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Novel human α_{1a} -adrenoceptor single nucleotide polymorphisms alter receptor pharmacology and biological function

Beilei Lei,

Department of Anesthesiology, Duke University Medical Center, Box 3094 Durham, NC, 27710, USA

Department of Pharmacology/Cancer Biology Duke University Medical Center, Durham, NC, USA

Daniel P. Morris,

Department of Anesthesiology, Duke University Medical Center, Box 3094 Durham, NC, 27710, USA

Department of Pharmacology/Cancer Biology Duke University Medical Center, Durham, NC, USA

Michael P. Smith,

Department of Anesthesiology, Duke University Medical Center, Box 3094 Durham, NC, 27710, USA

Department of Pharmacology/Cancer Biology Duke University Medical Center, Durham, NC, USA

Laura P. Svetkey,

Department of Medicine, Duke University Medical Center, Durham, NC, USA

Mark F. Newman,

Department of Anesthesiology, Duke University Medical Center, Box 3094 Durham, NC, 27710, USA

Jerome I. Rotter,

Department of Medicine, Cedars-Sinai Medical Center and the University of California, Los Angeles, CA, USA

Department of Pediatrics, Cedars-Sinai Medical Center and the University of California, Los Angeles, CA, USA

Department of Human Genetics, Cedars-Sinai Medical Center and the University of California, Los Angeles, CA, USA

Thomas A. Buchanan,

Department of Medicine, University of Southern California, Los Angeles, CA, USA

Stephen M. Beckstrom-Sternberg,

Translational Genomics Research Institute, Phoenix, AZ, USA

Eric D. Green, and

Genome Technology Branch and NIH Intramural Sequencing Center, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA

Debra A. Schwinn

Department of Anesthesiology, Duke University Medical Center, Box 3094 Durham, NC, 27710, USA, e-mail: schwi001@mc.duke.edu, Tel.: +1-919-6814781, Fax: +1-919-6814776

Department of Pharmacology/Cancer Biology Duke University Medical Center, Durham, NC, USA

Abstract

We identified nine naturally-occurring human single nucleotide polymorphisms (SNPs) in the α_{1a} -adrenoceptor (α_{1a} AR) coding region, seven of which result in amino acid change. Utilizing rat-1 fibroblasts stably expressing wild type α_{1a} AR or each SNP at both high and low levels, we investigated the effect of these SNPs on receptor function. Compared with wild type, two SNPs (R166K, V311I) cause a decrease in binding affinity for agonists norepinephrine, epinephrine, and phenylephrine, and also shift the dose-response curve for norepinephrine stimulation of inositol phosphate (IP) production to the right (reduced potency) without altering maximal IP activity. In addition, SNP V311I and I200S display altered antagonist binding. Interestingly, a receptor with SNP G247R (located in the third intracellular loop) displays increased maximal receptor IP activity and stimulates cell growth. The increased receptor signaling for α_{1a} AR G247R is not mediated by altered ligand binding or a deficiency in agonist-mediated desensitization, but appears to be related to enhanced receptor-G protein coupling. In conclusion, four naturally-occurring human α_{1a} AR SNPs induce altered receptor pharmacology and/or biological activity. This finding has potentially important implications in many areas of medicine and can be used to guide α_{1a} AR SNP choice for future clinical studies.

Keywords

α_{1a} -Adrenoceptor; Polymorphism; Single nucleotide polymorphisms; Signal transduction; Inositol phosphate; Receptor/G protein coupling; Human

Introduction

α_1 -Adrenoceptors (α_1 ARs) belong to a superfamily of G protein-coupled receptors (GPCRs) that share a common overall structure with seven hydrophobic transmembrane (TM) helices. α_1 ARs are activated by endogenous catecholamines norepinephrine (NE) and epinephrine, thereby mediating actions of the sympathetic nervous system. Stimulation of α_1 ARs predominantly activates the $G_{q/11}$ protein, resulting in hydrolysis of membrane phospholipids via phospholipase C β ; resultant second messengers include inositol triphosphate (IP₃) and diacylglycerol, which mobilize intracellular calcium and activate protein kinase C, respectively (Graham et al. 1996; Michelotti et al. 2000).

Three α_1 AR subtypes have been identified based on results from pharmacological and molecular cloning studies— α_{1a} (formerly α_{1c} , located in chromosome 8p), α_{1b} (located in chromosome 5q) and α_{1d} (formerly $\alpha_{1a/d}$, located in chromosome 20p). These subtypes are present in a wide variety of organs and tissues including human brain, liver, prostate, vascular smooth muscle, and myocardium (Price et al. 1994; Rudner et al. 1999). α_{1a} ARs have been shown to play important roles in the dynamic component of benign prostatic hyperplasia (Roehrborn and Schwinn 2004) and in the development of myocardial hypertrophy (Autelitano and Woodcock 1998; Rokosh et al. 1996). Previous studies from our laboratory demonstrated that the α_{1a} AR subtype predominates in human resistance vessels, which mediate sympathetically derived vasoconstriction (Rudner et al. 1999). Consistent with α_{1a} AR expression patterns (Rudner et al. 1999), Rokosh and Simpson (2002) have used a gene knockout approach to verify that the α_{1a} AR subtype is a vasopressor in resistance arteries and is required to maintain normal arterial blood pressure. Taken together these findings suggest that human α_{1a} ARs contribute to blood pressure homeostasis and potentially the pathogenesis of diseases such as hypertension.

