The \( \alpha_2 \)-adrenergic receptors (\( \alpha_2 \)ARs) play a critical role in modulating neurotransmitter release in the central and peripheral sympathetic nervous systems. A polymorphism of the \( \alpha_2 \)AR subtype localized to human chromosome 4 (the pharmacologic \( \alpha_2c \)AR subtype) within an intracellular domain has been identified in normal individuals. The polymorphism (denoted Del322–325) is because of an in-frame 12-nucleotide acid deletion encoding a receptor lacking Gly-Ala-Gly-Pro in the third intracellular loop. To delineate the functional consequences of this structural alteration, Chinese hamster ovary cells were permanently transfected with constructs encoding wild-type human \( \alpha_2 \)cAR and the polymorphic receptor. The Del322–325 variant had decreased high affinity agonist binding (\( K_D = 7.3 \pm 0.95 \) versus \( 3.7 \pm 0.43 \) nM; \%\( R_m \) = \( 31 \pm 4 \) versus \( 49 \pm 4 \)) compared with wild-type indicating impaired formation of the agonist-receptor-G protein complex. The polymorphic receptor displayed markedly depressed epinephrine-promoted coupling to \( G_{\alpha} \), inhibiting adenyl cyclase by \( 10 \pm 4.3\% \) compared with \( 73 \pm 2.4\% \) for wild-type \( \alpha_2c \)AR. This was also for the endogenous ligand norepinephrine and full and partial synthetic agonists. Decreased agonist-promoted coupling to the stimulation of MAP kinase (–71% impaired) and inositol phosphate production (–60% impaired) was also found with the polymorphic receptor. The Del322–325 receptor was \( \sim 10 \) times more frequent in African-Americans compared with Caucasians (allele frequencies 0.381 versus 0.040). Given this significant loss of function phenotype in several signal transduction cascades and the skewed ethnic prevalence, Del322–325 represents a pharmacogenetic locus and may also be the basis for interindividual variation in cardiovascular or central nervous system pathophysiology.

A Four Amino Acid Deletion Polymorphism in the Third Intracellular Loop of the Human \( \alpha_2c \)-Adrenergic Receptor Confers Impaired Coupling to Multiple Effectors

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94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for seven minutes. Attempts to directly sequence this product resulted in ambiguous reads, so the product was ligated into the vector PCR2.1-TOPO (Invitrogen) and TOP 10 cells were transformed, multiple colonies from each transformation were expanded, and their isolated DNA was sequenced using an automated sequencer in the forward and reverse directions using dye terminator chemistry with vector T7 and M13 primers. As is discussed below, a 12-bp deletion was found in some individuals beginning at nucleotide 964 (Fig. 1A). This results in the loss of amino acids 322–325 and thus this polymorphic receptor is denoted Del322–325. This deletion is in the loss of a Nci I restriction site at nucleotide 964 (forward strand), and thus a rapid detection method was devised. Smaller (384 and 372 base pair) PCR products were produced using 5′-AGCCCCGAGGACGCGGCA-3′ as the sense primer and the aforementioned antisense primer (same reaction conditions as above), and genomic DNA derived from blood samples as the template. Within this fragment there are either five or six Nci I restriction sites depending on the presence or absence of the deletion, providing for the pattern shown in Fig. 1C. This rapid detection technique was applied to additional DNA samples providing genotypes at this locus from a total of 146 individuals.

Constructs and Cell Transfection—To create the polymorphic α2C-AR construct the larger (723 bp) PCR product described above amplified from one individual was digested and subcloned into the Bpu1102 I and Eco47 III sites of the wild-type α2C-AR expression vector pBCl2BI (15). The integrity of the construct was verified by sequencing. Chinese hamster ovary cells (CHO-K1) were transiently perfomed with 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 (15). Selection of positive clones was carried out in 1.0 mg/ml G418, and expression of the α2C receptors from individual clones was determined by radioligand binding as described below. Cells were grown in monolayers in Ham’s F-12 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 80 μg/ml G418 (to maintain selection pressure) at 37 °C in a 5% CO2 atmosphere.

