Coupling of β₂-Adrenergceptor to Gᵢ Proteins and Its Physiological Relevance in Murine Cardiac Myocytes

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Abstract—Transgenic mouse models have been developed to manipulate β-adrenergceptor (βAR) signal transduction. Although several of these models have altered βAR subtypes, the specific functional sequelae of βAR stimulation in murine heart, particularly those of β₂-adrenergceptor (β₂AR) stimulation, have not been characterized. In the present study, we investigated effects of β₂AR stimulation on contraction, [Ca²⁺]ᵢ transient, and L-type Ca²⁺ currents (Iᵥ) in single ventricular myocytes isolated from transgenic mice overexpressing human β₂AR (TG4 mice) and wild-type (WT) littermates. Baseline contractility of TG4 heart cells was increased by 3-fold relative to WT controls as a result of the presence of spontaneous β₂AR activation. In contrast, β₂AR stimulation by zinterol or isoproterenol plus a selective β₁-adrenergceptor receptor (β₁AR) antagonist CGP 20712A failed to enhance the contractility in TG4 myocytes, and more surprisingly, β₂AR stimulation was also ineffective in increasing contractility in WT myocytes. Pertussis toxin (PTX) treatment fully rescued the β₂AR stimulations [Ca²⁺]ᵢ, and contractile responses to β₂AR agonists in both WT and TG4 cells. The PTX-rescued murine cardiac β₂AR response is mediated by cAMP-dependent mechanisms, because it was totally blocked by the inhibitory cAMP analog Rp-cAMPS. These results suggest that PTX-sensitive G proteins are responsible for the unresponsiveness of mouse heart to agonist-induced β₂AR stimulation. This was further corroborated by an increased incorporation of the photoactive GTP analog [γ-³²P]GTP azidoanilide into α subunits of G₁₂ and G₁₃ after β₂AR stimulation by zinterol or isoproterenol plus the β₂AR blocker CGP 20712A. This effect to activate Gᵢ proteins was abolished by a selective β₁AR blocker ICI 118,551 or by PTX treatment. Thus, we conclude that (1) β₂ARs in murine cardiac myocytes couple to concurrent Gᵢ and Gᵢ₃ signaling, resulting in null inotropic response, unless the Gᵢ signaling is inhibited; (2) as a special case, the lack of cardiac contractile response to β₂AR agonists in TG4 mice is not due to a saturation of cell contractility or of the cAMP signaling cascade but rather to an activation of β₂AR-coupled Gᵢ proteins; and (3) spontaneous β₂AR activation may differ from agonist-stimulated β₂AR signaling. (Circ Res. 1999;84:43-52.)

Key Words: β₂-adrenergceptor ■ inhibitory G protein ■ cardiac contractility ■ L-type Ca²⁺ current ■ mice, transgenic

Current opinion suggests that gene therapy may hold great promise for treatment of cardiovascular diseases that lead to chronic heart failure. The assimilation of rapid advances in mouse genetics into the realm of cardiovascular research has provided pharmacologists and physiologists a tremendous range of new opportunities to unravel the molecular secrets that govern cardiovascular structure and function in health and disease. Several lines of transgenic mice have been generated to target key proteins that govern transmembrane signal transduction or modulate the contractile properties of myocardial cell.¹⁻⁹ One such model that has drawn substantial attention is a transgenic mouse model overexpressing the human β₂-adrenergceptor (β₂AR) in a cardiac specific manner (TG4). In this model, while baseline myocardial contractility is successfully enhanced relative to wild-type (WT) littermates,¹ cardiac responsiveness to an acute administration of the β-adrenergceptor (βAR) agonist isoproterenol (ISO) is totally lacking both in vivo and in isolated atria.¹⁻⁷ These observations have led to a conclusion that β-adrenergceptor modulation in the TG4 mouse heart is saturated in the basal state because of a greater number of β₂ARs in the spontaneous active state (R* state) in the absence of agonists.¹⁻⁷

Because recent studies in other mammalian species have shown that physiological responses and signal transduction mechanisms of β₂AR subtype stimulation are distinctly dif-

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different from those of β2-adrenergic receptor (β2AR) stimulation,10–15 it is essential to characterize the individual βAR subtypes in murine heart for optimal genetic manipulation of their signaling pathways. However, a close inspection of the studies in murine models to date1–3,6–8 surprisingly reveals whether β2AR agonists can elicit a contractile response in murine myocardium has been a matter of debate. Specifically, in WT mice, the mixed βAR agonist ISO in the presence of the β2AR antagonist CGP 20712A (CGP) has virtually no positive inotropic effect.7 In addition, in β2AR knockout mice, the mixed βAR agonist ISO fails to increase cardiac contractility.10 Thus, it is possible that an inability of β2AR stimulation by agonists to increase contractility in mouse heart per se masquerades as the observed “saturation” of β2-adrenergic signaling in the TG4 mouse.

