A Gain-of-function Polymorphism in a G-protein Coupling Domain of the Human β₁-Adrenergic Receptor*

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The β₁-adrenergic receptor (β₁AR) is a key cell surface signaling protein expressed in the heart and other organs that mediates the actions of catecholamines of the sympathetic nervous system. A polymorphism in the intracellular cytoplasmic tail near the seventh transmembrane-spanning segment of the human β₁AR has been identified in a cohort of normal individuals. At amino acid position 389, Gly or Arg can be found (allele frequencies 0.26 and 0.74, respectively), the former previously considered as the human wild-type β₁AR. Using site-directed mutagenesis to mimic the two variants, CHW-1102 cells were permanently transfected to express the Gly-389 and Arg-389 receptors. In functional studies with matched expression, the Arg-389 receptors had slightly higher basal levels of adenyl cyclase activities (10.7 ± 1.2 versus 6.1 ± 0.4 pmol/min/mg). However, maximal isoproterenol-stimulated levels were markedly higher for the Arg-389 as compared to the Gly-389 receptor (63.3 ± 6.1 versus 20.9 ± 2.0 pmol/min/mg). Agonist-promoted [35S]guanosine 5'-O-(thiotriphosphate) binding was also increased with the Arg-389 receptor consistent with enhanced coupling to Gs and increased adenyl cyclase activation. In agonist competition studies carried out in the absence of guanosine 5'-[(βγ-imido)triphosphate], high affinity binding could not be resolved with the Gly-389 receptor, whereas Arg-389 displayed an accumulation of the agonist high affinity receptor complex (R₄H = 26%). Taken together, these data indicate that this polymorphic variation of the human β₁AR results in alterations of receptor-Gₛ interaction with functional signal transduction consequences, consistent with its localization in a putative G-protein binding domain. The genetic variation of β₁AR at this locus may be the basis of interindividual differences in pathophysiologic characteristics or in the response to therapeutic βAR agonists and antagonists in cardiovascular and other diseases.

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The abbreviations used are: βAR, β₁-adrenergic receptor; Arg-389, β₁AR polymorphism with Arg at amino acid 389; Gly-389, β₁AR polymorphism with Gly at amino acid 389; 125I-CYP, 125I-labeled cyanopindolol; Gpp(NH)p, guanosine 5'-[(βγ-imido)triphosphate]; GTP[S], guanosine 5'-O-(thiotriphosphate); PCR, polymerase chain reaction.
membranes were resuspended in buffer (75 mM Tris, 12.5 mM MgCl₂, 2 mM EDTA, pH 7.4) and saturation binding experiments performed with ³²P-labeled CYP as described (12), with nonspecific binding defined by co-incubation with 1 μM propranolol. Reactions for these and other radioligand experiments were terminated by dilution and vacuum filtration over glass fiber filters that were washed twice with 10 mM Tris buffer.

Miscellaneous—Protein concentrations were determined by the copper-bichromonic acid method (15). Curve fitting was carried out using PRISM software (GraphPad, San Diego, CA). Statistical comparisons were made by paired or unpaired t tests, as appropriate, with significance considered when p < 0.05. Data are presented as mean ± S.E. of the indicated number of independent experiments.

RESULTS

Sequencing results from DNA derived from 30 individuals from a defined healthy cohort revealed variance in the examined β₁AR region (encompassing the coding block from the second transmembrane domain to the last amino acid in the carboxyl terminus of the receptor) in only one location (Fig. 1), at nucleotide 1165, where guanine or cytosine could be found, resulting in a Gly or Arg at amino acid 389. Using BsmF1 restriction digests, additional genotyping at this locus was carried out for a total of 50 samples. While the wild-type human β₁AR had previously been reported (8) as Gly at amino acid 389, we found a higher frequency of Arg in our normal population. The allele frequencies were 0.74 for Arg and 0.26 for Gly.

To ascertain whether the Arg in this position conferred a distinct phenotype compared with the Gly-389 receptor (which we (11) and others (16) had previously characterized), site-directed mutagenesis was carried out to mimic Arg-389, and the two cDNAs were subcloned into mammalian expression vectors. The final constructs were identical except for the single nucleotide difference. These constructs were used to establish β₁AR constructs by calcium phosphate precipitation as described (5) to mimic the Arg-389, and directed mutagenesis was carried out to mimic Arg-389, and additional genotyping at this locus was made by paired or unpaired t tests, as appropriate, with significance considered when p < 0.05. Data are presented as mean ± S.E. of the indicated number of independent experiments.