Adenylyl Cyclase Activities—α2C-AR inhibition of adenylyl cyclase was determined in membrane preparation from CHO cells stably expressing the two receptors using methods similar to those previously described (15). Briefly, membranes (20 μg) were incubated with 27 μM phenolpentoyrivate, 0.6 μM GTP, 0.1 mM cAMP, 0.12 mM ATP, 50 μg/ml myokinase, 0.05 mM ascorbic acid, and 2 μCi of [a-32P]ATP in a buffer containing 40 mM HEPES, pH 7.4, 1.6 mM MgCl2, and 0.8 mM EDTA for 30 min at 37 °C. Reactions were terminated by the addition of a stopping solution containing excess ATP and cAMP and ~100,000 dpm of [3H]cAMP. Labeled cAMP was isolated by gravity chromatography over alumina columns with [3H]cAMP used to quantify column recovery. Activities were measured in the presence of water (basal), 5 μM forskolin, and 5 μM forskolin with 10 mM GppNHp as the agonist. Results are expressed as percent inhibition of forskolin-stimulated activity.

MAP Kinase Activation—Activation of p44/42 MAP kinase was determined by quantitative immunoblotting using a phosphospecific antibody. Briefly, confluent cells were incubated overnight at 37 °C and 5% CO2 in serum-free media prior to treatment with media alone (basal), epinephrine (10 μM), or thrombin (1 μM) for 5 min. Cells were washed three times with phosphate-buffered saline and then lysed in radiommune precipitation buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 5 mM NaF) containing protease inhibitors (10 μg/ml benzamidine, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, and 5 μg/ml leupeptin). Western blots of these whole cell lysates were performed essentially as described previously (18) except that polyvinylidene difluoride membranes (Amersham Pharmacia Biotech) were used and incubated with phospho-p44/42 MAP kinase E10 antibody and (after stripping) with the p44/42 MAP kinase monoclonal antibody (both from New England Biolabs, Beverly, MA) at dilutions of 1:2000 for 1 h at room temperature. Washed membranes were subsequently incubated with anti-mouse fluorescein-linked immunglobulin followed by incubation with fluorescently labeled secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Fluorescent signals were quantitated by real time acquisition using a Molecular Dynamics STORM imager.

Inositol Phosphate Accumulation—Total inositol phosphate levels in intact cells were determined essentially as described previously (19). Briefly, confluent CHO cells stably expressing each of the α2C-ARs were incubated with [3H]myoinositol (5 μCi/ml) in media lacking lactating calf serum for 16 h at 37 °C in 5% CO2 atmosphere. Subsequently, cells were washed and incubated with phosphate-buffered saline for 30 min followed by a 30-min incubation with 20 mM LiCl in phosphate-buffered saline. Cells were then treated with phosphate-buffered saline alone (basal), 10 μM epinephrine, or 5 units/ml thrombin for 5 min, and inositol phosphates were extracted using a solution containing 0.1 M formic acid and 1 M formate.

Radioligand Binding—Expression of mutant and wild-type α2C-AR was determined using saturation binding assays as described (21) with 12 concentrations (0.5–30 nM) of [3H]yohimbine and 10 μM phentolamine used to define nonspecific binding. For competition studies, membrane fractions were incubated in 50 mM Tris-HCl, pH 7.4, 10 mM MgSO4, 0.5 mM EDTA with 2.0 mM [3H]yohimbine and 16 concentrations of the indicated competitor in the presence of 100 μM GppNHp for 30 min at 37 °C. Reactions for the above radioligand binding studies were terminated by dilution with 4 volumes of ice cold 10 mM Tris-HCl, pH 7.4, buffer and vacuum filtration over Whatman GF/C glass fiber filters.

Miscellaneous—Protein determinations were by the bocky bicinchoninic acid method (22). Data from adenylyl cyclase and radioligand binding assays were analyzed by iterative least-square techniques using Prizm software (GraphPad, San Diego, CA). Agreement between genotypes was observed, and those predicted by the Hardy-Weinberg equilibrium were assessed by a χ² test with one degree of freedom. Comparisons of results from biochemical studies were paired by t tests, and significance was considered when p < 0.05. Data are provided as means ± standard errors.

