 5-methylurapidil had similar potencies at α_{1a}- and α_{1d}-ARs and about 10-fold lower affinity at the α_{1b}-AR. We conclude that the complex α_{1A}- and α_{1B}-like pharmacology observed in native SK-N-MC cells is due to expression of all three subtypes in different proportions, independently of cell-specific processing or environmental factors, and that the α_{1a}-AR cDNA encodes the pharmacologically defined α_{1A} subtype.

α_1-ARs comprise a heterogeneous family (1). Two natively expressed subtypes (α_{1A} and α_{1B})[1] can be distinguished pharmacologically, whereas three subtypes (α_{1a}, α_{1b}, and α_{1d}) have been cloned (2–5). The α_{1b}-AR cDNA clone appears to encode the natively expressed, pharmacologically defined, α_{1B} subtype. Earlier studies using heterologous expression systems suggested that neither the α_{1a}- nor α_{1d}-AR clones (previously defined as α_{1C} and α_{1A} or $\alpha_{1A/D}$, respectively) encoded a subtype identical to the native α_{1A}-AR. The uncertain relationship between the cloned and native subtypes has been a source of much confusion. However, more recent reports detailing the cloning and expression of the rat homolog of the bovine α_{1a} (previously α_{1C}) subtype provide strong evidence that the α_{1a}-AR cDNA encodes the pharmacological α_{1A}-AR subtype (6, 7). The functional role of the native α_{1D}-AR remains to be defined.

Human SK-N-MC neuroepithelioma cells express at least two α_1-AR subtypes, with pharmacological properties similar

This work was supported in part by National Institutes of Health Grants NS21325 (K.P.M.) and HL48252 (T.J.M.), a National Research Service Award (HL08448) from the National Institutes of Health (T.A.E.), and research grants from the Scientific Research Fund of the Ministry of Education, Science, and Culture of Japan, the Japan Health Science Foundation, and the Ministry of Human Health and Welfare (A.H. and G.T.).

[1] Throughout this paper, we use the standardized nomenclature system for α_1-AR subtypes recently recommended by the IUPHAR Committee on the Classification of Adrenoceptors. In this system, the cloned subtypes are designated with lowercase letters as α_{1a}, α_{1b}, and α_{1d}, which correspond to the clones previously defined as α_{1C}, α_{1B}, and α_{1A} (or $\alpha_{1A/D}$ and α_{1D}), respectively. The corresponding pharmacological subtypes are designated with uppercase letters and are defined as α_{1A}, α_{1B}, and α_{1D}, respectively.

ABBREVIATIONS: AR, adrenergic receptor; CEC, chloroethylclonidine; 5-MU, 5-methylurapidil; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; InsP, inositol phosphate; IPTG, isopropyl-β-D-thiogalactoside; NE, norepinephrine; PBS, phosphate-buffered saline; RT, reverse transcription; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; BE, BE 2254; bp, base pair(s); $[Ca^{2+}]_i$, intracellular calcium concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase(s).

to those of α_{1A}- and α_{1B}-ARs (8, 9). This is, to date, the only cell line where α_{1A}-ARs have been found and should be helpful in defining the molecular properties of this subtype. However, contradictory data exist regarding which α_1-AR genes are expressed in these cells. Northern blots of SK-N-MC mRNA hybridized to hamster α_{1b} and rat α_{1d} but not bovine α_{1a} cDNA (9), and nuclease protection assays showed α_{1b} mRNA in these cells (10). However, other investigators have found α_{1a} and α_{1d} but not α_{1b} mRNA in these cells by RNase protection assays (11). The relationship of the mRNA transcripts to the endogenous subtypes in these cells is not clear.

Cell-specific modifications or microenvironments have been suggested to alter the binding specificity of α_1-ARs (12), although there is little precedent for this with other G protein-coupled receptors (13). Direct comparison of the expressed human clones with endogenously expressed receptors in their "native" environment might help clear up the confusion. The human α_{1a} homolog has recently been cloned and expressed (14), and the expression, but not the functionality, of the human homolog of the rat α_{1d} cDNA has recently been reported (15). None of these clones has yet been expressed in cells known to be capable of expressing an α_{1A}-like pharmacology.

To directly compare the products of the human clones and the native subtypes, we have cloned the human α_{1d}-AR and studied its functional properties when transfected into human embryonic kidney cells. We have also expressed all three human clones (10, 14–17) in SK-N-MC cells, which endogenously express both pharmacologically defined α_{1A}- and α_{1B}-AR subtypes. Putative cell-specific modifications, processing, or environments that might contribute to the apparent drug specificities of the expressed receptors should be available in these cells, allowing quantitative comparison between the cloned and native subtypes.

Experimental Procedures

Materials. SK-N-MC and human embryonic kidney 293 cells were obtained from the American Type Culture Collection (Rockville, MD). The gene fusion construct for the human α_{1b}-AR and the cDNA for the rat α_{1d}-AR were generously provided by Dr. R. Graham (Cleveland Clinic, Cleveland, OH). Materials were obtained from the following sources: phentolamine mesylate (Ciba-Geigy, Summit, NJ); CEC, 5-MU, (+)-niguldipine, and WB 4101 (Research Biochemicals, Natick, MA); prazosin hydrochloride (Pfizer, Groton, CT); oxymetazoline hydrochloride (Schering Corp., Bloomfield, NJ); hygromycin B (Boehringer Mannheim, Indianapolis, IN); BE [[2-β-(4-hydroxyphenyl)ethylaminomethyl]tetralone] (Beiersdorf AG, Hamburg, Germany); [^{3}H]inositol (20–40 Ci/mmol) (American Radiolabelled Chemicals, St. Louis, MO); carrier-free Na^{125}I (Amersham, Chicago, IL); fura-2/acetoxymethyl ester (Molecular Probes, Eugene, OR); fetal bovine serum and trypsin/EDTA (GIBCO-BRL, Gaithersburg, MD); and digitonin, (−)-NE bitartrate, yohimbine hydrochloride, Dulbecco's modified Eagles's medium, penicillin, streptomycin, and all other chemicals (Sigma Chemical Co., St. Louis, MO).

cDNA library preparation. cDNA was prepared from SK-N-MC cell poly(A)$^+$ RNA using random nonamers and oligo(dT) primers (Superscript kit; GIBCO-BRL). After addition of *Bst*XI adapters, size-fractionated cDNAs (>1.0 kb) were cloned into the *Bst*XI sites of the pCDM8 expression vector. A library of 800,000 recombinants was obtained by electroporation of MC1061/P3 *Escherichia coli*.

Library screening. The SK-N-MC cDNA library (8×10^5 clones) and a human genomic library (10^6 clones) in Lambda FixII (Stratagene, La Jolla, CA) were plated at 50,000 clones/plate, and duplicate nitrocellulose filter lifts were hybridized overnight at 42° in 50% formamide, 6× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 5× Denhardt's solution, 100 μg/ml sheared salmon sperm DNA, 1% SDS, with a random-primed (Prime-It; Stratagene), [^{32}P]dCTP-labeled probe. Filters were washed three times at medium (50° for 15 min with 1× SSC/0.1% SDS) or high (65° for 15 min with 0.1× SSC/0.1% SDS) stringency, and positive clones were identified and purified.

DNA sequencing. The cloned cDNA, genomic DNA, and constructs were sequenced by the dideoxy chain termination procedure (Sequenase; United States Biochemical Co., Cleveland, OH), using double-stranded plasmid template, to obtain sequences for both strands.

RT-PCR. Total RNA from wild-type SK-N-MC cells was treated with RNase-free DNase I (Stratagene), and 10-μg samples were reverse transcribed using Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL). After heating at 94° for 5 min to terminate the reactions, samples were stored at −20° until used.