Our previous studies have shown that in native rat cardiac myocytes, pertussis toxin (PTX) pretreatment selectively potentiates the positive inotropic effect of β2AR but not β1AR stimulation, suggesting that β2AR dually couples to Gs and to PTX-sensitive inhibitory G proteins.10 If the coupling of β2AR-Gs protein in murine heart were highly efficient, it might be expected to completely negate the Gs-mediated positive inotropic effect. Thus, a strong coupling of β2AR, G proteins in murine heart may explain the apparent and mysterious loss of the positive inotropic effect of agonist-induced β2AR stimulation in both WT and TG4 mice.

Cardiac membranes were prepared by homogenizing WT and β2AR overexpressing transgenic mouse (TG4) ventricles in ice-cold lysis buffer (20 mmol/L Tris-HCl [pH 7.4], 250 mmol/L sucrose, 1 mmol/L EDTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 0.1 mmol/L PMSF). The samples were centrifuged at 10 000 g for 10 minutes at 4°C. The supernatant was centrifuged at 100 000 g for 2 hours at 4°C. The pellet was resuspended up to a final protein concentration of 4.5 to 5 mg/mL in a buffer containing (in mmol/L) CsCl 100, TEACl 20, NaCl 10, HEPES 10, MgATP 5, and EGTA 5; the pH was adjusted to 7.2 with CsOH. In some experiments, Rp-cAMPS (100 μmol/L), an inhibitory cAMP analog, was included in the patch pipette solution and dialyzed into the cell for more than 10 minutes, as previously described.11 The superfusion solution was the same as that used for cell length and [Ca2+]i transient measurements. ICa was elicited from a depolarization from −40 to 0 mV and measured as the difference between the peak inward current and the current at the end of a 300-ms pulse.

**Materials and Methods**

**Measurements of Cell Contraction and [Ca2+]i Transient**

Mouse ventricular myocytes were isolated from hearts of 2- to 3-month-old male transgenic mice overexpressing human β2AR (TG4) and WT littermates via a modified enzymatic technique.8 Cells were then perfused with HEPES buffer solution consisting of (in mmol/L) CaCl2 1.0, NaCl 137, KCl 5, dextrose 15, MgSO4 1.3, NaH2PO4 1.2, and HEPES 20 (pH 7.4) and were electrically stimulated at 0.5 Hz at 23°C. Cell length was monitored from the brightfield image of the cell by an optical edge-tracking method using a photodiode array (Reticon Model 1024 SAQ) with a 3-ms time resolution. Cell contraction was indexed by the percent reduction of cell length after electrical stimulation. In some experiments, cells were loaded with the fluorescent Ca2+ indicator Fluo-3 by incubation in 10 μmol/L Fluo-3 AM (Molecular Probes) for 10 minutes followed by a 20-minute wash.10 A laser scanning confocal microscope (Zeiss LSM410) was used to acquire fluorescence images every 2.09 ms along a line focused 5 to 10 μm into the cell. Both the Ca2+ signal and cell contraction were directly measured from the line-scan images using IDL (Research System) software. Criteria for viable mouse myocytes have been described in our previous study8 and include (1) a rod shape, (2) clearly defined sarcomeric striations, (3) a clear negative staircase after a rest period of ~1.0 minute, and (4) a stable steady-state contraction amplitude for at least 5 minutes before drug administration.

**Photolabeling of Membrane Proteins**

Cardiac membranes were prepared by homogenizing WT and β2AR overexpressing transgenic mouse (TG4) ventricles in ice-cold lysis buffer (20 mmol/L Tris-HCl [pH 7.4], 250 mmol/L sucrose, 1 mmol/L EDTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 0.1 mmol/L PMSF). The samples were centrifuged at 10 000 g for 10 minutes at 4°C. The supernatant was centrifuged at 100 000 g for 2 hours at 4°C. The pellet was resuspended up to a final protein concentration of 4.5 to 5 mg/mL in a buffer containing 20 mmol/L Tris-HCl (pH 7.4) and 1 mmol/L EDTA. Membranes were aliquoted and stored at −80°C.