In addition to tissue-specific differences in both AR subtype distribution and expression levels, naturally-occurring human receptor polymorphisms have been shown to modulate

sympathetically mediated physiologic responses. Most data in this regard originate from β ARs (β_1 , β_2 and β_3) and α_2 ARs (α_{2a} , α_{2b} , α_{2c} ; Kirstein and Insel 2004; Small et al. 2002; Snapir et al. 2001; Svetkey et al. 1996). More limited genetic variant studies have been performed within the α_1 AR family, including identification of rare, nonfunctional, truncated α_{1a} ARs resulting from incomplete splicing of the two exons (Hawrylyshyn et al. 2004). The only polymorphic site in the full-length human α_{1a} AR functionally analyzed to date, R492C, is located in the carboxyl terminal portion of the receptor and was discovered via a *Pst*I restriction fragment length polymorphism (RFLP; Hoehe et al. 1992). Since this polymorphism has no effect on receptor behavior in vitro (Shibata et al. 1996), it is not surprising that no association has been shown for this variant and several diseases—benign prostatic hyperplasia (Shibata et al. 1996), depression (Bolonna et al. 2000), or essential hypertension (Xie et al. 1999). More recently, Sofowora et al. also demonstrated that this polymorphism has no impact on agonist-mediated veno-constriction in vivo (Sofowora et al. 2004). Therefore, it is important to identify and characterize other human α_{1a} AR polymorphisms.

In order to more fully define naturally-occurring polymorphisms for the human α_{1a} AR, we systematically resequenced the entire α_{1a} AR coding region in 281 individuals. Nine single nucleotide polymorphisms (SNPs) were identified including the previously described R492C variant (named as R347C throughout this report based on position of the α_{1a} AR protein sequence). Seven SNPs alter amino acid in the encoded human α_{1a} AR protein. We hypothesized that these SNPs might induce changes in receptor biological characteristics and function, which may influence variations in sympathetically mediated diseases. To examine this possibility, we employed rat-1 fibroblasts stably expressing either wild type (WT) receptor or receptors with each of the seven amino acid-altering SNPs and tested each for biological function including ligand binding, signaling, desensitization, and receptor–G protein interaction properties. Our findings indicate that four of seven naturally-occurring α_{1a} AR SNPs induce altered α_{1a} AR pharmacology and/or biological activity, a finding that may have important clinical implications and can be used to guide α_{1a} AR SNP choice for future clinical studies.

Materials and methods

Materials

Drugs and reagents were obtained from the following sources: (–)-epinephrine, ((–)-norepinephrine, oxymetazoline, phenylephrine, prazosin, 5-methylurapidil, phentolamine (Sigma, St. Louis, MO, USA); 125 I-(2- β -(4-hydroxyphenyl)-ethylaminomethyl)-tetralone ([125 I]HEAT), [3 H]inositol, [3 H]thymidine, [35 S]GTP γ S (Perkin-Elmer Life Sciences, Boston, MA, USA); Dulbecco's modified Eagle medium (DMEM) and G418 (Gibco, Grand Island, NY, USA); and fetal bovine serum (FBS, Hyclone, Logan, UT, USA).

SNP identification

The study was approved by the Institutional Review Board of Duke University. We employed a systematic sequencing strategy to identify SNPs in α_{1a} AR coding region. Genomic DNA was obtained from 281 individuals (562 chromosomes) purposefully inclusive of multiple ethnic populations (Black, Hispanic, White, American Indian); sources of DNA included the Coriell SNP discovery panel (Coriell Institute, Camden, NJ; $n=90$, enriched for minorities) and individuals from hypertension clinics and hospital settings in Los Angeles, CA ($n=40$) and Durham, NC ($n=151$). Five overlapping PCR amplicons (400–500 bp each) were generated from 1.5 kb α_{1a} AR gene (including 5' and 3' regions immediately adjacent to two exons), followed by direct double-stranded sequencing of PCR products. SNPs were identified from sequence traces using PolyPhred/Phrap (<http://www.phrap.org>; Nickerson et al. 1997). SNP authenticity was confirmed by manually examining each sequence trace identified by Consed (<http://www.genome.washington.edu>; Gordon et al. 1998) with only the three most stringent

matches used and by confirming the presence of the SNP in both forward and reverse reads. This was followed by confirmation after a separate PCR reaction using at least one of the following criteria: RFLP analysis, resequencing, presence of the SNP in $n \geq 5$ individuals in the data set, or a subcloning the PCR product into PCRII plasmid and transformation into One Shot INVαF' *E. coli* cells (Invitrogen, Carlsbad, CA, USA) (plasmid DNA was then isolated from individual colonies followed by DNA sequencing to identify the SNP sequence of each individual allele).

In vitro site-directed mutagenesis of the α_{1a} AR

Site-directed mutagenesis was utilized to introduce mutations corresponding to each SNP into hemagglutinin-tagged human α_{1a} AR previously placed in the expression vector pcDNA3 (Price et al. 2002). Mutagenesis was performed using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene) as recommended by the manufacture. All mutations were confirmed by DNA sequencing (Duke University DNA Analysis Facility).

