Polymorphic Deletion of Three Intracellular Acidic Residues of the α2B-Adrenergic Receptor Decreases G Protein-coupled Receptor Kinase-mediated Phosphorylation and Desensitization*

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A polymorphic variant of the human α2B-adrenergic receptor (α2BAR), which consists of a deletion of three glutamic acids (residues 301–303) in the third intracellular loop was found to be common in Caucasians (31%) and to a lesser extent in African-Americans (12%). The consequences of this deletion were assessed by expressing wild-type and the Del301–303 receptors in Chinese hamster ovary and COS cells. Ligand binding was not affected, although a small decrease in coupling efficiency to the inhibition of adenylyl cyclase was observed with the mutant. The deletion occurs within a stretch of acidic residues that is thought to establish the milieu for agonist-promoted phosphorylation and desensitization of the receptor by G protein-coupled receptor kinases (GRKs). Agonist-promoted phosphorylation studies carried out in cells coexpressing the α2ARs and GRK2 revealed that the Del301–303 receptor displayed ~56% of wild-type phosphorylation. Furthermore, the depressed phosphorylation imposed by the deletion was found to result in a complete loss of short term agonist-promoted receptor desensitization. Thus the major phenotype of the Del301–303 α2BAR is one of impaired phosphorylation and desensitization by GRKs, and thus the polymorphisms renders the receptor incapable of modulation by this key mechanism of dynamic regulation.

1 The abbreviations used are: AR, adrenergic receptor(s); GRK, G protein-coupled receptor kinase; PCR, polymerase chain reaction; bp, base pair(s); CHO, Chinese hamster ovary.

2Adrenergic receptors (α2AR) are cell surface receptors for catecholamines that bind to the Gαi/Gq family of G proteins, coupling to multiple effector systems including inhibition of adenylyl cyclase activity (1). α2AR are widely expressed within the central and peripheral nervous system (2–4) and partici-pate in a broad spectrum of physiologic functions such as regulation of blood pressure both centrally and within the vasculature, sedation, analgesia, regulation of insulin release, renal function, and cognitive and behavioral functions (5–12). Three human α2AR subtypes have been cloned and characterized (α2A, α2B, and α2C). The α2BAR has a distinct pattern of expres-sion within the brain, liver, lung, and kidney, and recent studies using gene knockouts in mice have shown that disruption of this receptor affects mouse viability (13), blood pressure responses to α2AR agonists (13), and the hypertensive response to salt loading (14).

Like the α2AR subtype (15, 16), the α2BAR undergoes short term agonist promoted desensitization (17). This desensitiza-tion is due to phosphorylation of the receptor, which evokes a partial uncoupling of the receptor from functional interaction with Gαi (18, 19). Such phosphorylation appears to be due to G protein-coupled receptor kinases (GRKs), a family of serine/threonine kinases that phosphorylate the agonist-occupied conformation of many G protein-coupled receptors (20). The process serves to finely regulate receptor function providing for rapid adaptation of the cell to its environment. Desensitization may also limit the therapeutic effectiveness of administered agonists. For the α2BAR, phosphorylation of serines/threonines in the third intracellular loop of the receptor is dependent on the presence of a stretch of acidic residues in the loop that appears to establish the milieu for GRK function (18). In this report we delineate the phenotype of a common polymorphism of the α2BAR (21, 22), which consists of a deletion of three glutamic acid residues in this region; such a variation has a pronounced effect on receptor phosphorylation leading to a loss of agonist-promoted desensitization.