RESULTS AND DISCUSSION

From the initial sequencing of α2C-AR third intracellular loop PCR products from 40 chromosomes, one nonsynonymous sequence variant was identified (Fig. 1). This consisted of an in-frame 12-nucleotide (GGGGCGGCGCGC, sense strand) deletion beginning at nucleotide 964. This results in a loss of Gly-Ala-Gly-Pro at amino acid positions 322–325 within the third intracellular loop of the receptor (Fig. 2). Other than this deletion, the remaining encoded sequence was identical to that shown in Fig. 2. The frequencies of the wild-type and the Del322–325 polymorphic α2C-ARs are shown in Table I. The polymorphism is rare in Caucasians with an allele frequency of 0.040. In contrast, the frequency is ~10-fold higher (0.381) in African-Americans. The distribution of homozygous and heterozygous alleles was not different than that predicted from the Hardy-Weinberg equilibrium (p > 0.8). No other nonsynonymous polymorphisms were found in the third loop sequence. However, five synonymous single nucleotide variations were found at nucleic acids 868, 871, 933, 996, and 1167. The consequences of this polymorphism on receptor function were evaluated by permanently expressing the human wild-type α2C-AR and the Del322–325 receptor in CHO cells and examining multiple signaling pathways. As indicated, multiple clones with similar expression levels were utilized for these studies. Saturation radioligand binding studies using the α2C-AR antagonist [3H]yohimbine revealed that Del322–325 had a slightly, but statistically significant, lower affinity for the radioligand compared with wild-type α2C-AR (Kd = 3.8 ± 0.55 versus 2.0 ± 0.14 nM, n = 5, p = 0.03, Table II). In competition studies with the agonist phentolamine, however, no differences in the Kd values were found between the Del322–325 polymorphism and the wild-type α2C-AR (11.1 ± 1.8 versus 10.4 ± 1.2 nM, n = 5). In competition studies with the agonist epinephrine, carried out in the absence of GTP, high and low affinity binding was detected with both receptors. However, the high affinity dissociation constant, Kd, of the Del322–325 mutant was greater (i.e. lower affinity) compared with the wild-type receptor (7.3 ± 0.95 versus 3.7 ± 0.43 nM, n = 4, p = 0.01). The percentage of receptors in the high affinity state was less with the mutant receptor (%RH2 = 31 ± 4 versus 49 ± 4, p = 0.01). The Kd values were not different (584 ± 71 versus 416 ± 75 nM). Taken together, this suggested that functional coupling might be
depressed with the Del322–325 receptor because of impaired formation of the high affinity agonist-receptor-Gi/Go complex. Indeed, the location of the deletion in the third intracellular loop of the receptor is within 15 residues of the sequence RRGGRR. This is a motif (BBXRB or BBXBB) that has been identified in a number of receptors as a Gi coupling domain (23, 24). We considered that the deletion of the two glycines or the proline in the Del322–325 receptor may induce conformational changes affecting this region or other Gi protein coupling domains. Functional studies examining agonist-promoted inhibition of forskolin-stimulated adenylyl cyclase activities were carried out in lines with the wild-type α2C-AR and the Del322–325 receptor at expression levels of 1375 ± 141 versus 1081 ± 157 fmol/mg (n = 5, p > 0.05) and a second set of lines with lower expressions of 565 ± 69 versus 519 ± 51 fmol/mg (n = 5, p > 0.05), respectively. The results of these studies are shown in Table II and Fig. 3. As can be seen, there is a marked functional difference between the two receptors. In the higher expressing lines (Fig. 3A), wild-type α2C-AR exhibited a maximal inhibitory response of 60 ± 3%. In contrast, the Del322–325 polymorphic receptor achieved a maximal inhibition of 31 ± 2% (n = 5, p < 0.001), which represents an ~50% impairment of function. Of note, the EC50 values for these responses (2.6 ± 0.74 versus 1.2 ± 0.37 nM, respectively) were not different. Results from studies with the lower expressing lines revealed an even more striking phenotypic difference between the two receptors. As is shown in Fig. 3B, at these more physiologic levels of expression, agonist-promoted inhibition of adenylyl cyclase with wild-type α2C-AR was 73 ± 2.4%. In marked contrast, the Del322–325 receptor exhibited very little inhibition (10 ± 4.3%, n = 5, p < 0.001). With the low expression Del322–325 line, the EC50 in some experiments could not be calculated because of the minimal response. Analysis of the composite curve of the mean data from all experiments with this line revealed an EC50 of 29.6 nM. This is in contrast to 4.3 nM calculated in a like manner for the low expressing wild-type line. A similar degree of impairment was also observed with the endogenous agonist norepinephrine (Table III). Agonist-promoted functional activities of the two higher expressing receptors were also explored with full and partial synthetic α2C-AR agonists with diverse structures. (Because some of these agents were weak partial agonists, only the high expressing lines could be used.) As is shown in Table III, the Del322–325 receptor has depressed agonist-promoted coupling to inhibition of adenylyl cyclase with all the agonists tested. Similar to the responses observed with epinephrine and norepinephrine, the Del322–325 receptor showed ~50% impairment in the maximal inhibition of adenylyl cyclase compared with the wild-type α2C-AR for UK14304 (full agonist) as well as BHT-933, guanabenz, clonidine, and oxymetazoline (partial agonists), with no significant differences observed in the EC50 values for these responses.