Cell culture and transfection

Rat-1 fibroblasts were cultured in DMEM supplemented with 10% FBS at 37°C. Cells were transfected with either the WT (reference sequence with all major alleles, GenBank accession number L31774) or each of seven individually mutated α_{1a} ARs in pcDNA3 by calcium phosphate precipitation. For stable transfection, clones resistant to G418 (0.8 mg/ml) were isolated and tested for receptor expression. Clones at high and low expression levels were chosen for investigation of receptor function. Low receptor expression level was defined a priori as <0.5 pmol/mg total protein, while high-level expression was defined as >1.5 pmol/mg protein. Transient transfection with identical amounts of each DNA (3 μ g/10 cm plate) was also used to test the effect of SNPs on receptor expression and ligand binding.

Membrane preparation and radioligand binding

Rat-1 membrane preparation and ligand binding assay using the α_1 AR selective antagonist, [125 I]HEAT, were performed as previously described (Schwinn et al. 1995). For saturation binding isotherms, [125 I]HEAT concentrations ranged from 10 to 600 pM. Competition curves were performed with a final [125 I]HEAT concentration of 60 pM. Nonspecific binding was measured in the presence of 1 μ M prazosin. Curves were fitted using noniterative regression analysis with Prism 3.0 (Graphpad, San Diego, CA, USA).

Measurement of intracellular inositol phosphate production

Rat-1 cells expressing either the WT or mutated α_{1a} AR grown on 12-well plates were labeled with [3 H]inositol for 20–24 h with 2.5 μ Ci/ml in complete DMEM. Measurement of intracellular inositol phosphate (IP) production was performed under serum-free conditions. After labeling, cells were stimulated for 20 min with various concentrations of NE in DMEM containing 20 mM LiCl. In desensitization experiments, cells were pretreated for 10 min with 10 μ M NE or vehicle, quickly rinsed with DMEM once, placed in DMEM with 20 mM LiCl, immediately stimulated by NE addition, and then incubated for 20 min. Total inositol phosphates were extracted and separated as described previously (Price et al. 2002). For all experiments, membranes were collected for receptor quantitation and cells were counted at the time of assay.

[35 S]GTP γ S binding

Confluent cells were washed twice with cold PBS, harvested, and homogenized in cold 50 mM Tris buffer (pH 7.4) using a Kinematica polytron. The homogenate was centrifuged for 20 min at 42,000 $\times$ g and the membrane pellet resuspended in the same buffer and centrifuged two additional times. [35 S]GTP γ S binding experiments were initiated by the addition of 20- μ g

membranes to an assay buffer (50 mM Tris, pH 7.4, 120 mM NaCl, 3 mM MgCl, 0.2 mM EDTA, 3 μ M GDP, 0.1 nM [35 S]GTP γ S) containing 10 μ M NE or vehicle (basal) or 10 μ M GTP γ S (to define nonspecific binding). Reactions were incubated for 20 min at 37°C and terminated by rapid filtration through GF/B filters using a cell harvester. Filters were washed three times with ice-cold dH₂O and counted with a liquid scintillation counter.

[3 H]Thymidine incorporation

Cells plated in 24-well plates at 1×10^4 cells/well were cultured in complete DMEM for 48 h with 1 μ Ci [3 H]thymidine included during the last 4-h incubation. Then cells were harvested and [3 H]thymidine incorporation was quantified as described previously (Cornwell et al. 1994).

Measurement of cellular total protein

Cells plated in 12-well plates at 5×10^4 cells/well were cultured in complete DMEM. After washing once with PBS, cells were harvested by 250 μ l of lysis buffer (1% nonidet P-40 and 0.5% sodium deoxycholate) at 4-, 24-, 48-, and 72-h points with side-by-side cell count. Fifty microliters of samples were used for total protein measurement by BCA protein assay reagent kit (Pierce, Rockford, IL, USA).

Statistical analysis

Results are expressed as the mean $\pm$ SEM, compiled from n replicate experiments each performed in duplicate or triplicate. Statistical significance was analyzed by ANOVA and where significance was identified, Student's t tests were used to determine exact p values. All calculations were performed using GraphPad Prism 3.0 (GraphPad Software) with $p < 0.05$ considered significant.

Results

Human α_{1a} AR SNPs

Nine α_{1a} AR coding region SNPs were identified and confirmed by our laboratory in this study. Throughout this manuscript, amino acid numbers are referenced relative to the initiator methionine (M=1). Receptors with SNPs are designated with the number of the polymorphic residue preceded by the wild type amino acid and followed by the SNP amino acid. Nucleotide numbers are relative to the ATG (A=1) in an analogous fashion. As seen in Table 1, SNPs located at nucleotides 15 and 1,203, do not induce amino acid change and were not investigated for their pharmacological characteristics here. The other seven SNPs at nucleotides 460, 497, 599, 739, 931, 1,039, and 1,395 alter encoded residues at amino acid positions 154, 166, 200, 247, 311, 347, and 465 of the human α_{1a} AR protein, respectively. Figure 1 shows the location of each SNP relative to putative agonist and antagonist binding sites and salt bridge in the human α_{1a} AR. In general α_{1a} AR coding region SNPs are relatively rare except for SNPR347C with $f(-)=0.46$. Our study demonstrates that this common SNPR347C is less frequent in blacks, $f(-)=0.267$, than in whites, $f(-)=0.565$, and hispanics, $f(-)=0.525$, consistent with a previous report (Xie et al. 1999). Although SNPs S154A, I200S, G247R, and E465D are rare, they have recently been reported by other groups (see Table 1), but have never been functionally characterized. Because of these low frequencies, it is also not possible to directly test association with hypertensive disease states in this pilot population.