Functional Human β₁AR Polymorphism

Radioisotopes binding parameters of the two polymorphic β₁ARs

Studies were carried out in the presence of 100 μM GTP, ISO, isoproterenol; NE, norepinephrine; EPI, epinephrine.

| Receptor  | ¹²⁵I-CYP Kᵦ | ISO | NE | EPI |
|-----------|--------------|-----|----|-----|
| Gly-389   | 60.9 ± 2.0   | 25.9 ± 4.8 | 131.7 ± 27.4 | 457.5 ± 66.6 |
| Arg-389   | 60.9 ± 8.9   | 26.8 ± 3.5 | 136.0 ± 15.6 | 385.4 ± 37.4 |

25 °C. To assess high and low affinity agonist receptor binding, membranes were prepared as described above, except two additional centrifugations were included before the addition of reaction buffer to assure the removal of endogenous GTP. Membranes were incubated with 40 μM ¹²⁵I-CYP and 18 concentrations of isoproterenol in the presence or absence of the nonhydrolyzable GTP analog Gpp(NH)p at 100 μM for 1 h at 37 °C. Competition data were fit to one-site and two-site models by an iterative least squares technique as described previously (13). A two-site model was considered valid if by F-test the fit was statistically better (p < 0.05) than that obtained with a one-site model.

Adenylyl Cyclase Activity Measurements—Membranes were incubated with 30 mM Tris, pH 7.4, 2.0 mM MgCl₂, 0.8 mM EDTA, 120 μM ATP, 60 μM GTP, 2.8 mM phosphoenolpyruvate, 2.2 μg of myokinase, 100 μM cAMP, and 1 μCi of [³²P]ATP for 30 min at 37 °C as described (11). [³²P]cAMP was separated from [³²P]ATP by chromatography over alumina columns. A [³²P]cAMP standard included in the stop buffer accounted for individual column recovery. Activities were determined in the presence of vehicle (basal), the indicated concentrations of agonists, or 100 μM forskolin.

Radioisotopic binding—Transfected COS cells were washed three times with phosphate-buffered saline, lysed in hypotonic buffer (5 mM HEPES, 1 mM EDTA, pH 8), detached by scraping with a rubber policeman, and centrifuged for 10 min at 42,000 × g. Pellets were resuspended in the same buffer and centrifuged two additional times. [³²P]GTP-S binding was carried out by a modification of the method of Befort et al. (14). The reaction consisted of membranes in 10 mM HEPES, pH 7.40, 5 mM MgCl₂, 100 mM NaCl, 1 mM EDTA buffer, with 1.0 μM GDP and 180 μM [³²P]GTP-S in the presence of water (basal), 100 μM unlabeled GTP-S (to define nonspecific binding), or 10 μM isoproterenol for 2.5 h at 30 °C. Unbound [³²P]GTP-S was separated using vacuum filtration over glass fiber filters that were washed twice with 10 mM Tris buffer.

RESULTS

Sequencing results from DNA derived from 30 individuals from a defined healthy cohort revealed variance in the examined β₁AR region (encompassing the coding block from the second transmembrane domain to the last amino acid in the carboxyl terminus of the receptor) in only one location (Fig. 1), at nucleotide 1165, where guanine or cytosine could be found, resulting in a Gly or Arg at amino acid 389. Using BsmF1 restriction digests, additional genotyping at this locus was carried out for a total of 50 samples. While the wild-type human β₁AR had previously been reported (8) as Gly at amino acid 389, we found a higher frequency of Arg in our normal population. The allele frequencies were 0.74 for Arg and 0.26 for Gly.

To ascertain whether the Arg in this position conferred a distinct phenotype compared with the Gly-389 receptor (which we (11) and others (16) had previously characterized), site-directed mutagenesis was carried out to mimic Arg-389, and the two cDNAs were subcloned into mammalian expression vectors. The final constructs were identical except for the single nucleotide difference. These constructs were used to establish permanent lines of transfected CHW cells, which endogenously
lack βAR expression. As indicated, studies were carried out on several clonal lines from each transfection at matched levels of receptor expression. Radioligand binding studies (Table I) revealed essentially identical dissociation binding constants for 

competition binding studies with isoproterenol, epinephrine, and norepinephrine in the presence of GTP showed no differences between the two receptors in binding affinities for these agonists.