Oligonucleotide primers were constructed from the published cDNA sequences. Sequences of the primers were as follows: α_{1a}, 5′-ATGCTCCAGCCAAGAGTTCA-3′ (sense, bases 1417–1437) and 5′-TCCAAGAAGAGCTGGCCTTC-3′ (antisense, annealing to bases 1898–1918) (14); α_{1b}, 5′-CTGTGCGCCATCTCCATCGATCGCTAC-3′ (sense, bases 406–432) and 5′-ATGAAGAAGGGTAGCCAGCACAAGATGAA-3′ (antisense, annealing to bases 907–935) (10); α_{1d}, 5′-CTCTGCACCATCTCCGTGGACCGGTAC-3′ (sense, bases 563–589) and 5′-AAAGAAGAAAGGGAACCAGCAGAGCACGAA-3′ (antisense, annealing to bases 1073–1102); β-actin, 5′-ATCATGTTTGAGACCTTCAACACCCCAGCC-3′ (sense, bases 2158–2187) and 5′-AAGAGAGCCTCGGGGCATCGGAACCGCTCA-3′ (antisense, annealing to bases 2550–2579) (18). β-Actin was included to control for the efficiency of RNA isolation and cDNA synthesis. The predicted sizes of the amplified human α_{1a}-, α_{1b}-, and α_{1d}-AR and β-actin PCR products were 502, 530, 540, and 421 bp, respectively.

PCR was carried out as described previously (14), using *Thermus aquaticus* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT), for 35 cycles in a Perkin Elmer Cetus thermal cycler. The amplification profiles consisted of denaturation at 94° for 1 min, primer annealing at 55° for 30 sec, and extension at 72° for 1 min. For positive controls, 0.1 ng of cDNA fragments was amplified. Negative control reactions without template were routinely included with both primer sets. PCR products were gel purified, blunt ended, subcloned into Bluescript II KS+, and sequenced to confirm their identity.

Preparation of expression vectors. The full length α_{1d}-AR fusion construct was subcloned into the Epstein Barr virus-based expression vector pREP9 (Invitrogen, San Diego, CA) and designated pREP9α_{1d}. The full length human α_1-AR coding sequences were each subcloned into the operator vector (pOPRSVICAT) of the inducible LacSwitch system (Stratagene). The *Not*I fragment of pOPRSVICAT containing the chloramphenicol acetyltransferase reporter gene was replaced with the multiple cloning site of pBluescript KS+ (where an additional *Not*I site had been inserted 5′ to the *Xho*I site) to facilitate the insertion of the genes of interest. The full length human α_{1a}-AR gene fusion construct (14), the α_{1b}-AR cDNA (10), and the α_{1d}-AR cDNA/gene fusion construct were each inserted into this plasmid, to create the plasmids pOPα_{1a}, pOPα_{1b}, and pOPα_{1d}, respectively.

Cell culture. Both SK-N-MC and 293 cells were propagated in 75-cm^2 flasks in a humidified 7% CO_2 incubator, in Dulbecco's modified Eagle's medium containing glucose (4.5 g/liter), streptomycin (100 mg/liter), and penicillin (10^5 units/liter) and supplemented with 10% fetal bovine serum. Confluent cells were subcultured at a ratio of 1:10 (SK-N-MC) or 1:5 (293). For measurements of [^{3}H]InsP formation, 35-mm Primaria dishes were seeded at a density of 300,000 cells/2 ml. In studies involving radioligand binding and Ca^{2+}, 100-mm dishes were seeded at a density of 3×10^6 cells/10 ml for both cell lines.

Transfections. Plasmids were purified by polyethylene glycol precipitation and transfected into 293 or SK-N-MC cells by calcium

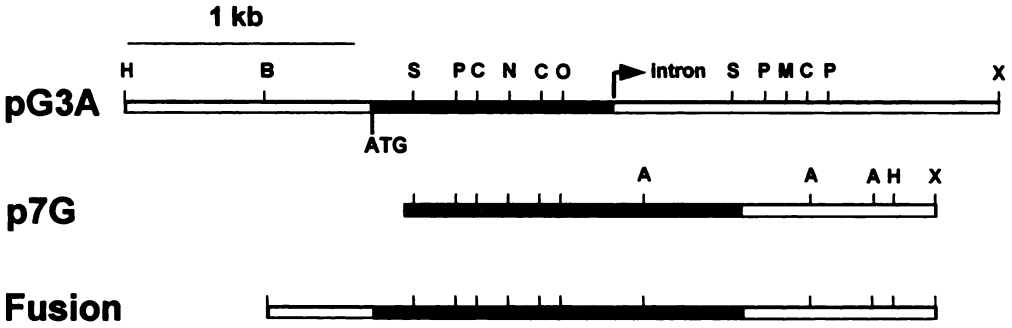

Fig. 1. Restriction maps of the human 4-kb genomic clone pG3A (*top*), the 2.5-kb cDNA clone p7G (*middle*), and the full length fusion construct (*bottom*). The putative open reading frame in pG3A is *black*, that in p7G is *shaded*, and the fusion construct shows the portions of the open reading frame derived from the genomic (*black*) and cDNA (*shaded*) clones ligated at the *Not*I site. The translation initiation start site is shown. The restriction enzymes were as follows: *A*, *Acc*I; *B*, *Bam*HI; *C*, *Nco*I; *H*, *Hind*III; *M*, *Sma*I; *N*, *Not*I; *O*, *Xho*I; *P*, *Pst*I; *S*, *Sst*I; *X*, *Xba*I.

phosphate precipitation. After recovery, the cells were exposed to Geneticin (250 μg/ml for 293 cells, 400 μg/ml for SK-N-MC cells) for several weeks to select resistant cells, which were propagated in the presence of antibiotic. For inducible expression with the LacSwitch system, SK-N-MC cells were transfected with the repressor vector (p3'SS) and cells resistant to 200 μg/ml hygromycin were selected and propagated. These resistant cells (SK-N-MC/LR) were then transfected with the individual operator vectors (pOPα_{1a}, pOPα_{1b}, and pOPα_{1d}). Cells for radioligand binding assays, [^{3}H]InsP formation assays, and fura-2 measurements of $[Ca^{2+}]_i$ were plated at lower (1/10) antibiotic concentrations before assays.

^{125}I-BE binding. Cells were washed twice in PBS (20 mM sodium phosphate, 154 mM NaCl, pH 7.6) and harvested by scraping. Cells were homogenized with a Polytron homogenizer, and membranes were collected by centrifugation at 30,000 × *g* for 10 min, washed, and resuspended in PBS. ^{125}I-BE binding was performed with membrane preparations as described previously (9). Saturation curves were analyzed by Scatchard analysis, and displacement curves were analyzed by nonlinear regression for the best one-site or two-site fit, using a partial *F* test. Differences with $p < 0.05$ were considered significant.

CEC inactivation. Membranes were suspended in 10 mM Na-HEPES, pH 7.4, and incubated for 10 min at 37°, with or without 0.1–100 μM CEC (19). Incubations were terminated by dilution with cold PBS and centrifugation at 30,000 × *g* for 10 min. Membranes were resuspended by homogenization or sonication in PBS, centrifuged again, and resuspended in PBS. Saturation analysis of specific ^{125}I-BE binding, or total and nonspecific binding of an 80% saturating concentration (200 pM) of ^{125}I-BE, was determined in duplicate.

[^{3}H]InsP formation. Accumulation of [^{3}H]InsPs was determined in confluent 35-mm dishes. Cells were prelabeled with *myo*-[^{3}H]inositol (2 μCi/plate) for 3–4 days and the production of [^{3}H]InsPs was determined as described previously (9). In some experiments cells were incubated for 30 min in Krebs-Ringer bicarbonate buffer, without or with 10 or 100 μM CEC, and washed three times, and [^{3}H]InsP formation was determined as described.