Pharmacological characterization

In order to examine whether these natural polymorphic α_{1a} ARs have altered ligand binding characteristics and would therefore be candidates for larger association studies, we first performed saturation binding isotherms to determine the dissociation constant (K_d) for the

antagonist [125 I] HEAT, followed by competition assays designed to determine binding affinities for a series of α_1 AR agonists and antagonists. Binding measurements with [125 I] HEAT on membranes from a WT α_{1a} AR high-expressing clone indicates a receptor density of 1.77 ± 0.24 pmol/mg protein and a K_d value of 42.1 ± 6.5 pM (Table 2). At similar expression levels (1.53–2.37 pmol/mg), receptors containing each SNP display K_d values for [125 I]HEAT not significantly different from WT; these findings suggest that alterations of amino acids in these seven SNPs do not affect the overall receptor [125 I]HEAT binding site (Table 2).

Competition binding analysis with the classic α_{1a} AR subtype selective agonist oxymetazoline shows no change in the affinity for receptors containing any SNP (Table 2). In contrast, SNPs R166K and V311I, in TM 4 and 7, respectively, cause a significant decrease in receptor affinity for the agonists NE, epinephrine, and phenylephrine (K_I increased approximately 3-fold). Competition experiments using antagonists demonstrate that α_{1a} AR V311I also has a 3-fold higher affinity for α_{1a} AR subtype selective antagonist 5-methylurapidil (Table 2). In addition, a SNP in TM5 (I200S) decreases receptor binding affinity for the antagonist phentolamine (K_I increased approximately 3-fold). No change in affinity for any variant is noted for the classic nonsubtype selective α_1 AR antagonist prazosin.

To exclude the possibility that receptor densities or clonal differences might be responsible for altered binding characteristics of receptors containing SNPs, we next tested ligand binding for each receptor from distinct low-expressing clones (receptor densities 0.21–0.44 pmol/mg protein). As shown in Table 3, binding constants obtained are essentially identical to those observed for receptors present in high-expressing clones, with exactly the same polymorphic receptors (R166K, V311I, I200S) displaying similar alterations in agonist and antagonist affinity. Therefore, our findings are consistent across multiple clones and expression levels. Finally, we tested receptor expression and binding affinities by transient transfection with identical amounts of each DNA. Receptor densities are high and fairly consistent for all the transiently expressed receptors (1.30–1.98 pmol/mg protein), indicating that all SNPs have no major effects on receptor expression or stability. The binding results obtained from transient transfection membranes are essentially the same as those observed for individual stable clones.

α_1 AR signal transduction

To investigate whether these SNPs affect receptor activation, we tested each receptor's ability to stimulate IP formation in response to challenge with the endogenous agonist NE. For stable clones at high expression, neither basal IP release (without agonist stimulation, Fig. 2a) nor efficacy of NE (maximal activity, Fig. 2b) is altered with respect to α_{1a} AR WT for receptors with any SNP. However, the same receptors that display decreased affinity for NE, α_{1a} AR R166K and V311I also display 2.0-fold and 2.4-fold decreases in the potency of NE-stimulated IP formation, respectively, compared with WT receptor, 50% effective concentration (EC_{50}) = 0.16 ± 0.01 and 0.19 ± 0.02 μ M for R166K and V311I, respectively, versus 0.08 ± 0.01 μ M, (Fig. 2c). As observed at high expression levels, receptors containing SNP V311I or SNP R166K at low expression levels display a significantly increased EC_{50} for NE, demonstrating that decreased agonist binding for this receptor consistently translates into less effective IP production (i.e., requires more agonist for half maximal activity). This shift in dose-response curve to the right for R166K and V311I (reduced potency) confirms the pharmacologic alteration in these two SNPs.

Investigation of IP signaling properties at the low-expression level (Fig. 3) indicates that basal and maximum IP production for polymorphic receptors are the same as the WT receptor with the exception of α_{1a} AR G247R (in the third intracellular loop), which displays a significantly higher maximum activity (confirmed in three totally independent clones). This maximal activity is close to that observed with high-expression clones; consequently the higher activity of α_{1a} AR G247R appears to reflect a native ability to achieve full signaling with less receptor.