Basal, isoproterenol-, and forskolin-stimulated adenylyl cyclase activities were determined using membranes from lines expressing Gly-389 at 159 ± 14 fmol/mg and Arg-389 at 151 ± 13 fmol/mg (Fig. 2A). As shown, basal activities were slightly higher with the Arg-389 receptor than with the Gly-389 receptor (10.7 ± 1.2 versus 6.1 ± 0.4 pmol/min/mg, n = 4, p < 0.02). The most dramatic differences, however, were seen with agonist stimulation. Maximal isoproterenol-stimulated activities were higher with the Arg-389 receptor than with the Gly-389 receptor (63.3 ± 6.1 versus 20.9 ± 2.0 pmol/min/mg, n = 4, p < 0.01). Similarly, expressing the responses as fold increases over basal levels, the Arg-389 receptor displayed a greater stimulation of adenylyl cyclase than the Gly-389 receptor (6.1 ± 0.3-fold versus 3.3- ± 0.1-fold). In contrast, stimulation of adenylyl cyclase by forskolin was not consistently different between the lines (104 ± 11 versus 120 ± 10 pmol/min/mg). Thus, normalizing the data to the extent of stimulation of adenylyl cyclase by forskolin still revealed a significantly greater agonist stimulation by the Arg-389 variant than the Gly-389 variant (Fig. 2B). The EC_{50} values for isoproterenol stimulation of adenylyl cyclase for Gly-389 and Arg-389 receptors were not different (115 ± 5 versus 132 ± 5 nM). Results from an additional set of studies carried out on other lines at higher expression levels are shown in Fig. 2, C and D (expression was 246 ± 32 and 255 ± 11 fmol/mg for Gly-389 and Arg-389, respectively). Again, the maximal extent of stimulation by agonist was greater with the Arg-389 variant. These differences in adenylyl cyclase stimulation between the two polymorphic receptors were also observed in responses to epinephrine and norepinephrine (data not shown).

To investigate the basis of these differences between the two receptors, studies were undertaken to assess interactions between agonist, receptor, and G_{α} with [35S]GTPγS binding experiments and agonist competition studies in the absence and presence of Gpp(NH)p. For [35S]GTPγS binding, we found that adequate signals were reproducibly obtained with high levels of transient receptor expression (~10 pmol/mg) in COS-7 cells, which were co-transfected with G_{α}. Results of these studies are shown in Fig. 3. Maximal isoproterenol-stimulated [35S]GTPγS binding was found to be greater in membranes bearing the Arg-389 versus the Gly-389 receptor, consistent with the adenylyl cyclase studies, which also showed enhanced agonist-stimulated function with the Arg-389 receptor.

A well recognized characteristic of agonist binding to receptors such as the βAR is the increase in high affinity binding states, indicative of receptor-G-protein interactions (17–19). Interestingly, we have previously reported (13) that in competition studies carried out in the absence of Gpp(NH)p, agonist-promoted accumulation of a βAR (Gly-389) high affinity fraction is not readily resolved. This result is likely because of the similar affinities of the high and low affinity sites consistent with relatively less free energy transfer during signal transduction. We wondered whether the Arg-389 receptor would in
fact have enhanced agonist-promoted high affinity binding, because this receptor has increased functional coupling to $G_s$. Isoproterenol competition studies were thus carried out in parallel, in the absence and presence of Gpp(NH)p, with washed membranes from CHW cells expressing each receptor. These results are provided in Fig. 4 and Table II. As shown, the displacement curves in the absence of a guanine nucleotide from the Gly-389 experiments were relatively steep (mean pseudo-Hill coefficient, 0.87 ± 0.03) and were best resolved statistically to a one-site model. Gpp(NH)p had no effect on agonist competition with the Gly-389 receptor (Fig. 4).