$[Ca^{2+}]_i$ determinations. $[Ca^{2+}]_i$ was determined with fura-2 as described previously (9). In brief, cell suspensions were prepared (3–4 × 10^6 cells/ml) and loaded for 15 min with 1 μM fura-2/acetoxymethyl ester. The cells were then washed and resuspended in balanced salt solution (130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 20 mM HEPES, 10 mM glucose, 0.1% bovine serum albumin) (2 × 10^6 cells/ml), and 3-ml aliquots were used for each assay. Fura-2 measurements were performed in a Perkin-Elmer (Beaconsfield, Buckinghamshire, England) LS 50 luminescence spectrofluorometer. The excitation wavelengths were 340 and 380 nm and the emission wavelength was 510 nm (all with 5-nm bandwidths). Calibration of the fluorescence signals for calculation of $[Ca^{2+}]_i$ was performed for every aliquot by equilibration of intracellular and extracellular Ca^{2+} with 30 μM digitonin (R_{max}), followed by addition of 300 mM EGTA, 1 M Tris, pH 9.0 (R_{min}), using a K_d of 225 nM for fura-2.

Results

Isolation of a partial α_1-AR cDNA from SK-N-MC cells. A 2.5-kb cDNA clone (p7G) was isolated from an SK-N-MC cDNA library screened with a full length rat α_{1d}-AR probe (4). Restriction analysis and sequencing of p7G (Fig. 1) showed similarity to the sequence reported previously for the human α_{1d}-AR cDNA (20), except at the 5′ end. No specific binding sites were obtained when this clone was expressed in 293 cells (data not shown), and divergence from the previously reported sequence (20) and the absence of a translation initiation codon suggested that the clone was incomplete at the 5′ end. A number of additional hybridization and PCR strategies were used unsuccessfully in an attempt to isolate a full length cDNA or an overlapping partial cDNA containing the missing 5′ end of the protein coding region.

Isolation of a partial overlapping genomic clone. A human placental genomic library was screened at high stringency with a ^{32}P-labeled 650-bp PCR fragment generated from p7G with sense (5′-CTATTTCATCGTGAACCTGCC-3′) and antisense (5′-AGAGTCTTGGCCGCTTTCTTC-3′) primers corresponding to the putative second and sixth transmembrane domains of the receptor coding region. Three positive clones were identified, each of which yielded a 4-kb *Hind*III/*Xba*I fragment that hybridized strongly to the PCR probe. This fragment was subcloned into pBluescript KS+ and designated pG3A. The restriction map of this clone showed a partial overlap with p7G (Fig. 1, *top*), and sequencing showed an identical 489-bp region overlapping with p7G and including a *Not*I restriction site. Comparison of the restriction maps for the cDNA and genomic clones suggests the presence of at least one intron in the 3′ region of the genomic sequence, in a position analogous to that of the splice site in the sixth transmembrane domain of the human α_{1b} gene (10).

Construction of a full length human cDNA/genomic α_{1d}-AR fusion construct. A full length cDNA/gene α_{1d}-AR fusion construct was constructed by ligation of the genomic clone 5′ from the *Not*I site to the cDNA clone 3′ from this site (Fig. 1) and was subcloned into the expression vector pcDNA1-Amp (yielding pcDNAα_{1d}). Sequencing of this construct showed an open reading frame of 1716 bp[2] encoding a 572-amino acid protein; the nucleotide and amino acid sequences are 99% and 100% identical, respectively, to those of the recently deposited human α_{1a} sequence (GenBank acces-

[2] The sequence of the fusion construct has been deposited in the GenBank database (accession number D29952).

TABLE 1

Inhibition of specific ^{125}I-BE binding in 293α_{1d} cells

Inhibition curves were analyzed by nonlinear regression of results from two experiments performed in duplicate (data not shown). All curves were best fit by a one-site model. K_i was calculated using the Cheng-Prusoff equation (24).

Drug	−log K_i
	M
Prazosin	9.57 ± 0.05
WB 4101	9.18 ± 0.04
5-MU	7.80 ± 0.02
Phentolamine	7.62 ± 0.03
Yohimbine	6.78 ± 0.05
(+)-Niguldipine	6.70 ± 0.10
(−)-NE	6.57 ± 0.06
Oxymetazoline	6.46 ± 0.04

sion number U03864). There also exist approximately 600-bp 5′ and 700-bp 3′ untranslated regions that have not been sequenced (Fig. 1). Amino acid identities, within the putative transmembrane domains, with other subtypes are as follows: α_{1a}, 68%; α_{1b}, 74%; rat α_{1d}, 98%; rat α_{1b}, 75%; bovine α_{1a}, 66%. There is 78% total amino acid identity with the the rat α_{1d}-AR but only 41% and 44% identity with the human α_{1a}- and α_{1b}-ARs, respectively. The α_{1d}-AR has a long amino terminus with potential *N*-linked glycosylation sites at residues 65 and 72. Several serine and threonine residues in the carboxyl terminus and intracellular loops may be potential targets for protein kinases A and C.

Stable expression of α_{1d} in 293 cells. To study the pharmacological and functional properties of this receptor, the full length α_{1d} construct was subcloned into pREP9 (yielding pREP9α_{1d}) and transfected into 293 cells. Geneticin-resistant cells were selected and propagated, and the level of expression of specific ^{125}I-BE binding sites was 304 fmol/mg of protein, with a K_d of 96 pM. Displacement of specific ^{125}I-BE binding from membranes showed that the recombinant α_{1d}-AR had a high affinity for prazosin and WB 4101 but a lower affinity for 5-MU and phentolamine and an even lower affinity for (+)-niguldipine, oxymetazoline, and yohimbine (Table 1). Pretreatment of membranes for 10 min with CEC (10 μM) reduced the density of binding sites by 71% (data not shown).

NE caused a concentration-dependent increase in [^{3}H]InsP formation in 293α_{1d} cells, with an EC_{50} of 12 nM and a maximal 3.8-fold increase over basal levels (Fig. 2, *left*). NE did not stimulate [^{3}H]InsP formation in untransfected 293

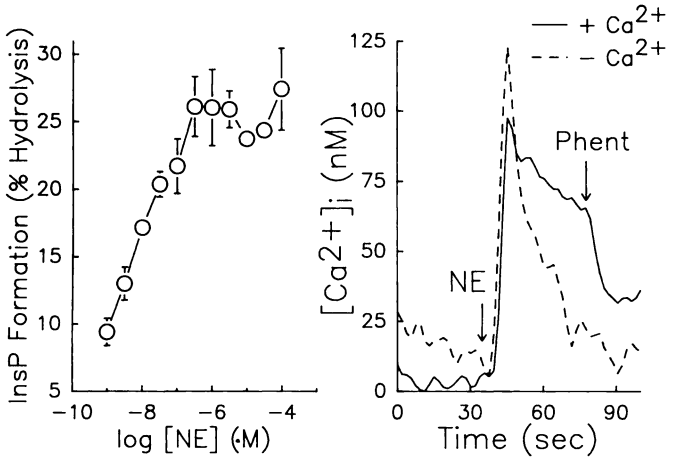

Fig. 2. Activation of InsP formation and mobilization of intracellular Ca^{2+} by the expressed human α_{1d}-AR in 293α_{1d} cells. *Left*, NE increases [^{3}H]InsP formation in a concentration-dependent manner in 293α_{1d} cells. Results are expressed as percentage hydrolysis of the *myo*-[^{3}H]inositol incorporated into the lipid pool. Each *point* represents the mean ± standard error of three or four experiments performed in duplicate. *Right*, fura-2 measurements of NE-stimulated increases in $[Ca^{2+}]_i$ in 293α_{1d} cells are shown. Cells were harvested and prepared for measurements of $[Ca^{2+}]_i$ with fura-2, in the presence of 1.5 mM free extracellular Ca^{2+} (+ Ca^{2+}) or in Ca^{2+}-free buffer with 2 mM EGTA (− Ca^{2+}), for 30 sec before addition of 100 μM NE. After 45 sec, 10 μM phentolamine (*Phent*) was added to cells incubated in the presence of Ca^{2+}. Data are from a single experiment representative of two experiments.