[γ-32P]GTP-azidoalidilide ([γ-32P]GTP-Aza) was synthesized and purified according to the procedure described previously8 with some modifications. Briefly, 100 μL of 30 mg/mL 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (N-DEC) (Fluka, Buchs, Switzerland) solution in 0.15 mol/L MES (pH 5.5) and 2 to 3 mCi of lyophilized [γ-32P]GTP-azidoalidilide ([γ-32P]GTP-Aza) was mixed for 10 minutes at room temperature. Then 50 μL of 4-azidoalidilide (40 mg/mL in 1,4-dioxane) was added to this mixture and kept at 25°C to 28°C for 3 hours with constant mixing. The synthesized [γ-32P]GTP-Aza was purified on a C-18 Sep-Pak Cartridge (Waters) and dried on a Speed-Vac Centrifuge. The purity of the final product was >90%, checked by thin-layer chromatography on PEI cellulose with 1 mol/L LiCl. The dried [γ-32P]GTP-Aza was stored at −25°C. All synthesis procedures were performed in a dark room with red light illumination.

Membrane proteins (40 to 50 μg) were preincubated at 25°C for 10 minutes in 20 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 0.2 mmol/L EDTA, 1.0 mmol/L benzamidine, 2 mmol/L MgCl2, 1.0 mmol/L EDTA, and 50 mmol/L GDP to load G protein α subunits. βAR agonists or antagonists and 5 to 10 μCi of [γ-32P]GTP-Aza were then added to the samples and incubated for 4 minutes. The reaction was terminated by putting the samples on ice. All the subsequent procedures were performed at 4°C. After centrifugation (14 000 g for 10 minutes), membrane pellets were carefully resuspended in 50 μL of ice-cold buffer (20 mmol/L Tris-HCl [pH 7.4], 1 mmol/L EDTA, and 1 mmol/L dithiothreitol), transferred to individual dispemples in aluminum foil, and irradiated with a UV lamp (254 nm, 100 W) for 10 minutes at a distance of 10 cm. The irradiated samples were centrifuged at 14 000 g for 30 minutes.
Immunoprecipitation of G Protein α Subunits

Immunoprecipitation of G protein α subunits was performed as previously described. Pellets of photolabeled membranes were solubilized in 40 μL of 2% SDS (wt/vol) at room temperature. Precipitation buffer (103 μL) containing 1% (wt/vol) Triton X-100, 1% (wt/vol) deoxycholate, 0.5% (wt/vol) SDS, 150 mmol/L NaCl, 1 mmol/L diethiothreitol, 1 mmol/L EDTA, 0.2 mmol/L PMSF, 10 μg/mL aprotinin, and 10 mmol/L Tris-Cl (pH 7.4) was added, and the solubilized membranes were centrifuged at 14 000g for 5 minutes at 4°C. Antiserum (5 to 20 μL) was added to the supernatant. The samples were incubated overnight at 4°C under constant rotation. After adding washed protein A Sepharose beads, the samples were centrifuged at 14 000g for 5 minutes and washed with buffer A (1% [wt/vol] Igepal, 0.5% [wt/vol] SDS, 600 mmol/L NaCl, and 50 mmol/L Tris-HCl [pH 7.4]) and buffer B (300 mmol/L NaCl, 10 mmol/L EDTA, and 100 mmol/L Tris-Cl [pH 7.4]). The pellets of protein A Sepharose were dried with a Speed-Vac centrifuge. After a 15-minute incubation at room temperature, the samples were boiled for 10 minutes and centrifuged at 14 000g for 5 minutes. Thereafter, 20 μL of supernatants was subjected to SDS-PAGE electrophoresis according to Laemmli. The separating gel contained 9% acrylamide and 6 mol/L urea. Gels were stained with Coomassie blue. Photolabeled proteins were visualized by autoradiography.

