The Myocardium-protective Gly-49 Variant of the β₁-Adrenergic Receptor Exhibits Constitutive Activity and Increased Desensitization and Down-regulation

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The β₁-adrenergic receptor (β₁AR) is a major mediator of catecholamine effects in human heart. Patients with heart failure who were hetero- or homozygous for the Gly-49 variant of the β₁AR (Gly-49-β₁AR) showed improved long-term survival as compared with those with the Ser-49 genotype. Here, the functional consequences of this polymorphism were studied in cells expressing either variant. The Gly-49-β₁AR demonstrated characteristic features of constitutively active receptors. In cells expressing the Gly-49-β₁AR, both basal and agonist-stimulated adenylyl cyclase activities were higher than in cells expressing the Ser-49 variant (Ser-49-β₁AR). The Gly-49-β₁AR was more sensitive to the inhibitory effect of the inverse agonist metoprolol and displayed increased affinity for agonists. Isoproterenol potency for adenylyl cyclase activation was higher on membranes expressing the Gly-49-β₁AR than on those expressing the Ser-49-β₁AR. After incubation with saturating concentrations of catecholamines or sustained stimulation, the Gly-49 variant showed a much higher desensitization, which largely prevailed over constitutive activity in terms of cAMP accumulation. The Gly-49-β₁AR also displayed a more profound agonist-promoted down-regulation than the Ser-49 variant. The stronger regulation of the Gly-49-β₁AR could explain the beneficial effect of the Gly-49 genotypes on survival, further supporting the concept that β₁AR desensitization is protective in heart failure.

β₁AR is the principal subtype of β-adrenergic receptors (βARs) regulating human heart rate and contractility (1). Stimulation of myocardial β₁AR by catecholamines results in the activation of the heterotrimeric G protein, which, in turn, activates the adenylyl cyclase (AC) and promotes the production of cAMP. During chronic heart failure (HF) the persistent compensatory increase of catecholamines causes β₁AR desensitization and down-regulation. The resulting partial loss of β₁AR function is believed to be an adaptive mechanism to counteract the cardiotoxicity of chronic adrenergic signaling (2). This hypothesis is supported by the fact that β₁-adrenergic blockade has proved to be effective in the treatment of chronic HF (3, 4).

Two loci of allelic polymorphism were recently identified in the coding region of the human β₁AR at codons 49 and 389 (5–8), causing amino acid variability within the extracellular N-terminal and the intracellular C-terminal regions, respectively. The two natural variants of the β₁AR at position 389, Arg-389-β₁AR and Gly-389-β₁AR, displayed different patterns of coupling to the G protein and of AC activation in vitro, but no correlation was found between either variant and susceptibility for (7), or severity of (9), myocardial disease. In contrast, the allelic distribution of β₁AR polymorphism at codon 49 was found to be associated with long term survival of patients with chronic HF (5). Patients with the Ser-49 genotype showed a high mortality rate (46%) after 5 years, whereas patients either homozygous or heterozygous for the Gly-49 variant showed a mortality rate of only 23%.

Because of the major role of β₁AR in both normal and failing myocardium, the beneficial effect of the Gly-49-β₁AR on survival in HF patients might be due to specific features of this variant in terms of receptor signaling and/or regulation. To address this question, we compared the pharmacological and functional properties of Ser-49-β₁AR and Gly-49-β₁AR after stable transfection in human embryonic kidney 293 cells. Our data show that the Gly-49 and Ser-49 variants differ in terms of coupling to the downstream adenylyl cyclase pathway and agonist-dependent regulation. They also provide a plausible explanation for the myocardial-protective effect of the Gly-49-β₁AR in patients with chronic HF.