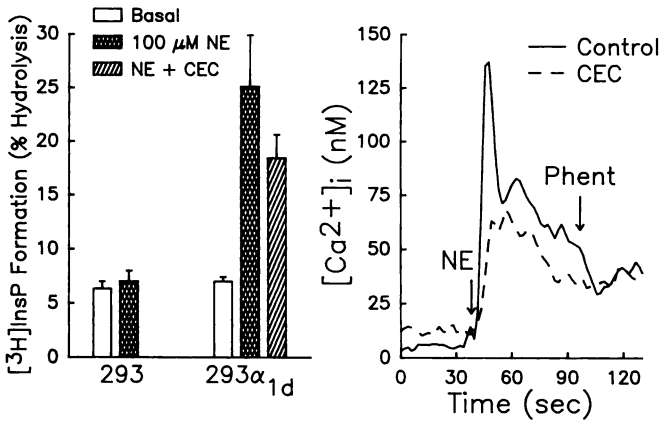

Fig. 3. Evidence that pretreatment of 293α_{1d} cells with CEC only partially blocks NE-stimulated InsP and Ca^{2+} responses. *Left*, effect of NE on [^{3}H]InsP formation in untransfected 293 cells and 293α_{1d} cells. Prelabeled 293α_{1d} cells were pretreated with 100 μM CEC for 30 min, washed three times, and incubated for 1 hr in the absence (*Basal*) or presence (*100* μM NE) of NE. Pretreatment with CEC caused a 35% decrease in the InsP response stimulated by 100 μM NE (*NE + CEC*). Results are expressed as percentage hydrolysis of the *myo*-[^{3}H]inositol incorporated into the lipid pool. Each *bar* represents the mean ± standard error of two or three experiments performed in duplicate. *Right*, fura-2 measurements of NE-stimulated increases in $[Ca^{2+}]_i$ in 293α_{1d} cells. Cells were incubated for 30 min in the presence or absence of 100 μM CEC, washed, and then exposed to 100 μM NE, followed by 10 μM phentolamine (*Phent*), in the presence of 1.5 mM free extracellular Ca^{2+}. Results are from a single experiment representative of two experiments.

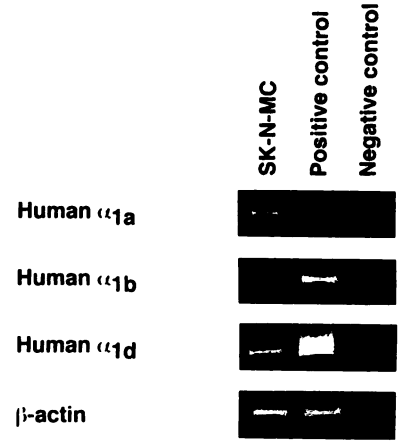

Fig. 4. RT-PCR assay for expression of three α_1-AR subtype mRNAs in human SK-N-MC cells. RT-PCR was performed with the primer sets described in the text. For positive controls, 0.1 ng of the respective cDNA fragments was amplified. No template cDNA was included with the primer sets for the negative controls. β-Actin served as a control for the efficiency of RNA isolation and cDNA synthesis.

TABLE 2

Effect of IPTG induction on total α_1-AR density (B_{max}) and affinity (K_d) in stably transfected SK-N-MC/LR cells expressing recombinant human α_1-AR subtypes

Cells were incubated in the absence (no IPTG) or presence (1 mM IPTG) of IPTG for 4–5 days and harvested, and membranes were prepared as described in the text. B_{max} and K_d values were determined by Scatchard analysis of saturation isotherms of ^{125}I-BE binding (Fig. 5). Each value represents the mean ± standard error of three or four independent experiments.

	B_{max}		K_d	
	No IPTG	1 mM IPTG	No IPTG	1 mM IPTG
	fmol/mg		*pM*	
Control	23.9 ± 3.8	23.5 ± 4.1	108 ± 36	110 ± 30
α_{1a}	52.6 ± 11.8	116.4 ± 23.0[a]	72 ± 20	64 ± 17
α_{1b}	57.0 ± 11.4[b]	102.2 ± 10.6[a]	69 ± 12	50 ± 10
α_{1d}	44.6 ± 6.2[b]	72.8 ± 14.3[b]	114 ± 42	102 ± 19

[a] Values significantly different from the respective control values, $p < 0.01$, by Students t test.
[b] $p < 0.05$.

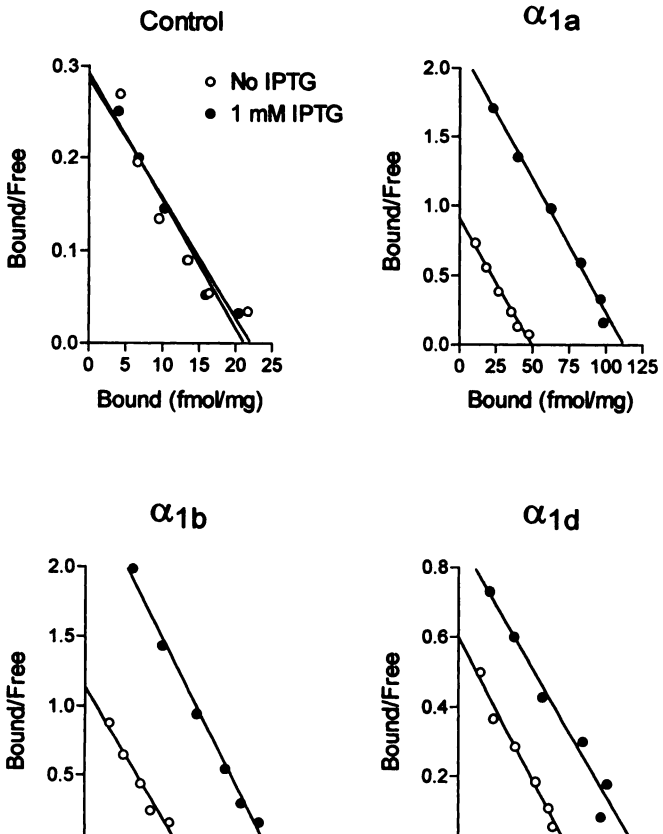

Fig. 5. Effect of IPTG on the expression of α_1-AR subtypes in non-transfected SK-N-MC/LR cells (control) or SK-N-MC/LR cells stably transfected with α_{1a}, α_{1b}, or α_{1d} operator vectors. Cells were incubated in the absence (○) or presence (●) of 1 mM IPTG for 4–5 days and harvested, and membranes were prepared as described in the text. Saturation isotherms for ^{125}I-BE binding were analyzed by Scatchard analysis as described in the text. Each *point* is the mean of duplicate determinations from four experiments.