Effects of human α_{1a} AR SNPs on norepinephrine-induced desensitization

A characteristic of many GPCRs is the tendency of these receptors to elicit less signal with continuing agonist exposure (i.e., to desensitize). Because human α_{1a} ARs have been shown to desensitize in response to agonist stimulation (Price et al. 2002), we tested the ability of rat-1 fibroblasts stably expressing α_{1a} AR WT or each SNP to respond to a subsequent challenge with NE following an initial NE pretreatment. As expected for the wild type α_{1a} AR, compared with vehicle-pretreated groups, pretreatment with NE results in 33.5 ± 3.2 and $31.0 \pm 3.1\%$ lower IP production at high- and low-expression levels, respectively (Price et al. 2002). There is no difference in agonist-induced desensitization between α_{1a} AR WT and receptors with any SNP at either high- or low-expression levels (Fig. 4). The finding that α_{1a} AR G247R desensitizes normally strongly suggests that a failure to desensitize is not responsible for the increased signaling observed.

Effects of human α_{1a} AR SNP G247R on receptor–G protein interaction

Another possibility for the increased signaling of α_{1a} AR G247R is that the SNP has enhanced receptor–G protein interaction(s). To test this hypothesis, receptor–G protein coupling was measured by [35 S]GTP γ S (a nonhydrolyzable GTP analog) binding. When treated with 10 μ M NE, membranes from cells expressing α_{1a} AR G247R display a 2.1 ± 0.3 -fold greater increase in [35 S]GTP μ S binding compared with membranes from WT receptor cells ($p < 0.05$, $n = 4$).

Effects of human α_{1a} AR SNPs on cell growth

Several laboratories have suggested that α_1 AR subtypes may modify cell growth. In order to test if any of the SNPs affect cell growth, equal numbers of rat-1 cells (70,000 cells/well) were plated into 12-well plates for cell counts done side-by-side with IP assays. No difference in cell number is apparent among high-expression stable clones (48 h incubation). But among low-expression cells, clones expressing α_{1a} AR G247R (three distinct clones) always grow faster and have a higher cell count (≈ 2 -fold) after incubation for 48 h compared with cells expressing α_{1a} AR WT (Fig. 5a). This was confirmed by [3 H]thymidine incorporation measurements (2.1 ± 0.2 -fold higher in SNP cells, $n = 3$, $P < 0.05$). Side-by-side cell count and protein assays were used to further investigate α_{1a} AR G247R-induced growth at 4, 24, 48, and 72 h. All clones expressing α_{1a} AR G247R have higher growth rates than WT clones (Fig. 5b) and display a proportional increase in total protein (Fig. 5c) suggesting proliferative rather than hypertrophic effects under these conditions.

Discussion

The present study is the first to report that naturally-occurring human α_{1a} AR SNPs induce altered receptor pharmacology and/or biological activity. Seven of nine naturally-occurring SNPs identified in the α_{1a} AR coding region result in amino acid substitutions. These seven SNPs were investigated for biological behavior in rat-1 fibro-blasts and four SNPs were found to alter ligand binding and/or receptor activation. SNP R166K in TM4 and V311I in TM7 reduce binding affinity for NE, epinephrine, and phenylephrine, an effect that is translated into reduced potency of NE in activating the receptor. Complementing this finding, α_{1a} AR V311I and I200S display altered antagonist binding. Quite surprisingly, cells expressing SNP G247R demonstrate increased proliferative ability as well as increased efficacy (increased maximal activity) to NE-stimulated IP production, probably via enhanced receptor–G protein coupling.

Although current knowledge regarding naturally-occurring α_{1a} AR polymorphisms is fairly limited, investigation into mechanisms underlying AR agonist binding and receptor activation has been extensive. Mutagenesis studies suggest that natural agonists, epinephrine and NE, bind to residues in TM3 through TM6 in α_{1a} AR (Fig. 1; Hwa et al. 1995; Hwa and Perez

1996; Piascik and Perez 2001; Porter et al. 1996; Waugh et al. 2000). In the α_{1a} AR, two phenylalanine residues, F163 in TM4 and F187 in TM5 were found to be involved in agonist-specific binding interactions (Waugh et al. 2000). Interestingly, the mutation in α_{1a} AR R166K, which causes a consistent reduction in agonist binding affinity, sits immediately above F163 almost one full helical turn earlier in the sequence (Fig. 1), suggesting that R166K may be acting indirectly through influence on nearby agonist binding residues.

The other polymorphism V311I in TM7 is located only two amino acids carboxy-terminal to K309, a conserved residue in GPCRs, which plays a key role in maintaining the inactive conformation of GPCRs via a salt bridge formed with the highly conserved aspartate in TM3 (D106 of α_{1a} AR; Porter et al. 1996). Upon agonist binding, this aspartate is believed to interact with the amine group of epinephrine or NE (Porter et al. 1996; Porter and Perez 1999; Strader et al. 1987), disrupting the salt bridge and allowing the receptor to shift into the active conformation. Importantly, the decrease in agonist binding affinity of the receptor with V311I substitution, directly translates into decreased potency (increased EC_{50}) for NE-induced activation. This could reflect the fact that the substitution is inhibiting the essential activation step of salt bridge disruption. The V311I substitution also occurs immediately between two phenylalanine residues, F308 and F312, that frequently play a role in antagonist binding for the α_{1a} AR (Waugh et al. 2001). Thus, it could be the case that the binding pocket for 5-methylurapidil is altered by the V311I substitution, resulting in increased affinity for 5-methylurapidil. Despite the fact that SNP I200S in TM5 is not near the three consecutive residues (Q177, I178, N179) of the second extracellular loop involved in phentolamine binding (Zhao et al. 1996; see Fig. 1), it causes lower affinity for the antagonist phentolamine, which might arise from some subtle changes in conformation induced by this substitution.