EXPERIMENTAL PROCEDURES

Construction and Expression of the Ser-49 and Gly-49 Variants of β₁AR—The deoxycadenosine at position 145 in the coding sequence for Ser-49 of the human β₁AR gene was changed for a deoxyctydine by site-directed mutagenesis, using the method of Kunkel (10), to generate a codon for glycine at position 49. The mutation was confirmed by dideoxy sequencing. Both Ser- and Gly-encoding β₁AR cDNAs were subcloned into the mammalian expression vector pCDNAs. Nearly confluent human embryonic kidney 293 cells were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, 100 μg/ml streptomycin, 100 units/ml penicillin, and 1 mM glutamine at 37 °C and transfected with 8 μg of vector coding for either the Ser-49-β₁AR or the Gly-49-β₁AR. Clones resistant to 1 mg/ml G418 were screened for β₁AR expression by radioligand binding assay using [125I]-CYP (Amersham Biosciences, specific activity, 2200 Ci/mmol) as ligand.

Preparation of Human Myocardial Membranes—Fresh human left ventricular myocardium specimens were obtained from two patients with chronic HF during implantation of left ventricular mechanical support. The patients were enrolled in the national registry for dilated cardiomyopathy, approved by the Ethics Committee at Göteborg Uni-
versity, and had given informed consent. Crude membranes were prepared by homogenization in ice-cold buffer (30 mM Tris, pH 7.4, 0.6 mM EDTA, with protease inhibitors as described below), filtered through gauze fiber, and immediately analyzed for AC activity.

Receptor Radioligand Binding—Radioligand binding assays were performed in 50 μl of buffer, prepared as described previously (11), or on whole cells in a final volume of 250 μl of 5 mM Tris, pH 7.4, 12 mM MgCl₂, and 0–400 pm ¹²⁵I-CYP. Nonspecific binding was determined in the presence of 10 μM alpenrol. Membranes were incubated for 45 min at 30 °C, filtered through Whatman GF/C filters, and washed three times with the same ice-cold buffer. Protein concentrations were determined in the BCA protein assay kit (Pierce). Competition binding experiments were performed with 80 pm ¹²⁵I-CYP and increasing concentrations of various agonists and antagonists. Ligand affinities were calculated using Prism 3.0 (GraphPad Software, San Diego, CA).

Measurements of Adenylyl Cyclase Activity in Membranes and Intact Cells—Non-exposed cells or cells exposed to 10 μM isoproterenol (ISO) and 10 μM ascorbic acid or 10 μM ascorbic acid alone for 20 min at 37 °C were washed three times with ice-cold phosphate-buffer saline and harvested mechanically using a pipette. After centrifugation at 800 × g, cells were resuspended in ice-cold lysis buffer containing protease inhibitors (5 mg/liter soybean trypsin inhibitor, 5 mg/liter leupeptin, and 10 mg/liter benzamidin) and membranes were prepared (11). The AC activity was determined in 2–5 μg of membranes resuspended in 60 μl of 0.2 M Na₂HPO₄, 0.05 M MgCl₂, 0.12 mM ATP, 0.053 mM GTP, 0.1 mM cAMP, 0.1 mM 3-isobutyl-1-methylxanthine (IBMX), 2.8 mM phosphoenolpyruvate, 1 μCi of [α-³²P]ATP, 0.2 unit of pyruvate kinase, 1 unit of myokinase, and [³H]cAMP (60,000 cpm). Enzyme activity was determined in the absence or presence of 0.1 μM to 100 μM ISO or 100 μM metoprolol for 15 min at 37 °C. Membranes incubated with 10 μM forskolin were used as positive controls. The reaction was stopped with 200 μl of ice-cold 0.5 M HCl, followed by 5 min of boiling. Samples were buffered with 0.2 ml of 1.5 M imidazole, pH 7.5, and finally applied to Al₂O₃ columns. [α-³²P]cAMP was eluted with 3 ml of 10 μM imidazole, pH 7.5, and counted in a β-counter (LKB Wallac). Dose-response curves of AC activity were analyzed using Prism 3.0 (GraphPad Software).

Intracellular cAMP was measured in intact cells exposed to the indicated concentrations of ISO, norepinephrine (NE), or metoprolol, in the presence of IBMX for 10 min at 37 °C (dose-response experiments), or in the absence of IBMX, for 0–8 h at 37 °C, with fresh ligands added every other hour (time-course experiments). Ascorbic acid (10 μM) was added in the incubation medium to prevent catecholamine oxidation. After incubation, cells were washed three times with ice-cold phosphate-buffer saline and lysed in 300 μl of ice-cold 5 mM Tris, pH 7.4, 5 mM EDTA. The suspension was boiled for 3 min and centrifuged. The cAMP contained in the supernatant was measured with a [³H]cAMP assay kit (Amersham Biosciences). Protein concentrations were determined with the BCA protein assay kit (Pierce).