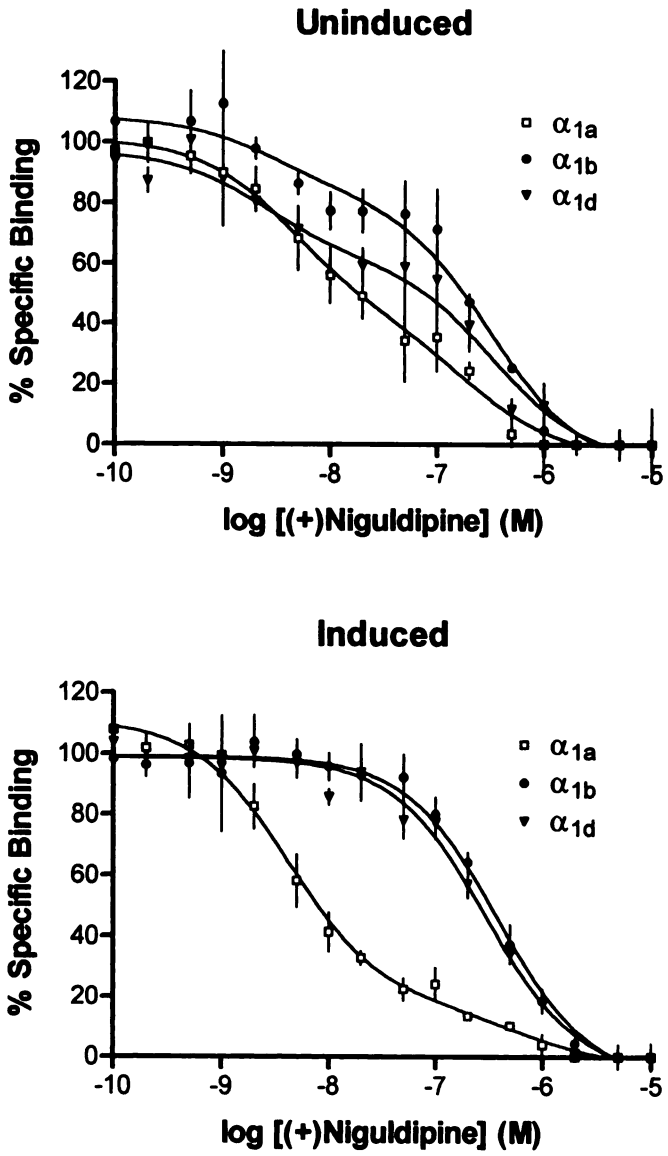

Fig. 6. Effect of IPTG induction on the inhibition of specific ^{125}I-BE binding by (+)-niguldipine in membrane preparations of SK-N-MC/LR cells stably transfected with α_{1a}-, α_{1b}-, or α_{1d}-ARs. Cells were incubated in the absence (*Uninduced*) or presence (*Induced*) of 1 mM IPTG for 4–5 days and harvested, membranes were prepared, and radioligand binding was measured as described in the text. Each *point* is the mean ± standard error from three experiments performed in duplicate.

cells (see Fig. 4). In the presence of 1.5 mM extracellular Ca^{2+}, NE (100 μM) caused a rapid transient increase in $[Ca^{2+}]_i$ in fura-2-loaded 293α_{1d} cells, followed by a sustained increase in $[Ca^{2+}]_i$ (Fig. 2, *right*). The increase in $[Ca]_i$ was also observed in the absence of extracellular Ca^{2+}, although the sustained component was lost. Addition of the α-AR antagonist phentolamine after NE stimulation blocked this response (Fig. 2, *right*). Pretreatment of confluent 293α_{1d} cells with 100 μM CEC for 30 min only partially reduced the effects of NE on both [^{3}H]InsP formation and $[Ca^{2+}]_i$ (Fig. 3).

RT-PCR analysis of α_1-AR subtypes in SK-N-MC cells. RT-PCR was used to identify mRNA expression of the three human α_1-AR subtypes in SK-N-MC cells. mRNA for both α_{1a}- and α_{1d}-AR subtypes was clearly expressed in SK-N-MC cells, whereas transcripts for the α_{1b}-AR were present at a very low, but detectable, level (Fig. 4). However, Southern blot analysis of the RT-PCR amplification products clearly showed the presence of a transcript for the α_{1b}-AR (data not shown).

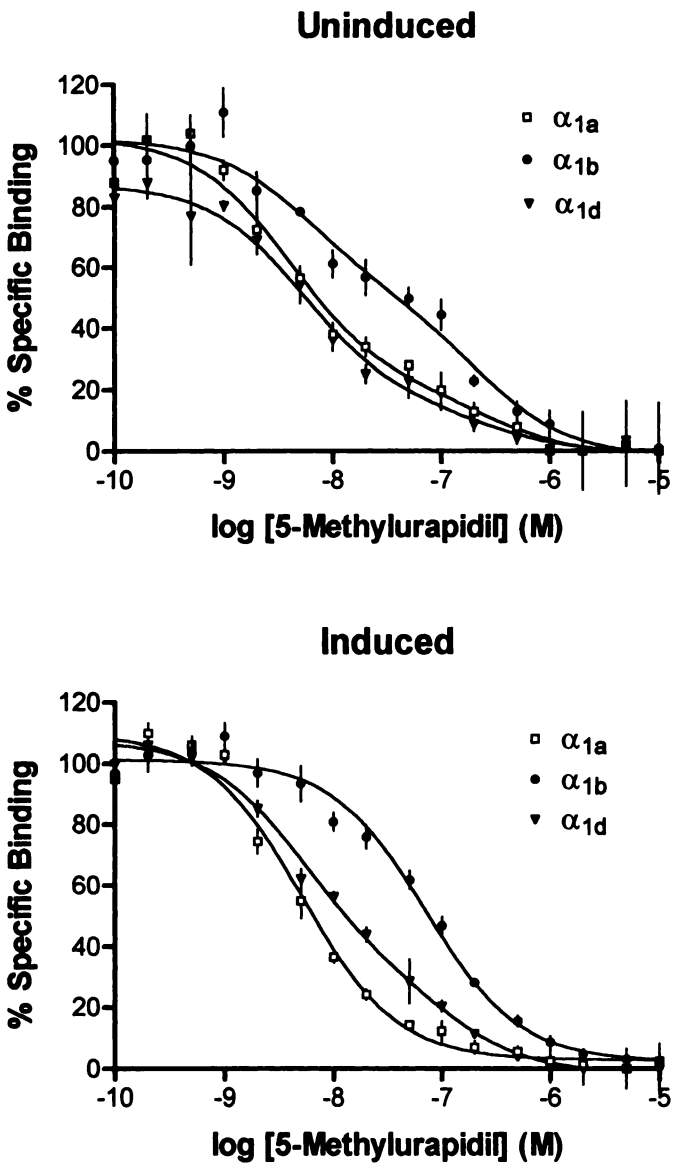

Fig. 7. Effect of IPTG induction on the inhibition by 5-MU of specific ^{125}I-BE binding in membrane preparations of SK-N-MC/LR cells stably transfected with α_{1a}-, α_{1b}-, or α_{1d}-ARs. Cells were incubated in the absence (*Uninduced*) or presence (*Induced*) of 1 mM IPTG for 4–5 days and harvested, membranes were prepared, and radioligand binding was measured as described in the text. Each *point* is the mean ± standard error from three experiments performed in duplicate.

Inducible expression of three human α_1-AR subtypes in SK-N-MC cells. The LacSwitch inducible expression system was used to compare expression of the three known human clones in human SK-N-MC cells, which are known to natively express α_{1A}- and α_{1B}-like AR subtypes (9) and the mRNA transcripts for all three cloned human α_1-AR subtypes. Thus, SK-N-MC cells should possess any cell-specific modification, processing, or microenvironmental factors that may contribute to the apparent pharmacological properties of the expressed receptors and allow for direct comparison of the cloned and native subtypes.

We previously used this IPTG-inducible LacSwitch system to study the relationship between α_{1B}-AR density and response in DDT$_1$ MF-2 cells.[3] This system has proven useful for controlling the level of receptor expression to examine the pharmacological properties of the receptor as well as the effect of receptor number on functional responses when only a single subtype is examined.[3] However, some caution is warranted in interpreting functional responses attributable to an induced subtype in a transfection system such as the SK-N-MC cell line, where other natively expressed α_1-AR subtypes exist and may contribute to the response. In addition, the effect of increased receptor expression on the coupling of various signal transduction pathways at "normal" or overexpressed levels of the receptor subtype of interest must be considered when this system is used. This effect can be controlled to some extent with this system by comparison of the responses elicited in transfected cells under uninduced and induced conditions.

SK-N-MC cells stably transfected with the constitutively active *lac* repressor vector (SK-N-MC/LR) were transfected with the *lac* operator vector containing each of the three human constructs (pOPα_{1a}, pOPα_{1b}, and pOPα_{1d}) and were selected with Geneticin. Resistant cells were propagated and exposed to 0 or 1 mM IPTG for 4–5 days, a time and concentration previously shown to give maximal induction of gene expression.[3] ^{125}I-BE labeled a homogeneous population of binding sites in membrane preparations from control and transfected cells, and IPTG had no effect on the density or affinity of endogenously expressed α_1-ARs (Table 2). Cells transfected with the three operator plasmids showed increased α_1-AR density in the absence of IPTG (Fig. 5; Table 2), probably due to leakiness in control of the operator vector. Addition of IPTG increased receptor density further and caused 3–5-fold increases in total α_1-AR density in cells transfected with each of the three operator vectors. After IPTG induction, a significant proportion of the total α_1-AR population consisted of the recombinant subtype transfected into that cell line (α_{1a}, 80%; α_{1b}, 77%; α_{1d}, 67%).