One of the most interesting findings in this study is that receptors with a G247R substitution have the same binding characteristics as α_{1a} AR WT, but nevertheless display increased IP signaling and altered growth behavior at low expression levels (in several distinct clones). The absence of significant influence on the binding characteristics of this receptor is not too surprising since the G247R substitution is in the center of the third intracellular loop, a nonconserved region that can usually be altered in GPCRs without affecting ligand binding properties (Greasley et al. 2001). We have recently shown that the third intracellular loop of α_{1a} AR may play the central role in acute receptor desensitization rather than the lengthy COOH-terminus (Price et al. 2002). Thus, one potential explanation for increased IP signaling could be a deficiency in agonist-mediated desensitization allowing extended high-level IP production. However, under the assay conditions used in this study, we observed no decrease in the ability of receptor with G247R or any other SNP to desensitize following agonist exposure.

A second hypothesis that could account for increased IP signaling from α_{1a} AR G247R would be improved receptor-G protein coupling. Indeed, [35 S]GTP γ S binding experiments reveal that α_{1a} AR G247R membranes display higher NE-induced overall G protein activation than α_{1a} AR WT membranes (2.1-fold). This observation strongly suggests increased coupling efficiency is responsible for the increased signaling observed for α_{1a} AR G247R. The ability of SNPs to alter G protein-coupling has a precedent in the AR family as a SNP in the human β_1 AR (R389G) increases receptor coupling to Gs (Mason et al. 1999). Interestingly, functional results for α_{1a} AR G247R are very similar to in vitro data from β_1 AR polymorphism R389G, which shows 2-fold enhanced coupling to Gs and 3-fold increased maximal agonist-induced adenylyl cyclase activity without change in EC_{50} values as compared to the G389 β_1 AR (Mason et al. 1999). It is noteworthy that R389 β_1 AR has been shown to confer predisposition to heart failure (Mialet Perez et al. 2003; Small et al. 2002).

The finding that SNP G247R displays increased growth is consistent with previous studies suggesting a role for α_1 ARs in cell proliferation and hypertrophy (Erami et al. 2002; Mimura et al. 1995; Xiao et al. 2001). While increased coupling to $G\alpha_{q/11}$ is likely to play a role in the increased proliferative response caused by α_{1a} AR G247R, it needs to be recognized that cells with this polymorphic receptor display faster growth in media without exogenously-added agonist. Clearly, more work will be required to identify the precise mechanistic connection between signaling and increased growth. However, the fact that altered proliferative and signaling behaviors are observed in distinct clones independently isolated months apart demonstrates that the presence of α_{1a} AR G247R is responsible for these changes.

Clinically, α_{1a} ARs play important roles in the pathogenesis of many diseases such as benign prostatic hyper-plasia and myocardial hypertrophy, as well as contributing to blood pressure regulation. The SNPs that induce changes in receptor biological functions may influence variations in α_{1a} AR-mediated diseases. Altered binding affinities for several antagonists caused by SNP V311I and I200S, suggest the possibility that drug dose variance seen clinically might depend on infrequent polymorphisms.

Some may call into question the broader clinical relevance of rare SNPs. However, this viewpoint is being challenged by increasing evidence of rare, yet clinically important SNPs. Perhaps the most widely known example is the β_2 AR T164I polymorphism, $f(-)=0.005-0.02$ (Aynacioglu et al. 1999; Liggett et al. 1998), which shows blunted cardiac β_2 AR responsiveness (Brodde et al. 2001) and an association with a decreased survival rate in patients with congestive heart failure (Liggett et al. 1998), consistent with defective ligand binding and functional response in vitro ($\approx$ threefold lower affinity for agonists and twofold decrease in basal and agonist-stimulated cAMP formation; Green et al. 1993, 2001). In another study, Cohen et al. recently reported that multiple rare alleles contribute to low plasma levels of HDL cholesterol (Cohen et al. 2004). Furthermore, a rare SNP in the apolipoprotein A1 gene, +83C $\rightarrow$ T, $f(-)=0.041$, has been shown to be associated with the severity of coronary artery disease (Wang et al. 1996). Finally, a rare synapsin III SNP, S470N, $f(-)\approx 0.02$, has been reported to have a possible relationship to schizophrenia (Porton et al. 2004). Although it is not possible to produce significant association results for the rare α_{1a} AR SNPs from our present study in this pilot population, our findings for the first time report that naturally-occurring human α_{1a} AR SNPs induce altered receptor biological activity. Such detailed information is critical for geneticists to rationally choose candidate SNPs for use in association studies. In conclusion, the present study indicates that four naturally-occurring human α_{1a} AR SNPs induce altered α_{1a} AR pharmacology and/or biological activity, a finding that may have important clinical implications.