The Gly-389 receptor could not be fit to a two-site model with greater significance than a one-site model (see text for details), and thus high and low affinity binding parameters were not obtained. $R_{II}$, percent of receptors in high affinity states; NA, not applicable.

| Receptor | $K_i$ | $K_L$ | $K_H$ | $R_{II}$ |
|----------|-------|-------|-------|----------|
| Gly-389  | 116.6 ± 20.6 | NA | NA | NA |
| Arg-389  | 108.4 ± 14.0 | 68.9 ± 13.6 | 3.30 ± 1.03 | 26.2 ± 1.4 |

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DISCUSSION

These findings reveal in the normal human population the presence of two $\beta_1$AR genetic variants with significant differences in functional signaling. For purposes of consistency, and because of the fact that a number of structure/function studies have been published using the Gly-389 receptor (15, 20), we prefer to continue to designate this receptor as the "wild-type" human $\beta_1$AR, although the Arg variant appears to be more common. This is analogous to the precedent that has been set with the common $\beta_2$AR polymorphisms (5, 6). The site of the variability is 9 amino acids from the seventh transmembrane-spanning domain, in the intracellular portion of the tail prior to the proposed palmitoylated cysteine(s) (Fig. 5). This region is sometimes referred to as the fourth intracellular loop, but in this report will be termed the proximal portion of the cytoplasmic tail. By analogy with $\beta_2$AR (21, 22), $\alpha_2$AR (23), and other G-protein-coupled receptors, this region is considered important for receptor coupling to its cognate G-protein, $G_s$. Indeed, the difference between the Arg-389 and Gly-389 receptors is in functional coupling. Receptor-promoted binding of GTPγS to $G_s$, another indicator of agonist-initiated coupling to $G_s$, was similarly different between the two polymorphic $\beta_1$ARs. Consistent with these findings, agonist-promoted accumulation of the high affinity state in washed membrane preparations in the absence of a guanine nucleotide was detected with the Arg-389 receptor but could not be resolved in studies with the Gly-389 receptor. Based on the multistate model (18, 19) of receptor/G-protein interactions, as well as the above results, an elevated basal activity of adenylyl cyclase (i.e., spontaneous toggling to $R^*$ in the absence of agonist) should also be expected with the Arg-389 receptor if it has a greater efficiency of stabilizing the active conformation in the presence of agonist, which in fact was the case. The differences in basal activities that we observed were small but consistent, and the magnitude likely results from the relatively low ("physiologic") levels of expression utilized in the functional studies. As such, we consider the functional phenotype as shown in Fig. 2, A and C, to be indicative of signaling in cells that endogenously express the two polymorphic $\beta_1$ARs.

The residues in this region of the $\beta_1$AR among other species are shown in Fig. 5. The amino acid analogous to position 389 of the human, as well as the surrounding residues, are highly conserved in species sequenced to date, with the only deviation at position 389 being found in the human, where Gly was...
originally reported. Whereas we know nothing regarding genetic variability in these other species, the high degree of consistency in this region, its importance in G-protein linking, and the nonconservative (size and charge) nature of the Gly to Arg substitution are consistent with this variation having functional consequences.

As introduced earlier, $\beta_1$AR are expressed on a number of cell types in the body. In the heart, $\beta_1$AR represent the predominant $\beta$AR subtype and is expressed on myocytes of the atria and ventricles, where they act to increase the force and frequency of contraction in response to sympathetic stimulation. It is intriguing to consider the potential role of the $\beta_1$AR polymorphisms at position 389 in regulating cardiac function. As introduced earlier, $\beta_1$AR are expressed on a number of cell types in the body. In the heart, $\beta_1$AR represent the predominant $\beta$AR subtype and is expressed on myocytes of the atria and ventricles, where they act to increase the force and frequency of contraction in response to sympathetic stimulation. It is intriguing to consider the potential role of the $\beta_1$AR polymorphisms at position 389 in regulating cardiac function.

In conclusion, we have found polymorphic variation of the human $\beta_1$AR at amino acid 389, where Gly (previously considered wild-type) or Arg can be found. This variation alters receptor-$G_s$ coupling, manifested as significant differences between the two receptors in agonist-stimulated adenylyl cyclase activation. Such variation may represent a genetic basis for interindividual differences in disease susceptibility or phenotype, or the response to agents targeting the $\beta_1$AR.

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