Receptor Internalization and Receptor Down-regulation—Receptor internalization was determined by measuring the number of “sequestrated” receptors that became inaccessible to the hydrophilic membrane-impermeable ligand CGP-12177A after agonist stimulation, as previously described (12). The total number of receptors was measured using the membrane-permeable ¹²⁵I-CYP radioligand, and the amount of intracellular receptors was determined as the number of ¹²⁵I-CYP binding sites that could not be displaced by a 3.5-h incubation with 3 μM CGP-12177A at 13 °C. Surface receptors were calculated as the difference between total and intracellular receptors. Sequestered receptors correspond to the loss of surface receptors induced by agonist stimulation.

Receptor down-regulation was determined in nearly confluent cells incubated with 1 μM ISO and 10 μM ascorbic acid, for 0–20 h at 37 °C, in culture medium containing 5 μg/ml cycloheximide.

Statistical Analysis—Values are means ± S.E. or means ± S.D. as given in the figure legends. Significant differences between means were calculated with Student’s t test for either paired or unpaired observations. A value of p < 0.05 was considered statistically significant.

RESULTS

To test the hypothesis that the natural substitution of a serine by a glycine residue in the N-terminal extracellular domain of the β₁ AR may affect its functional properties, the cDNAs of the Gly-49-β₁ AR and of the Ser-49-β₁ AR were transfected in human embryonic kidney HEK-293 cells. Stable clones expressing variable amounts (−0.5 to −5 pmol/mg) of either β₁ AR variant were selected.

Ligand binding properties of the Ser-49 and Gly-49 variants of β₁ AR

| Ligand | Kᵢ High | Kᵢ Low |
|--------|--------|--------|
| ISO    | 0.43 ± 0.2 | 268 ± 17 | 23 ± 2.4 |
| NE     | 137 ± 26   | 3600 ± 676 | 27 ± 6.8 |
| ICYP   | 57 ± 4     | 3.2 ± 0.8 | |
| METO   | 54 ± 9     | 4.3 ± 0.5 | |

| Gly-49-β₁ AR | ISO | NE | ICYP | METO |
|--------------|-----|----|------|------|
| 0.23 ± 0.11  | 187 ± 16 | 2413 ± 708 | 17 ± 1.4 |
| 50 ± 16     | 62 ± 6  | 3.4 ± 0.5 | 72 ± 13 |

*p < 0.01 versus Ser-49-β₁ AR.

*p < 0.05 versus Gly-49-β₁ AR.

Ligand Binding Properties of Ser-49-β₁ AR and Gly-49-β₁ AR

Receptor down-regulation was determined on membranes of HEK-293 cells expressing the Ser-49 or the Gly-49 variant of β₁ AR for 45 min at 30 °C as described above. The EC₅₀ values for [α-³²P]cAMP were measured by direct binding assays. Competition binding isotherms were determined for isoproterenol (ISO), norepinephrine (NE), alpenrol (ALP), and metoprolol (METO) at 80 pm of ¹²⁵I-CYP. Data were fit to one-site and two-site models by non-linear least-squares regression analysis using Prism 3.0. Values are mean ± S.E. of three to six independent experiments performed in duplicate.
Inverse Agonism of Metoprolol on Ser-49-βAR and Gly-49-βAR—From the results above, it appears that the replacement of the Ser-49 residue of the human βAR by a Gly residue is associated with striking changes of receptor function, including constitutive activation of basal cAMP production and amplification of the response to ISO. According to previous studies on constitutively active receptors, β-adrenergic inverse agonists would be expected to cause a stronger inhibition of the basal AC activity in cells expressing various densities of the Gly-49-βAR or the Ser-49-βAR (13). Comparison of basal AC activity in cell clones expressing various densities of the Gly-49-βAR (○) or the Ser-49-βAR (●); on average, basal activity was 4-fold (3.9 ± 0.8) higher in Gly-49 cells than in Ser-49 cells (p < 0.001). 