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Fig. 1. Seven-transmembrane-spanning model of human α_{1a} AR showing the primary amino acid sequence. Key residues are colored including single nucleotide polymorphism (SNP) sites (yellow, with amino acid number listed next to SNP), and important residues for agonist binding (red) and antagonist binding (green). The salt bridge is formed between D106 (red and blue, also identified as an important residue in agonist binding) in TM3 and K309 (blue) in TM7. This model was based on the results of several mutagenesis studies (Chen et al. 1999; Hamaguchi et al. 1996; Hwa et al. 1995; Hwa and Perez 1996; Porter et al. 1996; Waugh et al. 2000, 2001; Zhao et al. 1996). *Sites of splice junctions (Hawrylyshyn et al. 2004)

Fig. 2.

Effect of human α_{1a} AR SNPs on inositol phosphate (IP) signaling in high-expression stable clones (receptor densities 1.53–2.37 pmol/mg protein). Cells labeled with [³H]inositol were treated for 20 min with increasing concentrations (10^{-8} to 10^{-4} M) of norepinephrine (NE). For each construct, binding assays, IP assays, and cell count determinations were performed in parallel plates. **a** Basal IP release (without agonist stimulation). **b** Maximal IP release in response to NE, normalized for receptor/well. **c** EC₅₀ values for NE stimulation of IP production. ** $P < 0.01$ compared with α_{1a} AR WT; $n = 3-8$

Fig. 3.

Effect of human α_{1a} AR SNPs on IP signaling in low-expression stable clones (receptor densities 0.21–0.44 pmol/mg protein). Cells labeled with [³H]inositol were treated for 20 min with various concentrations (10^{-8} to 10^{-4} M) of NE. For each construct, IP assay, binding assay, and cell number were measured in paralleled plates. **a** Basal IP release. **b** Maximal IP release in response to NE, normalized for receptor/well. **c** EC₅₀ values for NE stimulation of IP production. ** $P < 0.01$ compared with α_{1a} AR WT; $n = 3-6$

Fig. 4.

Effects of human α_{1a} AR SNPs on NE-induced desensitization. [3 H]Inositol-labeled rat-1 fibroblasts stably expressing WT α_{1a} AR or its SNPs at **a** high or **b** low expression levels were pretreated for 10 min with 10^{-5} M NE *or* vehicle, washed, then incubated with 10^{-5} M NE for 20 min in the presence of LiCl, and total IPs were quantitated. The extent of desensitization was expressed as a percentage of reduction of IP response in NE-pretreated cells compared with the response in vehicle-pretreated cells. $n=3-6$

Fig. 5.

SNP G247R stimulates cell growth. **a** Low-expression clones plated at the same density were incubated for 48 h in complete medium at 37°C, prior to trypsinization and cell counting. **b**, **c** Two distinct clones low-expressing α_{1a} AR WT or α_{1a} AR SNP G247R were plated in 12-well plates at 5×10^4 cells/well. After washing once with PBS gently, cells were trypsinized and counted (**b**) or harvested by 250 μ l of lysis buffer (1% nonidet P-40 and 0.5% sodium deoxycholate; **c**) at 4-, 24-, 48-, and 72-h points. Fifty microliters of samples were used for total protein measurement by BCA protein assay reagent kit (Pierce). ** $P < 0.01$ compared with α_{1a} AR WT; $n = 3-5$

Localization of naturally-occurring single nucleotide polymorphisms (SNPs) in human α 1aAR coding region. *TM* transmembrane region, *IL* intracellular loop

Amino acid Position	SNP Name (dbSNP ID, handle, entry date) ^a	Nucleotide position	Nucleotide change	Domain position	Allelic <i>f</i> (–)		All (with Coriell)	All (except Coriell)	Black	Hispanic	White
					Previous reference						
5	S5S	15	G→C	N-terminus			0.002	0.003	0.000	0.000	0.005
154	S154A (G2286a1 ^{bc})	460	T→G	TM4	0.010		0.007	0.005	0.000	0.013	0.005
166	R166K ^b	497	G→A	TM4			0.002	0.003	0.000	0.000	0.005
200	I200S ^b (rs2229125, WICVAR, 6/28/01)	599	T→G	TM5	0.028		0.005	0.005	0.000	0.000	0.010
247	G247R (rs3730287, WIPGA, 7/12/02)	739	G→A	IL3	0.013		0.003	0.003	0.000	0.023	0.000
311	V311I	931	G→A	TM7			0.002	0.000	0.000	0.000	0.000
347	R347C ^b (rs1048101, Lee, 9/13/00)	1,039	C→T	C-terminus	0.55		0.463	0.482	0.267	0.525	0.565
401	S401S	1,203	T→G	C-terminus			0.002	0.003	0.012	0.000	0.000
465	E465D ^b (rs2229126, WICVAR, 6/28/01)	1,395	A→T	C-terminus	0.01		0.030	0.021	0.023	0.025	0.020

Nucleotide number is based on the first nucleotide of the start codon being +1 of human α 1aAR cDNA (GeneBank accession number is L31774). SNPs are identified from 281 individuals: Coriell, *n*=90 (enriched for minorities but exact race not known); Black, *n*=43; Hispanic, *n*=40; White, *n*=101

^a All SNPs discussed in this manuscript were first identified in 2000 via resequencing by Dr. Debra A. Schwinn at the NIH (NHGRI)

^b Subsequently confirmed by identification in a distinct population (*n*=96) with lower urinary tract symptoms by our laboratory