MATERIALS AND METHODS

Polymorphism Detection—The sequence encoding the third intracel-lular loop of the human α2BAR (GenBank™ accession number AF005900) was examined for polymorphic variation by performing polymerase chain reactions (PCRs) to amplify this portion of the cDNA from genomic DNA derived from blood samples. In this paper the adenine of the initiator ATG codon of the open reading frame of the receptor is designated as nucleotide 1, and amino acid 1 is the encoded methionine. The human receptor consists of 450 amino acids. For initial examination, DNA from 39 normal individuals was utilized. Two overlapping fragments encompassing the third intracellular loop region were generated using the following primer pairs: fragment 1 (534 bp), 5′-GCTCTATCATCCCCCTTCTCGGT (sense) and 5′-AAAGCCCAAGCAGTGCTGGT (antisense) and fragment 2 (588 bp), 5′-CTGATCGCAGAGACCAACCC (sense) and 5′-AAAAGCCCAATGACCACACCAGC (antisense). The 5′ end of each sense and antisense primer also contained sequences corresponding to the M13 forward (5′-TGTAAAACGACGGCCAGC- CAGT) and M13 reverse (5′-CAGGAAACAGCTATGACC) universal sequencing primers, respectively. The PCR reactions consisted of ~100 ng of genomic DNA, 5 μmol of each primer, 0.8 μl dNTPs, 10% MeSO2, 2.5 units of Platinum Taq DNA polymerase (Life Technologies, Inc.), 20 μl of 5× buffer J (Invitrogen) in a 100-μl reaction volume. Reactions were started by an initial incubation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 58 °C (fragment 1) or 60 °C (fragment 2) for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. PCR reactions were purified using the QIAquick PCR purification system (Qiagen), and automated sequencing of both strands of each PCR product was performed using Applied Biosystems 370 sequencer using dye
primer methods. As discussed below, a 9 bp in-frame deletion beginning at nucleotide 901 was detected that resulted in a loss of three glutamic acid residues at amino acid positions 301–303. Thus, this polymorphism was denoted Del301–303. No other nonsynonymous or synonymous polymorphisms were identified. PCR amplification of 200- and 200-bp fragments flanking this polymorphic region allowed screening of additional DNA samples whose genotypes were distinguished by size when run on 4 % Nusieve agarose gels. PCR conditions were the same as described above except that buffer F was used with the following primers: 5'-AGAAGGAGGTTGGTTGGAG (sense) and 5'-ACCTATAGGCACCACGGGTCCT (antisense).

Constructs and Cell Transfection—To create the polymorphic α2AR construct, a 1585-bp PCR product encompassing the α2AR gene was amplified from a homozygous deletion individual using the following primers: 5'-GCGCGAATTCCTTCTCTAAC (sense) and 5'-CAAGGGTTTCTTAAGATAG (antisense). This fragment was digested and subcloned into the XcmI and BamHI sites of the wild-type α2AR sequence in the expression vector pBC12BI (17). The integrity of the construct was verified by sequencing. Chinese hamster ovary cells (CHO-K1) were stably transfected by a calcium phosphate precipitation technique as described previously using 30 μg of each receptor construct and 0.5 μg of pSVneo to provide for G418 resistance (23). Selection of positive colonies was carried out in 1.0 mg/ml G418, and expression of the α2AR from individual clonal lines was determined by radioligand binding as described previously (17). Matched concentrations of agonists were used to determine α2AR expression levels from individual clonal lines was determined by radioligand binding as described previously (24). Briefly, solubilized material was preincubated for 2 h at 4 °C with protein G-Sepharose beads to remove nonspecific binding. The supernatant was then incubated with protein G-Sepharose beads and a 1:200 dilution of antibody for 1 h at 4 °C. Following immunoprecipitation, the beads were washed three times by centrifugation and resuspension and then incubated at 37 °C for 1 h in SDS sample buffer. Proteins in the supernatant were then fractionated on a 10% SDS-polyacrylamide gel with equal amounts of receptor (based on radioligand binding) loaded in each lane. Signals were visualized and quantitated using a Molecular Dynamics PhosphorImager with Image Quant Software.

Miscellaneous— Protein determinations were by the copper bicinchoninic acid method (28). Data from adenylyl cyclase and radioligand binding assays were analyzed by iterative least square techniques using Pizam software (GraphPad, San Diego, CA). Agreement between genotypes observed and those predicted by the Hardy-Weinberg equilibrium was assessed by a Chi-squared test with one degree of freedom. Genotype comparisons were by Fisher’s exact test. Comparisons of results from biochemical studies were by t tests, and significance was considered when p < 0.05. Data are provided as the means ± S.E.