Desensitization of Ser-49-βAR and Gly-49-βAR—Functional differences between receptor variants may also arise from the duration of the cellular signal they generate, which, in turn, depends on desensitization mechanisms (15). Desensitization was first studied by comparing the AC activity in membranes from cells, expressing −1 pmol of receptor per milligram of protein, pre-stimulated or not with ISO for 20 min. In both cases, receptor desensitization was characterized by a reduction of maximal AC activity and by a rightward shift of dose-response curves (Fig. 2). Although a 2-fold increase of EC_{50} values was observed for both variants, there was a marked difference in the extent to which the two βAR variants were desensitized. Maximal AC activity was reduced by 57 ± 8% in membranes expressing the Gly-49-βAR, whereas the reduction was only 19 ± 3% for Ser-49-βAR (p < 0.01). The study of additional cell clones expressing various receptor densities of either βAR variant showed that the different degrees of desensitization observed in these experiments are indeed attributable to the intrinsic properties of each variant (Fig. 3).

To determine whether the different extent of desensitization between the two βAR variants observed in membrane preparations could affect the concentration of cAMP reached in intact cells, dose-response assays of agonist-promoted cAMP accumulation were carried out on cells from the same clones as in Fig. 3. The intracellular cAMP, accumulated during 10-min incubation with the phosphodiesterase inhibitor IBMX alone, was higher in Gly-49 cells than in Ser-49 cells (56 ± 11 versus 25 ± 5.2 pmol/mg, p < 0.05), which reflected well the differences of basal AC activity found between the two variants (Fig. 4). The intracellular cAMP concentration reached in Gly-49 and Ser-49 cells, after 10-min stimulation with 1 nM ISO (a concentration below the EC_{50} of AC stimulation, as determined in Fig. 1), was comparable. However, after stimulation with higher concentrations of ISO, cAMP was significantly lower in cells expressing the Gly variant than in Ser-49 cells. Similar results were obtained with the natural ligand NE: intracellular cAMP was significantly lower in cells expressing the Gly variant upon stimulation with NE at concentrations of 1 μM or above (data not shown).

The myocardium of patients with chronic HF is permanently
subjected to increased levels of endogenous catecholamines. We investigated whether, in the context of sustained stimulation with ISO or NE, the different desensitization profile of β₁AR variants would result in distinct cAMP responses in the HEK-293 model. Cells expressing either receptor variant were incubated for up to 8 h with saturating concentrations of agonists (Fig. 5). Maximal stimulation with ISO or NE promoted a comparable raise of intracellular cAMP at 2 min, for Ser and Gly (431 ± 40 and 335 ± 30 pmol/mg, respectively, in the presence of ISO; 428 ± 32 and 360 ± 27 pmol/mg, respectively, in the presence of NE). However, for longer incubation times with agonist, a significant decrease of intracellular cAMP was observed in Gly-49-β₁AR (left panel, n = 6) and Gly-49-β₁AR (right panel, n = 5) are expressed as percent of maximal AC value after subtraction of basal activity. In the left panel, basal and maximal AC values were 7.6 ± 1.4 and 67 ± 6 pmol/mg/min, respectively; in the right panel they were 31 ± 8.9 and 122 ± 14 pmol/mg/min, respectively. Values are mean ± S.E.

Fig. 2. Short-term desensitization of AC activity in membranes expressing the Ser-49 or Gly-49 variants of β₁AR. Intact cells were pre-stimulated (●) or not (○) with 10 μM ISO for 20 min in 37°C; membranes were prepared, and AC activity was determined at increasing concentrations of ISO as described under “Experimental Procedures.”

TABLE II

| Membranial AC activity | % change from basal | Intracellular cAMP level | % change from basal |
|------------------------|--------------------|-------------------------|--------------------|
| Basal | Metoprolol | Basal | Metoprolol |
| Ser-49-β₁AR | 8.3 ± 1.3 | 5.7 ± 0.8 | 21 ± 7.7 | 17 ± 4.3 | −42 ± 1.7 | −40 ± 9.1 |
| Gly-49-β₁AR | 25 ± 4.3 | 15 ± 2.4 | 54 ± 11 | 30 ± 1.0 | −14 ± 8.6 |

*p < 0.05 versus basal. 
‡p < 0.01 versus basal. 
* p < 0.05 versus Ser-49-β₁AR.