Potencies of (+)-niguldipine and 5-MU at human α_1-AR subtypes. Inhibition of specific ^{125}I-BE binding by (+)-niguldipine and 5-MU in membranes from both uninduced and IPTG-induced SK-N-MC/LR cells transfected with the three cloned human α_1-AR subtypes is shown in Figs. 6 and 7. Inhibition curves were best fit by a two-site model in both uninduced control and transfected cells (Table 3). The high (K_H) and low (K_L) affinity constants calculated for (+)-niguldipine and 5-MU were similar in all cases, although the proportions of sites differed. IPTG induction altered the inhibition curves such that (+)-niguldipine had similar low affinities in α_{1b}- and α_{1d}-AR-transfected cells, which were best fit by a one-site model. (+)-Niguldipine was about 100-fold more potent in α_{1a}-AR-transfected cells, with about 80% of the sites showing a high affinity similar to that in untransfected cells. IPTG induction also altered inhibition curves for 5-MU. However, in this case, α_{1b}-transfected cells showed a uniformly low affinity, α_{1a}-transfected cells showed a uniformly high affinity, and α_{1d}-transfected cells showed a two-site fit with 67% high affinity sites (Table 3).

Inactivation of human α_1-AR subtypes by CEC. The effects of pretreatment with increasing concentrations of

[3] T. A. Esbenshade, X. Wang, N. G. Williams, and K. P. Minneman. Inducible expression of α_{1B}-adrenoceptors in DDT$_1$ MF-2 cells: comparison of receptor density and response. Submitted for publication.

TABLE 3

Quantitative analysis of antagonist inhibition of ^{125}I-BE binding in transfected SK-N-MC cells

All curves were analyzed by nonlinear regression. Data are from Figs. 6 and 7. K_H and K_L refer to affinities for high and low potency sites, respectively. Percentage R_H is the percentage of total sites exhibiting high affinity. The p value refers to the probability that a two-site fit is better than a one-site fit. When a two-site fit was not statistically (NS) a better fit than a one-site fit, the data from the one-site fit were arbitrarily assigned to K_H or K_L.

	Uninduced				Induced		
	Control	α_{1a}	α_{1b}	α_{1d}	α_{1a}	α_{1b}	α_{1d}
(+)-Niguldipine							
$-\log K_H$ (M)	8.80 ± 0.22	8.66 ± 0.18	8.83 ± 0.64	8.93 ± 0.41	8.68 ± 0.04		
$-\log K_L$ (M)	6.89 ± 0.18	7.01 ± 0.21	6.81 ± 0.17	6.78 ± 0.21	6.54 ± 0.38	6.71 ± 0.05	6.82 ± 0.06
R_H (%)	45 ± 6	56 ± 8	22 ± 7	35 ± 7	82 ± 4	0	0
p	0.0001	0.0001	0.036	0.004	0.002	NS	NS
5-MU							
$-\log K_H$ (M)	8.69 ± 0.11	8.70 ± 0.18	8.53 ± 0.28	8.58 ± 0.14	8.59 ± 0.07		8.62 ± 0.18
$-\log K_L$ (M)	6.96 ± 0.24	6.85 ± 0.63	6.66 ± 0.36	6.91 ± 0.6		7.46 ± 0.05	7.30 ± 0.35
R_H (%)	69 ± 5	78 ± 9	49 ± 15	81 ± 9	100	0	67 ± 13
p	0.0001	0.046	0.015	0.036	NS	NS	0.006

CEC on ^{125}I-BE binding in membrane preparations from IPTG-induced SK-N-MC/LR cells expressing human α_{1a}-, α_{1b}-, or α_{1d}-ARs are shown in Fig. 8. The α_{1b}- and α_{1d}-ARs were inactivated by CEC with similar concentration dependencies, with an IC_{50} of approximately 1 μM (10 min, at 37°). Both subtypes were >80% inactivated by pretreatment with 30 μM CEC. The α_{1a}-AR showed about a 10-fold lower sensitivity to CEC (IC_{50} = 10 μM); however, 75% of binding sites were inactivated after pretreatment with 100 μM CEC. In intact SK-N-MC/LR cells expressing the α_{1a}-AR, pretreatment with CEC decreased NE-stimulated [^{3}H]InsP formation, but with only about 40% maximal inhibition (Fig. 8).

Discussion

The molecular nature of the natively expressed α_{1A}-AR has been controversial. The recent cloning and expression of the rat α_{1a}-AR cDNA strongly support the idea that this cloned subtype encodes the native α_{1A}-AR (6–7). However, unequivocal interpretation of available data is complicated by the possibility of species differences between clones, cell-specific post-translational modifications, differences in membrane microenvironments, and/or alternative splice variants, which might influence the binding properties of the expressed receptors. Comparing the three cloned human α_1-AR subtypes in cells capable of expressing both pharmacologically defined subtypes would remove many of these sources of potential confusion.

The sequence and expression of the human α_{1a}-AR (14) and α_{1b}-AR (10) have been reported, and a human α_{1d}-AR (20) sequence has been published. This α_{1d}-AR sequence was not expressed, contains an inverted repeat, and may be incomplete at the 5′ end. We therefore cloned a full length human α_{1d}-AR to directly compare the three human clones. We were unable to isolate a full length cDNA, probably due to the extremely high G/C content of the 5′ end (>90% G/C), but ligation of cDNA and genomic clones resulted in a full length fusion construct. While this report was being prepared, transfection of a human α_{1d} sequence into mouse fibroblasts was reported to result in α_1-AR binding sites (15), although functional responses were not studied. The sequence of that clone (GenBank accession number U03864) is essentially identical to ours.

The pharmacological properties of the α_{1d}-ARs stably expressed in human 293 cells are very similar to those of the rat α_{1d}-AR (4). Activation of the α_{1d}-AR increases InsP formation and mobilizes intracellular Ca^{2+} in 293 cells. The rat α_{1d} homolog couples to these responses relatively poorly (21); however, the human homolog couples with a relatively high efficiency, as indicated by the magnitude of the InsP and Ca^{2+} responses and the high potency of NE.

The SK-N-MC cell line is the only cell line known to endogenously express α_{1A}-ARs (9, 22), providing a useful transfection vehicle for direct comparison of the human clones. The human origin of these cells and their ability to express all known pharmacological subtypes suggest that species differences, cell-specific processing, or membrane microenvironment should not confuse receptor subclassification. We therefore transfected SK-N-MC cells with the three known human α_1-AR subtypes in IPTG-inducible vectors, to allow direct comparison of these subtypes.

Cells transfected with each subtype showed α_1-AR expression approximately twice that in untransfected cells. This likely represents constitutive uninduced activity of the operator vectors under these conditions. Inhibition curves for (+)-niguldipine and 5-MU in uninduced cells were fit best by a two-site model, indicating receptor heterogeneity. The proportion of high affinity sites depended on which subtype had been transfected, although the calculated K_i values did not vary. In comparisons of transfected/uninduced cells with native SK-N-MC cells, α_{1a}-AR-transfected cells had a larger proportion of high affinity sites for both compounds, α_{1b}-AR-transfected cells had a smaller proportion of high affinity sites for both (+)-niguldipine and 5-MU, and α_{1d}-AR-transfected cells had a smaller proportion of high affinity sites for (+)-niguldipine but a larger proportion of high affinity sites for 5-MU.