RESULTS AND DISCUSSION

Sequence analysis of the third intracellular loop of the α2AR gene from 78 chromosomes revealed a single sequence variant. This consisted of an in-frame 9-bp deletion (GAAGAGGAG) beginning at nucleotide 901 (Fig. 1A) that results in loss of three glutamic acid residues at amino acid positions 301–303 of the third intracellular loop of the receptor (Fig. 2). Using the rapid detection method (Fig. 1B), allele frequencies were determined in a larger population. The frequencies of the wild-type and the Del301–303 polymorphic α2AR are shown in Table I. The deletion polymorphism is more common in Caucasians than African-Americans, with allele frequencies of 0.31 and 0.12, respectively. The distribution of homozygous and heterozygous alleles in either population was not different than that predicted from the Hardy-Weinberg equilibrium (p > 0.8).

The consequences of this polymorphism on ligand binding and receptor function were evaluated by stably expressing the human wild-type α2AR and the Del301–303 receptor in CHO cells (Table II). Saturation radioligand binding studies revealed a small but statistically significant lower affinity for the α2AR antagonist [3H]Hypoxanthine for Del301–303 compared with the wild-type receptor (Kd = 5.1 ± 0.2 versus 3.8 ± 0.3 nM, respectively, n = 5, p < 0.05). Agonist (epinephrine) competition binding experiments carried out in the presence of quinuasin 5 (β-adrenergic)triphosphate revealed a small increase in the Kd for the pharmacological receptor (285 ± 8.7 versus 376 ± 66 nm, n = 5, p < 0.05). In similar studies carried out in the

| n | Homozygous | Heterozygous | Del301-303 homozygous | Del301-303 allele frequency |
|---|------------|-------------|-----------------------|---------------------------|
| Caucasian | 94 | 41 | 47 | 6 | 0.31 |
| African-American | 79 | 61 | 17 | 1 | 0.12 |
absence of guanine nucleotide, two-site fits were obtained for both receptors with no differences in the $K_L$ or the percentage of receptors in the high affinity state ($R_H$; Table II). However, a trend toward an increased $K_H$ was observed with the Del301–303 mutant. These results prompted additional studies with the partial agonist radioligand $[125I]$aminoclonidine. Saturation binding studies (in the absence of quanosine 5'-b-ami-
do-triphosphate) with concentrations of the ligand from 0.2 to 4 nM revealed a single site with a $K_d$ of 1 nM as reported by others (27). Comparison of the wild-type $\alpha_{2B}$AR and the Del301–303 receptor revealed essentially identical $K_d$ values for $[125I]$aminoclonidine ($1.33 \pm 0.12$ versus $1.22 \pm 0.07$ nM, respectively). Taken together, the data suggest that there is little, if any, effect of the deletion in the third intracellular loop on the conformation of the ligand binding pocket within the transmembrane spanning domains.

To address the functional consequences of the mutation, studies examining agonist-promoted inhibition of forskolin-stimulated adenylyl cyclase activities were carried out in lines expressing the wild-type $\alpha_{2B}$AR and the Del301–303 receptor at densities of 626 ± 54 and 520 ± 82 fmol/mg ($n = 7$, $p > 0.05$). The results of these studies are shown in Table III. As can be seen, the Del301–303 receptor displayed less inhibition of adenylyl cyclase (23.4 ± 2.2%) compared with wild-type $\alpha_{2B}$AR (28.5 ± 1.6%, $p < 0.05$). Furthermore, the polymorphic receptor had a greater EC50 (19.6 ± 5.5 versus 7.9 ± 2.1 mM, $p < 0.01$). Thus, the loss of the three glutamic acids in the third intracellular loop, which is known to contain regions important for G protein coupling, results in a modest decrease in agonist-mediated receptor function.