PTX Treatment

For contraction, [Ca\(^{2+}\)], transient, and I\(_c\) measurements, aliquots of cells were incubated with PTX (1.5 μg/mL at 37°C for at least 3 hours), as previously described. PTX-treated cells were compared with nontreated control myocytes from the same heart that had been kept at 37°C in the absence of PTX for an equal time. After PTX treatment, both PTX-treated and nontreated cells were kept at room temperature for the rest of the experimental day (≥6 to 8 hours). For biochemical measurements, mice were injected with PTX (150 μg/kg IP) 24 hours before the isolation of the hearts.

Materials

CGP was kindly supplied by Ciba-Geigy Corp, Basel, Switzerland; ICI 118,551 (ICI) was kindly supplied by Imperial Chemical Industries, London, UK; and zinterol was kindly supplied by Bristol-Myers, Evansville, Ind. Antibodies recognizing the α subunits of G\(_i\) and G\(_s\) were obtained from Du Pont New England Nuclear (Wilmington, Del). The antibody recognizing the α subunits of G\(_i\) was obtained from Santa Cruz Biotechnology, Calif. In our experiments, the antibodies against G\(_i\) (from Santa Cruz) dominantly react with G\(_i\), because in most experiments, the molecular weight (MW) of the G\(_i\) antibody-precipitated proteins is slightly greater than that precipitated by the G\(_i\) antibodies, as expected. However, these antibodies may also slightly cross-react with G\(_i\). In some experiments, double bands are visible, but the lower MW band, which has the same MW as that of the proteins precipitated by G\(_i\) antibodies (Figure 8C and 8D), is always much lighter. In addition, the G\(_i\) antibody-precipitated proteins are mainly G\(_i\), even though this antibody may cross-react weakly with G\(_i\). The reason for this is that in our preliminary experiments, we have found that the abundance of G\(_i\) in murine myocardium is much lower than that of G\(_i\) and G\(_i\) and is difficult to detect by Western analysis (data not shown). Control peptides of the G\(_i\) antibody were obtained from Santa Cruz. PTX, forskolin, ISO, and norepinephrine (NE) were purchased from Sigma, St. Louis, Mo. Rp-cAMPs was purchased from Biolog Life Science Institute, La Jolla, Calif.

Statistics

Data reported are mean±SEM. Statistical comparisons were made by Student t test or paired t test when appropriate. Two-factor ANOVA was used to analyze the overall drug dose response. The significance between groups is analyzed by Bonferroni. A P value of <0.05 was considered to be statistically significant.

Results

Enhancement of Baseline Contractility of TG4 Ventricular Myocytes

In single ventricular myocytes isolated from TG4 or WT mice superfused with normal HEPES buffer solution with 1.0 mmol/L Ca\(^{2+}\), basal contractility was measured in the absence of any βAR agonists. Figure 1 shows that baseline contraction amplitude of TG4 cells was enhanced by 3.2-fold relative to myocytes isolated from WT mice. The enhanced baseline contractility was markedly reduced by ICI (5×10\(^{-7}\) mol/L), a βAR inverse agonist (a class of receptor ligands that preferentially bind to inactive receptor, therefore driving the equilibrium between R and R* to the inactive conformation R), whereas ICI alone had no significant effect on WT cell basal contraction (Figure 1). However, there was no significant difference in the resting cell length between these 2 groups (144.5±5.6 μm, n=28 for TG4 versus 141.0±4.18 μm, n=22 for WT), consistent with the previous observations that the heart size is not changed in this transgenic model.

βAR Agonists Fail to Increase Contractility of Either TG4 or WT Ventricular Myocytes

Despite the overwhelming expression (~200-fold of WT) of βARs in TG4 myocytes, the selective βAR agonist zinterol, even at a maximal concentration (10\(^{-5}\) mol/L), did not increase contraction amplitude (Figure 1). On the contrary, the [Ca\(^{2+}\)]\(_t\) transient (not shown). The inability of βAR stimulation to further increase contraction amplitude in TG4 myocytes might suggest that the βAR signaling to augment cell contractility in TG4 cells is already at the maximal level in the absence of agonist so that βAR agonists would not be expected to further increase the contraction amplitude in these cells, as proposed previously. Alternatively, the unresponsiveness of TG4 cells might be due to some compensatory alterations, eg, a reduction in G\(_s\)-adenyl cyclase signaling, or to a defect in excitation-contraction coupling machinery in these transgenic mice. The adenyl cyclase activator forskolin was used to test these possibilities. If the
contractility in TG4 heart cells were saturated at baseline, no positive inotropic effect would be observed after forskolin treatment. To the contrary, Figure 2B illustrates that forskolin (10^{-6} mol/L) markedly and reversibly enhanced contraction amplitude in a representative TG4 ventricular myocyte. On average, forskolin increased TG4 cellular contraction by 2.4-fold (from 6.1±1.0% to 14.4±1.0% of resting cell length, n=5 cells from 3 hearts; P<0.01). This result indicates that both the excitation-contraction machinery and the βAR signaling cascade downstream of the cyclase remain intact and are not saturated at baseline in TG4 mice. Therefore, we hypothesized that the unresponsiveness of TG4 ventricular myocytes to βAR stimulation likely results from an impairment within the proximal βAR signaling cascade.