Exposure of the transfected SK-N-MC cells to IPTG increased the density of each subtype, such that the recombinant subtype comprised most (67–80%) of the total α_1-AR population. Analysis of the affinity constants for (+)-niguldipine and 5-MU in the induced cells clarifies the pharmacology of the expressed clones. Induction of the α_{1b} subtype results in uniformly low affinities for (+)-niguldipine and 5-MU. These affinity constants correspond to those for α_{1B}-ARs in native SK-N-MC cells (9), confirming that the α_{1b} gene encodes the pharmacologically defined α_{1B} subtype. Induction of the α_{1a} subtype results in predominantly high affinities for (+)-niguldipine and 5-MU, although a small

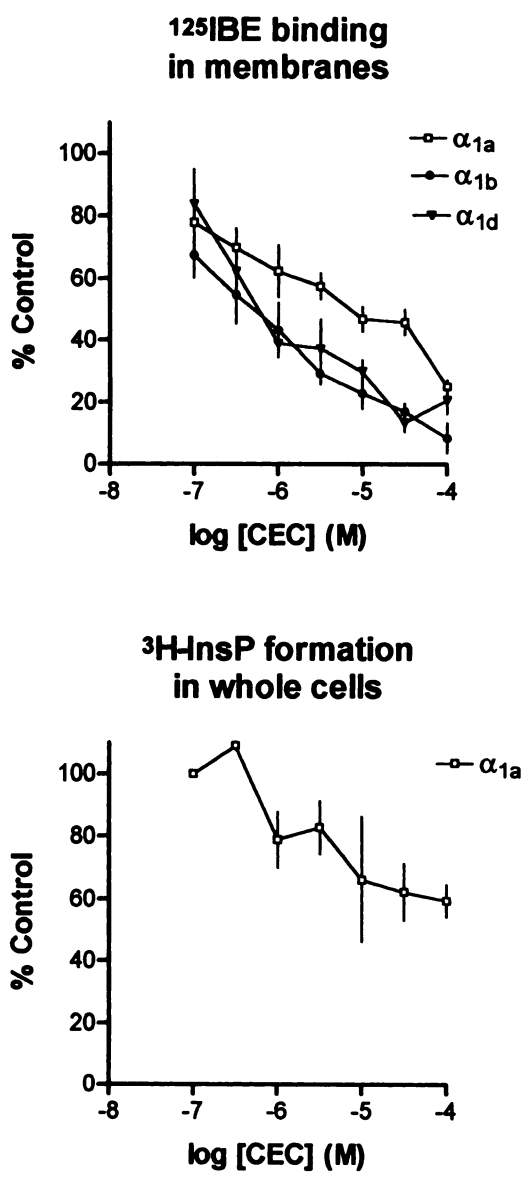

Fig. 8. Effect of CEC pretreatment on human α_1-AR subtypes in SK-N-MC/LR cells stably transfected with α_{1a}-, α_{1b}-, or α_{1d}-ARs and induced with 1 mM IPTG for 4–5 days. *Top*, membrane preparations were pretreated with increasing concentrations of CEC for 10 min at 37° in hypotonic buffer, washed, and resuspended in PBS, and the inhibition of binding by 200 pM ^{125}I-BE was determined as described in the text. Data are presented as a percentage of the specific control binding of 200 pM ^{125}I-BE to membranes not exposed to CEC pretreatment. Each *point* is the mean ± standard error from three experiments performed in duplicate. *Bottom*, prelabeled SK-N-MC/LR cells transfected with α_{1a}-ARs were pretreated with increasing concentrations of CEC for 30 min, washed three times, and incubated for 1 hr in the presence of 100 μM NE. Results are expressed as a percentage of the maximal response to NE in the absence of CEC. Basal and NE-stimulated percentages of hydrolysis were 8.2 ± 1.4% and 22.1 ± 1.8%, respectively. CEC had no effect on the basal level of [^{3}H]InsP formation (data not shown). Each *point* represents the mean ± standard error of three experiments performed in duplicate.

population of low affinity sites for (+)-niguldipine remain (probably representing endogenous expression of the other subtypes). These high affinity constants correspond to those for α_{1A}-ARs in native SK-N-MC cells (9), suggesting that the pharmacologically defined α_{1A}-AR is encoded by the α_{1a} gene. Induction of the α_{1d} subtype results in a uniformly low affinity for (+)-niguldipine, whereas 5-MU shows predominantly (67%) high affinity binding. This suggests that the α_{1d}-AR has a low (α_{1B}-like) affinity for (+)-niguldipine but a higher (α_{1A}-like) affinity for 5-MU. The high proportion of the recombinantly expressed subtype after IPTG induction makes it difficult to distinguish the native subtypes, which can be observed only with (+)-niguldipine in α_{1a}-AR-transfected cells and with 5-MU in α_{1d}-AR-transfected cells.

These data show that (+)-niguldipine has a very high affinity for the α_{1a} subtype and much lower, but similar, affinities for the α_{1b} and α_{1d} subtypes. 5-MU, on the other hand, has slightly higher affinity for the α_{1a} subtype than for the α_{1d} subtype but much lower affinity for the α_{1b} subtype. The differential affinity of 5-MU for the α_{1b} and α_{1d} subtypes explains complications in our previous analysis of α_1-AR binding sites in SK-N-MC cells (9). (+)-Niguldipine showed a lower proportion of high affinity sites (44%) than did 5-MU (69%) in native SK-N-MC cells, probably because these high affinity sites represent only the α_{1a}-AR for (+)-niguldipine but a mixture of α_{1a}- and α_{1d}-ARs for 5-MU. Interestingly, oxymetazoline and WB 4101 showed similar differences in their proportions of high affinity sites (44% and 79%, respectively) and oxymetazoline has an "α_{1B}-like" low affinity and WB 4101 an "α_{1A}-like" high affinity for the α_{1d} subtype (Table 1). This is compelling evidence that all three α_1-AR subtypes are endogenously expressed in SK-N-MC cells, with α_{1a}, α_{1b}, and α_{1d} comprising about 45%, 31%, and 24%, respectively, of the total native receptor population.

Pharmacological analysis predicts that all three genes are natively expressed in SK-N-MC cells, and thus all three mRNA species should be present. However, contradictory results have been reported. Northern blots show hybridization to the hamster α_{1b} and rat α_{1d} but not the bovine α_{1a} cDNA (9), whereas RNase protection assays show transcripts for the 5′ untranslated regions of the human α_{1b}-AR (10). Recent RNase protection assays, however, suggest transcripts for α_{1a}- and α_{1d}-ARs but not the α_{1b}-AR in SK-N-MC cells (11). Our RT-PCR experiments show that transcripts for α_{1a}- and α_{1d}-ARs are readily detectable at a considerably greater level, compared with those for the α_{1b}-AR. Overall, these reports are consistent with the endogenous expression of all three human genes in SK-N-MC cells, although the relationship of mRNA levels to protein expression for the different subtypes remains to be clarified.

The site-directed alkylating agent CEC has been a useful tool in differentiating α_1-AR subtypes, selectively inactivating α_{1B}-ARs but not α_{1A}-ARs (1, 19). However, use of CEC has often given conflicting results in tissues, isolated cells, and membranes, and inactivation is influenced by incubation conditions (19). Quantitative comparison of the expressed animal clones showed that the hamster α_{1b}-AR and rat α_{1d}-AR were equally sensitive to CEC, whereas the bovine α_{1a}-AR was about 10-fold less sensitive (23). Experiments here with the human clones gave essentially identical results, with the α_{1a}-AR being 10-fold less sensitive than the other two subtypes. However, CEC was generally less effective in inactivating α_1-AR-stimulated responses in transfected cells, causing only 40% inhibition of NE-stimulated InsP formation in α_{1a}-transfected SK-N-MC cells at a concentration that inactivated >75% of the binding sites in membranes. Similar results were obtained with 293α_{1d} cells. These results reemphasize the need for caution in using CEC

to differentiate α_1-AR subtypes (19). Highly selective competitive antagonists, when available, will be preferable to site-directed alkylating agents for receptor subclassification.