The deletion polymorphism occurs in a highly acidic stretch of amino acids (EDEAEEEESEESEESEE) within the third intracellular loop of $\alpha_{2B}$AR (Fig. 2). The structural importance of this region has been previously assessed and shown to be critical for short term agonist-promoted receptor phosphorylation leading to desensitization (18). These data and reports by others (29) suggest that this acidic environment is necessary for receptor phosphorylation by GRKs. Therefore, to investigate the consequences of this deletion polymorphism on receptor desensitization, agonist-promoted inhibition of adenylyl cyclase activity was determined in membranes from CHO cells expressing the wild-type and Del301–303 receptor after pretreatment with norepinephrine. In these experiments, cells were incubated with medium alone or medium containing agonist (10 μM norepinephrine) for 30 min at 37 °C and subsequently extensively washed at 4 °C; membranes were prepared, and agonist-mediated inhibition of adenylyl cyclase and norepinephrine as reported by others (27). Comparison of the wild-type $\alpha_{2B}$AR and the Del301–303 receptor revealed essentially identical $K_d$ values for $[125I]$aminoclonidine ($1.33 \pm 0.12$ versus $1.22 \pm 0.07$ nM, respectively). Taken together, the data suggest that there is little, if any, effect of the deletion in the third intracellular loop on the conformation of the ligand binding pocket within the transmembrane spanning domains.

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![Fig. 1](image1.png)

**Fig. 1. Identification of the human $\alpha_{2B}$AR variant.** Shown in A and B are representative automated sequence chromatograms identifying a deletion of the nucleotides GAAGAGGAG. In C, a rapid screening technique identifies homozygous and heterozygous PCR products by size.

![Fig. 2](image2.png)

**Fig. 2. Localization of the $\alpha_{2B}$AR polymorphism.** Shown are the fifth and sixth transmembrane spanning domains (TMD) and the third intracellular loop of the receptor.
change in the EC_{50} for the deletion receptor following agonist pretreatment (29.5 μM versus 31.2 μM). Desensitization was quantitated by examining adenylyl cyclase activities at a submaximal concentration of agonist (the EC_{50} for the control condition). At this concentration, wild-type α_{2β}AR inhibited adenylyl cyclase activity by 16.5 ± 3.9%; with agonist pre-exposure, inhibition at this same concentration of agonist was 7.6 ± 2.3% (n = 4, p < 0.05, Fig. 3C), amounting to ∼54% desensitization of receptor function. Submaximal inhibition of adenylyl cyclase for the Del301–303 receptor, however, was not different between control and agonist-treated cells (17.1 ± 3.0% versus 15.9 ± 1.7%, n = 4, p = ns). In another two cell lines with matched expression of −600 fmol/mg, the same desensitization phenotypes for wild-type and the Del301–303 polymorphic receptor were observed (data not shown).

We next performed whole cell phosphorylation studies of the wild-type and polymorphic α_{2β}AR under the same conditions used for desensitization. We hypothesized that agonist-promoted phosphorylation would be decreased in the polymorphic receptor. However, given that this receptor displays rightward-shifted dose-response curves for inhibition of adenylyl cyclase at base line, we also considered the possibility that the receptor is significantly phosphorylated in the basal state. Studies were carried out in cells cotransfected with the receptor and GRK2 (βARK1), a strategy that we have previously shown to be useful in identifying receptor-GRK interactions (30). The results of a representative study are shown in Fig. 4. The wild-type α_{2β}AR underwent a 5.84 ± 0.49-fold increase in phosphorylation with agonist exposure. In contrast, although the Del301–303 receptor displayed some degree of agonist-promoted phosphorylation, the extent was clearly less (3.28 ± 0.24-fold, p < 0.05 compared with wild type). Basal phosphorylation was equivalent between the two receptors. It is interesting to note that this partial loss of phosphorylation results in a receptor that fails to undergo any degree of functional desensitization. Although it might seem reasonable to assume that such phosphorylation would be associated with some degree of desensitization, several previous studies with the α_{2β}AR and α_{2γ}AR subtypes indicate that full (i.e., wild type) phosphorylation is necessary for the desensitization process (16, 18, 24). For the α_{2β}AR, we have shown that four serines in the third intracellular loop are phosphorylated after agonist exposure (16). Removal of serines by alanine substitution mutagenesis results in a proportional decrease in phosphorylation. Such partial phosphorylation (compared with wild type), however, was found to be insufficient to cause any detectable desensitization. In a previous study of the α_{2γ}AR, we deleted and substituted the entire aforementioned acidic region (18). Agonist-promoted phosphorylation was reduced by ∼50% in this mutant, and desensitization was ablated. These results are entirely consistent with the current work, where a restricted deletion resulted in a decrease in phosphorylation and a complete loss of desensitization. Finally, we have also recently shown that a chimeric α_{2α}/α_{2γ}AR, which undergoes agonist-promoted phosphorylation, fails to exhibit desensitization (24).