^c A Whitehead internal ID cited from website <http://www.cardiogenomics.org>

Table 2 Agonist and antagonist binding affinities of human α_{1A} AR wild type (WT) and SNPs from high-expression stable clones

	α_{1A} AR WT	S154A	R166K	I200S	G247R	V311I	R347C	E465D
Domain location								
Expression (pmol/mg)	1.77±0.24	TM4	TM4	TM5	IL3	TM7	C-terminus	C-terminus
[¹²⁵ I]HEAT(K_D , pM)	42.1±6.5	38.1±4.4	2.37±0.28 35.9±10.0	1.53±0.10 34.5±1.3	1.96±0.40 32.1±5.3	1.75±0.37 42.7±16.5	1.76±0.22 39.9±3.2	2.02±0.27 38.8±3.7
Agonists (p <i>K_i</i>)								
Norepinephrine	4.73±0.03	4.73±0.08	4.34±0.10**	4.90±0.08	4.61±0.03	4.34±0.06**	4.73±0.03	4.64±0.02
Epinephrine	5.01±0.02	4.87±0.06	4.64±0.04**	5.18±0.08	4.96±0.01	4.57±0.08**	5.01±0.05	4.91±0.04
Phenylephrine	4.69±0.04	4.60±0.08	4.44±0.01*	4.82±0.07	4.58±0.05	4.27±0.02**	4.69±0.04	4.75±0.06
Oxymetazoline	7.67±0.02	7.57±0.02	7.61±0.04	7.84±0.10	7.64±0.03	7.82±0.03	7.58±0.07	7.57±0.08
Antagonists (p <i>K_i</i>)								
Prazosin	9.39±0.10	9.34±0.09	9.19±0.08	9.69±0.10	9.40±0.14	9.20±0.06	9.30±0.07	9.61±0.10
Phentolamine	7.70±0.03	7.77±0.05	7.77±0.04	7.20±0.05**	7.60±0.07	7.60±0.04	7.75±0.01	7.84±0.01
5-Methylurapidil	8.44±0.04	8.45±0.10	8.38±0.08	8.40±0.07	8.45±0.07	8.87±0.06**	8.39±0.02	8.44±0.04

p*K_i* values for the binding of agonists and antagonists were determined in competition binding experiments on rat-1 cell membranes stably transfected to express α_{1A} AR WT or its SNPs (receptor density >1.5 pmol/mg protein). *K_D* values for the antagonist [¹²⁵I]HEAT and receptor densities were determined from saturation binding studies. Data are reported as the mean ± SEM of three to seven different experiments, each performed in duplicate or triplicate

* *P*<0.05 compared with α_{1A} AR WT

** *P*<0.01 compared with α_{1A} AR WT

Table 3 Agonist and antagonist binding affinities of human α_1 AR wild type and SNPs from low-expression stable clones

	α_1 AR WT	S154A	R166K	I200S	G247R	V311I	R347C	E465D
Domain location								
Expression (pmol/mg)	—	TM4	TM4	TM5	IL3	TM7	C-terminus	C-terminus
[¹²⁵ I]HEAT(K _d , pM)	0.36±0.01	0.37±0.09	0.44±0.05	0.21±0.06	0.33±0.03	0.29±0.03	0.36±0.01	0.26±0.05
Agonists (pK _i)	42.9±4.0	37.7±16.3	42.6±6.1	49.9±7.7	49.1±10.8	49.7±10.0	50.7±9.8	46.6±16.4
Norepinephrine	4.73±0.08	4.78±0.02	4.31±0.04**	4.82±0.07	4.77±0.09	4.32±0.04**	4.65±0.04	4.65±0.04
Epinephrine	5.02±0.07	4.97±0.02	4.62±0.03**	5.15±0.03	5.09±0.13	4.36±0.01**	4.97±0.08	4.90±0.10
Phenylephrine	4.68±0.08	4.72±0.07	4.37±0.04**	4.75±0.03	4.60±0.08	4.24±0.03**	4.62±0.03	4.66±0.11
Oxymetazoline	7.65±0.02	7.78±0.03	7.63±0.06	7.65±0.01	7.56±0.04	7.78±0.02	7.75±0.02	7.76±0.09
Antagonists (pK _i)								
Prazosin	9.51±0.11	9.36±0.09	9.32±0.08	9.54±0.04	9.40±0.14	9.50±0.03	9.47±0.12	9.22±0.11
Phentolamine	7.68±0.03	7.64±0.01	7.73±0.04	7.31±0.07**	7.60±0.07	7.49±0.10	7.70±0.05	7.76±0.11
5-Methylurapidil	8.42±0.11	8.38±0.07	8.41±0.15	8.43±0.07	8.45±0.07	8.94±0.06**	8.56±0.04	8.48±0.04

pK_i values for the binding of agonists and antagonists were determined in competition binding experiments on rat-1 cell membranes stably transfected to express α_1 AR WT or its SNPs (receptor density <0.5 pmol/mg protein). K_d values for the antagonist [¹²⁵I]HEAT and receptor densities were determined from saturation binding studies. Data are reported as the mean ± SEM of three to five different experiments, each performed in duplicate or triplicate

** P<0.01 compared with α_1 AR WT