We next explored coupling of these two receptors to the stimulation of inositol phosphate production. In CHO cells this response is ablated by pertussis toxin, indicating coupling via G α- and/or G βγ-proteins (25). The activation of phospholipase C is likely because of both Gαs and Gαi-associated Gi and/or Gαi-stimulation of the enzyme (25). As shown in Fig. 4, the loss of function phenotype of the Del322–325 receptor as delineated in adenylyl cyclase experiments was also observed in these inositol phosphate accumulation studies. Epinephrine-stimulated accumulation of inositol phosphates was 30 ± 3% over basal with the wild-type and sixth transmembrane-spanning domains (TMD) and the third intracellular loop. The polymorphism results in the loss of Gly-Ala-Gly-Pro at the indicated position. The third intracellular loop (ICL) is shown in a compact form for illustrative purposes and is not intended to represent known secondary structure.
**TABLE I**

Frequencies of the Del322–325 α2C-AR polymorphism

| Allele frequency | Del322–325 |
|------------------|------------|
| Caucasian        | 0.040      |
| African-American | 0.381      |

Shown are the number of individuals with each genotype out of a total of n individuals.

| Receptor          | n   | WT homozygous | Heterozygous | Del322–325 homozygous | Allele frequency Del322–325 |
|-------------------|-----|---------------|--------------|-----------------------|----------------------------|
| Caucasian         | 87  | 82            | 3            | 2                     | 0.040                      |
| African-American  | 59  | 23            | 27           | 9                     | 0.381                      |

**TABLE II**

Ligand binding properties and adenylyl cyclase activities of the wild-type and Del322–325 α2C-AR expressed in CHO cells

| Receptor        | Radioligand binding | Adenylyl cyclase activity |
|-----------------|----------------------|---------------------------|
|                 | Phenolamine Kᵢ   | Epinephrine | [³H]Yohimbine | Max inhibition | EC50 |
|                 | (nM)             | (nM)    | (fmol/mg)     | (nM)          | (nM)       |
| WTα2C           |                  |          |               |               |            |
|                 | nm               | nm      | nm            | %             | nm         |
|                 | 11.1 ± 1.8       | 3.7 ± 0.43 | 416 ± 75     | 49 ± 4        | 2.0 ± 0.14 |
|                 | Del322–325       |          |               |               |            |
|                 | 10.4 ± 1.2       | 7.3 ± 0.95* | 584 ± 71     | 31 ± 4*       | 3.8 ± 0.55* |

* p < 0.05 compared to the wild-type α2C-AR.