Taken together with our current work, these results indicate that the conformation of the third loop evoked by GRK mediated phosphorylation which provides for the binding of arrestins (which is the ultimate step that imparts uncoupling) is highly specific. Thus, a precise phosphorylation-dependent conformation is apparently required for arrestin binding to α_{2α}AR and subsequent functional desensitization. Perturbations of the milieu can thus have significant functional consequences, as occurs with the Del301–303 polymorphic α_{2β}AR.

Thus, the major signaling phenotype of the α_{2β}AR Del301–303 polymorphism is one of decreased agonist-promoted phosphorylation that results in a complete loss of the ability for the receptor to undergo agonist-promoted desensitization. In addition, the polymorphism imposes a small decrease in receptor coupling. The potential physiologic consequences of the polymorphism could be related to either or both of the above phenotypes. A receptor that fails to undergo desensitization would be manifested as static signaling despite continued activation of the receptor by endogenous or exogenous agonist. Such a lack of regulation by agonist may perturb the dynamic relationship between incoming signals and receptor responsiveness that maintains homeostasis under normal or pathologic conditions. Recently, Gayvaras and colleagues (14) have shown that α_{2β}AR+/− mice fail to display a hypertensive response to salt loading after subtotal nephrectomy. Thus, a polymorphic α_{2β}AR that fails to desensitize (i.e., does not display regulatable function) may predispose to salt-sensitive hypertension. Regarding the therapeutic response to α_{2α}AR agonists, the phenotype of the Del301–303 receptor indicates that individuals with this poly-

### Table II

Ligand binding properties of wild-type and Del301–303 α_{2β}AR expressed in CHO cells

| Receptor        | Saturation binding | Epinephrine competition | [3H]Aminoeclonidine |
|-----------------|--------------------|-------------------------|---------------------|
|                 | B_{max} [fmol/mg]  | K_{D} [nM]              | IC_{50} [nM]        | B_{max} [fmol/mg]  | K_{D} [nM] |
| Wild type       | 671 ± 56           | 3.8 ± 0.3               | 285 ± 8.7           | 3.0%               | 118 ± 27   |
| Del301–303      | 538 ± 79           | 5.1 ± 0.2*              | 376 ± 66*           | 4.1 ± 1.2          | 106 ± 20   |

* p < 0.05 compared with wild-type α_{2β}AR.

### Table III

Adenylyl cyclase activities of the wild-type and Del301–303 α_{2β}AR expressed in CHO cells

| Basal | Forskolin | Max Inhibition (%) | EC_{50} [μM] | Submax inhibition | Desensitization |
|-------|-----------|--------------------|-------------|------------------|-----------------|
|       | pmol/min/mg | pmol/min/mg | %  | %  | Ctrl | NE |
| Wild type | 2.0 ± 0.2       | 15.1 ± 0.9       | 28.5 ± 1.6 | 7.9 ± 2.1 | 165 ± 3.9 | 7.6 ± 2.3* | 54 |
| Del301–303 | 1.2 ± 0.1*   | 11.9 ± 0.9b       | 23.4 ± 2.2b | 19.6 ± 5.5b | 17.1 ± 3.0 | 15.9 ± 1.7b | 7  |

* p < 0.05 compared with control.

b p < 0.05 compared with wild-type α_{2β}AR.
morphism would display little tachyphylaxis to continued administration of agonists. In addition, the initial response to agonist might also be reduced based on the somewhat depressed coupling of the Del301–303 receptor.