Intracellular cAMP in cells expressing the Ser-49 or Gly-49 variants of β₁AR. Intact cells expressing −1 pmol/mg either the Ser-49-β₁AR (open bars) or the Gly-49-β₁AR (filled bars) were exposed to various concentrations of ISO for 10 min at 37°C, and cAMP accumulation was determined as described under “Experimental Procedures” (n = 3–6). Values are mean ± S.E. *, p < 0.05 versus Ser-49-β₁AR.
Intracellular cAMP was measured as described under “Experimental Procedures” (n = 3). Values are mean ± S.E. *, p < 0.05 versus 2 min.

The marked amplification of the response to the agonist and the moderate changes of $K_d$ and $K_{ass}$ values are less common, but they were reported for some GPCRs with constitutive activity, such as the Asn-111 mutant of the AT$_{1A}$ receptor (17).

According to the modified ternary complex model of GPCR activation (18), mutations causing constitutive activation are likely to facilitate the isomerization between active and inactive conformations of the receptor. Although most receptor-activating mutations lie in intracellular regions interacting with G proteins or in adjacent transmembrane domains, they can also occur in extracellular regions. For example, several mutations of the extracellular domain of the thyroid-stimulating hormone receptor were reported to cause constitutive activation of the receptor in patients with toxic adenomas (19). Polymorphism within the N-terminal region was also found for the $\beta_2$AR. The Gly-16-$\beta_2$AR, for example, showed a 5-fold increase of agonist sensitivity, although this variant did not display higher basal activity as in the case of the Gly-49-$\beta_2$AR. The mechanism, by which the Ser-49 → Gly substitution facilitates $\beta_2$AR activation, will remain speculative before structural studies. However, it is plausible that, because of the very different conformational preferences of glycine and serine residues (20), the Ser → Gly substitution may cause a sliding or a change in the orientation of the adjacent transmembrane domain and, consequently, in the overall conformation of the receptor. This hypothesis is consistent with the current model of GPCR stimulation by peptides, which activate the receptors by interacting with amino acid residues located in their extracellular regions (21).

A second striking feature of the Gly-49 variant of the $\beta_1$AR is its high propensity for desensitization. Increased desensitization was evident for high concentrations of catecholamines and/or sustained stimulation of the receptor. Under these conditions, the level of cAMP was indeed much lower in cells expressing the Gly-49 variant than in cells expressing the less active Ser-49-$\beta_1$AR. The association between constitutive activity and increased sensitivity to desensitization is probably not fortuitous, because a number of adrenergic receptor mutants generated in vitro were characterized by structural instability (22), increased phosphorylation (23, 24), constitutive desensitization (24), or changes in endocytosis (25). The higher level of Gly-49-$\beta_1$AR desensitization might be explained by higher affinities of protein kinase A and/or G protein receptor kinases (GRKs) for this variant, as reported for constitutively active $\beta_2$AR mutants (24). Previous studies indicated that phosphorylation by both protein kinase A and GRKs contribute to $\beta_2$AR desensitization (26). The observation, that the desensitization of the Gly-49-$\beta_2$AR was more noticeable in the case of sustained agonist activation or in the presence of agonist concentrations close to or above the $K_d$, suggests that GRKs are involved in this phenomenon, because it is well established that these kinases only phosphorylate receptors that are bound by the agonist.

Sustained activation by the agonist failed to induce any significant down-regulation of the Ser-49-$\beta_1$AR, whereas a substantial loss of receptor binding sites was observed in cells expressing the Gly-49 variant. GPCR down-regulation may be caused by the sorting of internalized receptors to the lysosomal compartment where they are degraded by resident proteases. The different down-regulation profile of the two $\beta_1$AR variants might thus be explained by their different internalization rate (no significant internalization for the Ser variant versus 15% internalization for the Gly variant). Alternatively, because of its relatively low level of internalization, the Gly-$\beta_1$AR might be directly degraded at the plasma membrane by proteases recognizing the activated conformation of the receptor. Such a
mechanism, which does not require receptor internalization, was reported for the β2AR (27).