In summary, expression of a human α_{1d}-AR cDNA/gene fusion construct results in receptors with pharmacological properties similar to those of the rat α_{1d}-AR, which efficiently couples to InsP formation and intracellular Ca^{2+} mobilization. Comparison of the three human α_1-AR subtypes expressed in SK-N-MC cells suggests that the α_{1a}-AR has a much higher affinity for (+)-niguldipine than does either the α_{1b}- or α_{1d}-AR and is least sensitive to inactivation by CEC. The α_{1a}- and α_{1d}-ARs have similar affinities for 5-MU (about 10-fold greater than that of the α_{1b}-AR). Quantitative analysis of radioligand binding sites produced by inducible expression of the three human clones in SK-N-MC cells provides strong evidence that the complex α_{1A}- and α_{1B}-like pharmacology observed in native SK-N-MC cells is due to expression of all three subtypes in different proportions and that the pharmacologically defined α_{1A} subtype is encoded by the α_{1a} gene.

Acknowledgments

We thank Donald M. Hastings III and Susanne Hollinger for excellent technical assistance. We also thank Drs. Diane Perez and Robert M. Graham for generously providing the rat α_{1d}- and human α_{1b}-AR clones and Dr. Debra Schwinn for helpful discussions.

References

1. Minneman, K. P., and T. A. Esbenshade. α_1-Adrenergic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.* **34**:117–133 (1994).
2. Cotecchia, S., D. A. Schwinn, R. R. Randall, R. J. Lefkowitz, M. G. Caron, and B. K. Kobilka. Molecular cloning and expression of the cDNA for the hamster α_1-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **85**:7159–7163 (1988).
3. Schwinn, D. A., J. W. Lomasney, W. Lorenz, P. J. Szklut, R. T. Fremeau, Jr., T. L. Yang-Feng, M. G. Caron, R. J. Lefkowitz, and S. Cotecchia. Molecular cloning and expression of the cDNA for a novel α_1-adrenergic receptor subtype. *J. Biol. Chem.* **265**:8183–8189 (1990).
4. Perez, D. M., M. T. Piascik, and R. M. Graham. Solution-phase library screening for the identification of rare clones: isolation of an α_{1D}-adrenergic receptor cDNA. *Mol. Pharmacol.* **40**:876–883 (1991).
5. Lomasney, J. W., S. Cotecchia, W. Lorenz, W. Y. Leung, D. A. Schwinn, T. L. Yang-Feng, R. J. Lefkowitz, and M. G. Caron. Molecular cloning and expression of the cDNA for the α_{1A}-adrenergic receptor, the gene for which is located on human chromosome 5. *J. Biol. Chem.* **266**:6365–6369 (1991).
6. Laz, T. M., C. Forray, K. E. Smith, J. A. Bard, P. J.-J. Vaysse, T. A. Branchek, and R. L. Weinshank. Cloned rat homolog of the bovine α_{1C}-adrenergic receptor shows the pharmacological properties of the classical α_{1A} subtype. *Mol. Pharmacol.* **46**:414–422 (1994).
7. Perez, D. M., M. T. Piascik, N. Malik, R. Gaivin, and R. M. Graham. 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. *Mol. Pharmacol.* **46**:823–831 (1994).
8. Fisher, S. K., and R. E. Landon. Identification of multiple phosphoinositide-linked receptors on human SK-N-MC neuroepithelioma cells. *J. Neurochem.* **57**:1599–1608 (1991).
9. Esbenshade, T. A., C. Han, T. J. Murphy, and K. P. Minneman. Comparison of α_1-adrenergic receptor subtypes and signal transduction in SK-N-MC and NB41A3 neuronal cell lines. *Mol. Pharmacol.* **44**:76–86 (1993).
10. Ramarao, C. S., J. M. Kincade-Denker, D. M. Perez, R. J. Gaivin, R. P. Riek, and R. M. Graham. Genomic organization of the human α_{1B}-adrenergic receptor. *J. Biol. Chem.* **267**:21936–21945 (1992).
11. Price, D. T., R. S. Chari, D. E. Berkowitz, W. Meyers, and D. A. Schwinn. Expression of α_1-adrenergic receptor subtype mRNA in rat tissues and human SK-N-MC neuronal cells: implications for α_1-adrenergic subtype classification. *Mol. Pharmacol.* **46**:221–226 (1994).
12. Bevan, J. W., M. A. Oriowo, and R. D. Bevan. Physiological variation in α-adrenoceptor mediated arterial sensitivity: relation to agonist affinity. *Science (Washington D. C.)* **234**:196–197 (1986).
13. Strader, C. D., M. R. Candelore, E. Rands, and R. A. F. Dixon. β-Adrenergic receptor subtype is an intrinsic property of the receptor gene product. *Mol. Pharmacol.* **32**:179–183 (1987).
14. Hirasawa, A., K. Horie, T. Tanaka, K. Takagaki, M. Murai, J. Yano, and G. Tsujimoto. Cloning, functional expression and tissue distribution of human cDNA for the α_{1C}-adrenergic receptor. *Biochem. Biophys. Res. Commun.* **195**:902–909 (1993).
15. Forray, C., J. A. Bard, J. M. Wetzel, G. Chiu, E. Shapiro, R. Tang, H. Lepor, P. R. Hartig, R. L. Weinshank, T. A. Branchek, and C. Gluchowski. The α_1-adrenergic receptor that mediates smooth muscle contraction in human prostate has the pharmacological properties of the cloned human α_{1C} subtype. *Mol. Pharmacol.* **45**:703–708 (1994).
16. Yang-Feng, T. L., F. Xue, W. Zhong, S. Cotecchia, T. Frielle, M. G. Caron, R. J. Lefkowitz, and U. Francke. Chromosomal localization of adrenergic receptor genes. *Proc. Natl. Acad. Sci. USA* **87**:1516–1520 (1990).
17. Yang-Feng, T. L., H. Han, J. W. Lomasney, and M. G. Caron. Localization of the cDNA for an α_1-adrenergic receptor subtype (ADRA1D) to chromosome band 20p13. *Cytogenet. Cell Genet.* **66**:170–171 (1994).
18. Nudel, U., R. Zakut, M. Shani, S. Neuman, Z. Levy, and D. Yaffe. The nucleotide sequence of the rat cytoplasmic β-actin gene. *Nucleic Acids Res.* **11**:1759–1771 (1983).
19. Minneman, K. P., C. Han, and P. W. Abel. Comparison of α_1-adrenergic receptor subtypes distinguished by chlorethylclonidine and WB 4101. *Mol. Pharmacol.* **33**:509–514 (1988).
20. Bruno, J. F., J. Whittaker, J. Song, and M. Berelowitz. Molecular cloning and sequencing of a cDNA encoding a human α_{1A}-adrenergic receptor. *Biochem. Biophys. Res. Commun.* **179**:1485–1490 (1991).
21. Salon, J. A., J. A. Bard, C. Forray, R. L. Weinshank, and T. A. Branchek. Distribution of G protein α subunits and their coupling to cloned adrenergic α_1 receptors in commonly used transfection hosts. *Soc. Neurosci. Abstr.* **19**:386.1 (1993).
22. Fisher, S. K., and R. E. Landon. Identification of multiple phosphoinositide-linked receptors on human SK-N-MC neuroepithelioma cells. *J. Neurochem.* **57**:1599–1608 (1991).
23. Han, C., S. Hollinger, T. L. Theroux, T. A. Esbenshade, and K. P. Minneman. ^{3}H-Tamsulosin binding to cloned α_1-adrenergic receptor subtypes expressed in human embryonic kidney 293 cells: antagonist potencies and sensitivity to alkylating agents. *Pharmacol. Commun.* **5**:117–126 (1995).
24. Cheng, Y., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Anal. Biochem.* **22**:3099–3108 (1973).

Send reprint requests to: Timothy A. Esbenshade, Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322.