Until recently, it has been difficult to differentiate \( \alpha_{2B}\)AR function from the other two subtypes in physiologic studies. With the development of knockout mice lacking each \( \alpha_{2A}\)AR subtype (5, 6, 13, 31), certain functions can now be definitively attributed to specific subtypes. Characterization of the \( \alpha_{2B}\)AR knockout mouse has indicated that the \( \alpha_{2B}\)AR subtype is expressed on vascular smooth muscle and is responsible for the hypertensive response to \( \alpha_{2A}\)AR agonists (13). This indicates that vascular \( \alpha_{2B}\)AR contribute to overall vascular tone and thus participate in systemic blood pressure regulation. This role may be more important, however, during adaptive conditions, such as salt loading, because resting blood pressure is normal in the heterozygous \( \alpha_{2B}\)AR/\( \alpha_{2B}\)AR mice (14). Whether the \( \alpha_{2B}\)AR/\( \alpha_{2B}\)AR mice have altered resting blood pressures has not been studied in detail because of high perinatal lethality of the homozygous knockout (13). However, neither the region of chromosome 2 near the \( \alpha_{2B}\)AR coding sequence nor the deletion polymorphism has been linked or associated with hypertension (21, 22, 32). However, no studies have assessed whether the polymorphism is associated with salt-sensitive hypertension or other phenotypes, or the response to \( \alpha_{2A}\)AR agonist.

In summary, we have delineated the signaling phenotype of a polymorphism of the \( \alpha_{2B}\)AR that results in a deletion of three glutamic acids in the third intracellular loop of the receptor. The polymorphism is prevalent in the human population, with a frequency that is 2-fold greater in Caucasians as compared with African-Americans. The polymorphic receptor displays wild-type agonist binding affinity but a small decrease in function in the resting state. However, the major phenotype is a significant decrease in agonist-promoted phosphorylation by GRKs, which results in a receptor that fails to display agonist-promoted desensitization. To our knowledge this is the first polymorphism of any G protein-coupled receptor to affect GRK-mediated phosphorylation.

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Fig. 3. The Del301–303 \( \alpha_{2B}\)AR fails to undergo short term agonist-promoted desensitization. Cells in culture expressing the two receptors were exposed to vehicle or 10 \( \mu \)M norepinephrine for 30 min at 37 °C and washed extensively; membranes were prepared, and adenylyl cyclase activities were determined as described under “Experimental Procedures.” In A and B, results of full dose-response studies are shown, which reveal that whereas the wild type undergoes desensitization manifested as a rightward shift in the curve, the Del301–303 mutant does not. In C, the percentage of inhibition of adenylyl cyclase at a submaximal concentration of norepinephrine in the assay (the E50 of the control membranes) is shown for both conditions, indicating an ~54% desensitization of wild-type \( \alpha_{2A}\)AR. The Del301–303 failed to display such desensitization. Results are from four independent experiments. See also Table III. *, \( p < 0.05 \) compared with control. WT, wild type.

Fig. 4. The Del301–303 \( \alpha_{2B}\)AR has impaired agonist-promoted phosphorylation. Cells coexpressing each receptor and GRK2 were incubated with \([\gamma^{32}P]o\)phosphate, exposed to 10 \( \mu \)M norepinephrine for 15 min, and receptor purified by immunoprecipitation as described under “Experimental Procedures.” Shown is an autoradiogram from a single experiment representative of four performed (see text for mean results). NE, norepinephrine.
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