The observation that the frequency of Gly-49-β2AR genotypes are not different between healthy controls and patients with congestive HF or patients with idiopathic dilated cardiomyopathy (5, 9), suggests that the functional properties of this variant are not sufficient, by themselves, to promote a myocardial disease. However, the improved survival of patients with chronic HF and of either a Gly-49/Ser-49 or a Gly-49/Gly-49 genotype, might reflect a direct contribution of the Gly-49-β2AR to the more favorable prognosis.

In patients with HF, the impairment of cardiac output is compensated by a chronic increase of the sympathetic drive, resulting in permanently high concentrations of blood catecholamines. A number of studies, however, indicated that chronic adrenergic signaling in general and its β2AR component in particular, are harmful for myocardial tissue. Norepinephrine, the β2AR-selective catecholamine, was found to be cardiotoxic and to produce cardiac myocyte injury at concentrations found in failing human heart (28). In transgenic mice, a 5- to 40-fold overexpression of cardiac β2AR was reported to induce heart failure within months (29, 30). Similarly, a 3-fold overexpression of Goα led to myocardial damage characterized by cellular degeneration, hypertrophy, and sudden death (31). In adult rat myocytes, the β2AR mediates apoptotic signal through Go stimulation, whereas the β2AR is anti-apoptotic via its coupling to Gi proteins (32). In the failing heart, β-adrenergic signaling is reduced as a consequence of β2AR desensitization and down-regulation and of increased activity of Gi proteins (33). It was proposed that this loss of β-adrenergic-transducing capacity might represent a protective/adaptive mechanism against catecholamine overload (2). The rationale for β-adrenergic receptor blockade, the current established treatment of compensated chronic HF (3, 4), is in close accordance with this concept.

Taken together, these data suggest that the protective effect of the Gly-49-β2AR in HF patients may depend on the higher sensitivity of this receptor variant to catecholamine-induced desensitization. In the context of the chronic adrenergic stimulation occurring in HF, the increased desensitization of the Gly-49 variant is likely to largely override its basal constitutive activity. This hypothesis is consistent with the much lower level of cAMP measured in cells expressing Gly-49-β2AR, compared with Ser-49-β2AR cells, upon sustained receptor activation. In addition, the observation that the inverse agonist metoprolol could decrease basal AC activity and cAMP content in cells expressing the Gly-49-β2AR suggests that inverse agonists might be more beneficial than neutral β-blockers in the treatment of patients with HF, which are homo- or heterozygous for the Gly-49 variant. Interestingly, during the time of the study, we could examine two myocardial specimens, one from a patient with Ser-49-β2AR (homozygous Ser-49 genotype), the other from a patient with Gly-49 (heterozygous Gly-49/Ser-49 genotype). Metoprolol displayed a significant β2AR inverse agonism in myocardial membranes from the patient with Gly-49 but not in membranes from the patient with Ser-49-β2AR (28 ± 5% versus 5 ± 5% reduction of basal AC activity, p < 0.005). Furthermore, basal and maximal AC activities were higher in membranes from the Gly-49 patient than in Ser-49 membranes (4 ± 0.2 versus 0.6 ± 0.1 and 13 ± 0.6 versus 2 ± 0.2 pmol/mg/min, respectively). The β2AR densities were 37 and 30 fmol/mg, respectively. Although these data must be considered with caution due to inherent variability in the human population, they are consistent with the results obtained in transfected cells and with a previous clinical study showing that patients with at least one Gly-49-β2AR allele tended to respond better to β-blockers than patients devoid of this allele. The majority of these patients being treated with the inverse agonist metoprolol (5).

In conclusion, we have reported the first example of polymor-
phism associated with constitutive adrenergic receptor activation (13, 34) and increased susceptibility to desensitization. Although without known consequences in healthy subjects, the functional properties of the variants may affect long term prognosis of patients with chronic HF.

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