EC50 data were derived from composite curve analysis. At high and low levels of receptor expression, the Del322–325 receptor showed 50% and 71% impairment, respectively, in the maximal inhibition of adenylyl cyclase as compared to the wild-type receptor.

**Fig. 3. Coupling of wild-type and Del322–325 α2C-ARs to the inhibition of adenylyl cyclase.** Membranes from CHO cells were prepared, and adenylyl cyclase activities were determined as described under “Materials and Methods” in the presence of 5.0 μM forskolin and the indicated concentrations of epinephrine. Results are shown as the percent inhibition of forskolin-stimulated activities (for all cell lines the fold stimulation by forskolin was ~10-fold over basal levels). A, results from two cell lines expressing the wild-type and Del322–325 receptors at 1575 ± 141 and 1081 ± 157 fmol/mg. B, results from a lower levels of expression in two other cell lines with densities of 565 ± 69 and 520 ± 51 fmol/mg, respectively. Results are from five experiments. p < 0.001 for the maximal inhibition compared with wild-type.

α2C-AR, compared with 11 ± 2% for the Del322–325 receptor (n = 4, p < 0.005), which amounts to an ~60% impairment of function for the polymorphic receptor. Expression levels for the two receptors for these experiments were 806 ± 140 and 733 ± 113, respectively.

Finally, agonist-mediated stimulation of MAP kinase was examined. The mechanism of G protein-coupled receptor-mediated stimulation of this pathway is multifactorial and appears to be both receptor and cell-type dependent (26). For the β2AR, coupling to Gi, internalization of the receptor and interaction with β-arrestin is required for this receptor to activate the MAP kinase cascade (27). Less is known about α2C-AR coupling to this pathway; however, it is clear that it is pertussis toxin-sensitive and that receptor internalization is not necessary (28). For the current studies, MAP kinase activation was assessed using quantitative immunoblots with an antibody specific for the activated (phosphorylated) form of extracellular signal-regulated kinase 1/2. The total amount of MAP kinase was not different between the two cell lines utilized (Fig. 5A). Agonist-promoted activation of MAP kinase was significantly different between the two receptors (Fig. 5), with results expressed both as the agonist-promoted fold increase over basal levels of activated MAP kinase and as the percent of the thrombin response. In five such experiments, MAP kinase activity in Del322–325-expressing cells in response to 10 μM epinephrine was 57.8 ± 7.0% of the WTα2C-AR response (p < 0.005). When normalized to the thrombin response, epinephrine-stimulated MAP kinase activity was 37 ± 5.7% for the polymorphic receptor compared with 128 ± 10.0% for the wild-type α2C-AR (p < 0.005).

Recent studies have begun to elucidate specific functions for the α2C-AR subtype. In situ mRNA and immunohistochemical analysis of α2C-AR expression has revealed a distinct pattern of expression in rat brain and spinal cord (29, 30). α2C-ARs have been localized primarily in the neuronal perikarya and to a lesser extent in the proximal dendrites, with high levels of receptor expression detected in the basal ganglia, olfactory tubercle, hippocampus, and cerebral cortex (29). These data along with studies of genetically engineered mice indicate that the α2C-AR subtype plays explicit roles in cognitive and behavioral functions. Studies of mice that overexpress or that have targeted inactivation of the α2C-AR gene have shown that this receptor is involved in the regulation of spontaneous motor activity as well as agonist-induced regulation of body temperature and dopamine metabolism (2). In addition, results indicating that activation of α2C-AR reduces hyperreactivity and...
Adenylyl cyclase assays were performed on membranes prepared from CHO cell lines expressing wild-type and Del322–325 α2C-AR at 1570 ± 79.9 and 1520 ± 27.6 fmol/mg, respectively, as described under "Materials and Methods."  

| Agonist          | Max inhibition WTα2C-AR | Max inhibition Del322–325 | EC_{50} WTα2C-AR | EC_{50} Del322–325 |
|------------------|-------------------------|---------------------------|------------------|-------------------|
| Norepinephrine   | 76.6 ± 1.60             | 34.9 ± 0.887              | 219 ± 13.7       | 224 ± 70.7        |
| UK 14304         | 67.6 ± 2.09             | 30.8 ± 4.68               | 131 ± 31.0       | 109 ± 24.1        |
| BHT-933          | 58.6 ± 1.41             | 26.7 ± 2.40               | 5500 ± 2110      | 4080 ± 2005       |
| Guanabenz        | 53.8 ± 1.99             | 30.0 ± 1.98               | 10.0 ± 1.06      | 7.89 ± 3.43       |
| Clonidine        | 38.5 ± 2.44             | 20.9 ± 1.28               | 262 ± 26.1       | 176 ± 43.8        |
| Oxytemazoline    | 27.0 ± 2.70             | 12.8 ± 1.40               | 32.2 ± 1.66      | 29.1 ± 0.565      |

* p < 0.05 compared to the wild-type α2C-AR (n = three or four experiments).

fig. 4. Stimulation of inositol phosphate accumulation by wild-type and Del322–325 α2C-ARs. Total inositol phosphate production in intact CHO cells was measured as described under "Materials and Methods." in response to a 5-min exposure to 10 μM epinephrine. Receptor expression was 806 ± 140 and 733 ± 113 fmol/mg, respectively, for these experiments. *, p < 0.005 compared with wild-type response (n = four experiments). IP, inositol phosphate.

fig. 5. Stimulation of MAP kinase by wild-type and Del322–325 α2C-ARs. Phosphorylation of MAP kinase was determined in CHO cells by quantitative immunoblotting with enhanced chemiluminescence using antibodies specific for phosphorylated extracellular signal-regulated kinase 1/2. The same blots were stripped and reprobed for total kinase expression, which was not significantly different between the two cell lines (A). Cells were studied after incubation with carrier (basal) (NT), 10 μM epinephrine (Epi), or 1 unit/ml thrombin (Thr). Results are depicted as the fold stimulation over basal normalized to the wild-type response (B) and the percent of the thrombin response (C).*, p < 0.005 compared with wild-type response (n = five experiments).

The presence of functionally distinct polymorphic α2C-ARs may account for interindividual variability in physiological responses or may be the basis of differences in clinical characteristics of diseases where α2C-AR function is important. In addition, the Del322–325 polymorphism could conceivably predispose individuals to the development of disease. The response to agonist or antagonist therapeutic agents may also vary depending on receptor genotype. In this regard individuals with Del322–325 might be more sensitive to antagonists because they have receptors that are less responsive to endogenous catecholamines. For agonists, the response or sensitivity would be predicted to be less for those with the polymorphic α2C-AR due to its impaired coupling. Given the relatively high frequency of the polymorphism in healthy African-Americans (Table I), modification of a disease or drug response is more likely than predisposition to disease, although all these possibilities need to be explicitly tested. We and others have recently shown that functional polymorphisms of the β2-AR appear to have one or more of the above effects in asthma, congestive heart failure, and obesity (35–37). Interestingly, Comings et al. (38) have found that increased levels of plasma norepinephrine levels in children with attention deficit hyperactivity disorder with learning disabilities were associated with polymorphisms near the coding regions of the α2A, α2C, and dopamine β-hydroxylase genes.
In summary, we have identified a polymorphic α2C-AR that consists of a deletion of four amino acids in the third intracellular loop of the receptor. Such a deletion has a significant impact on agonist-promoted formation of the active receptor-G protein ternary complex resulting in significantly altered functional signaling to inhibition of adenylyl cyclase, stimulation of inositol phosphate accumulation, and activation of MAP kinase. For all three effector pathways, the Del322–325 receptor displays markedly impaired coupling. The polymorphism is rare in Caucasians but is ~10-fold more prevalent in African-Americans with an allele frequency of 0.381. To our knowledge, this is the greatest racial difference in a polymorphism of any G protein-coupled receptor reported to date. Given the extreme phenotype, this locus should be considered a basis for individual variation in physiologic responses, disease predisposition or modification, and drug responsiveness.

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