To identify possible alterations of cardiac βAR signaling in TG4 mice, we next examined the effects of βAR stimulation in WT heart cells. Surprisingly, βAR stimulation by the mixed βAR agonist ISO (10^{-8} mol/L) plus the βAR blocker CGP was also unable to augment contraction amplitude in WT mouse ventricular myocytes (Figure 3). In contrast, the mixed βAR stimulation by ISO alone or βAR stimulation by ISO plus the βAR blocker ICI markedly enhanced contraction amplitude in these WT myocytes (Figure 3), consistent with previous in vivo observations that ISO has no significant effect on the contraction amplitude (n=5 to 8). *P<0.01 vs control and ISO+CGP groups.

**Figure 2.** Representative examples of contractile response to the selective β2AR agonist zinterol (ZINT) (A) or to the adenylyl cyclase stimulator forskolin (B) in TG4 ventricular myocytes. Each panel shows a typical continuous chart recording of cell contraction (top, upward deflection) and traces on an expanded time scale (bottom; contraction is plotted as downward deflection) obtained at time points as indicated. Note that zinterol at 10^{-6} mol/L failed to elicit a positive inotropic effect, whereas forskolin (10^{-6} mol/L) markedly enhanced the contraction amplitude in TG4 cardiomyocytes.

**Figure 3.** Isoproterenol (ISO)-induced contractile response in the absence and presence of the selective β2AR antagonist CGP 20712A (CGP, 10^{-8} mol/L) or the selective β1AR antagonist ICI 118,551 (ICI, 10^{-7} mol/L) in WT mouse ventricular myocytes. Note that even in WT myocytes, β2AR stimulation by ISO+CGP has no significant effect on the contraction amplitude (n=5 to 8).

**Rescue of Contractile and [Ca^{2+}]_i Responses to β2AR Stimulation by PTX Treatment**

Because reconstituted βARs can couple to both G_s and G_i in artificial systems, and in rat ventricular myocytes, PTX treatment selectively potentiates the β2AR-mediated contractile response. We hypothesized that a dual coupling of β2AR to an inhibitory G protein in addition to a G_s protein might also exist in intact mouse ventricular myocytes and negate the contractile response mediated by the coupling to a G_s protein. To test this hypothesis, cells were incubated with PTX to abrogate G_s/G_i function via ADP ribosylation. Indeed, PTX pretreatment unmasked a potent positive inotropic effect after β2AR stimulation in both TG4 and WT heart cells, as shown in Figure 4A and 4B, in a representative PTX-treated TG4 and WT ventricular myocyte, respectively. The zinterol-induced (10^{-8} mol/L) increase in contraction amplitude was completely abolished by the specific β2AR antagonist ICI (10^{-7} mol/L). In contrast, the β2AR antagonist CGP (10^{-8} mol/L) could not reverse the positive inotropic effect of zinterol (Figure 4C) but completely blocked the increase in contraction induced by the β2AR agonist NE (10^{-7} mol/L) (Figure 4D). These results indicate that the PTX-responded
The contractile response to zinterol is mediated by β2AR stimulation. The average dose response of contraction amplitude to the β2AR agonist zinterol is shown in Figure 5A and 5B for WT and TG4 cells, respectively. It is noteworthy that similar maximal contraction amplitude (~15% of resting cell length) is obtained after zinterol in both PTX-treated WT and TG4 cells. Also note that the dose-response curve for PTX-treated TG4 cells is shifted leftward relative to that for WT cells (EC50 is ~1.5×10^{-6} and 10^{-7} mol/L for TG4 and WT groups, respectively), consistent with the greater β2AR density in TG4 cells. In addition, Figure 6 shows that the positive inotropic effect of zinterol in both PTX-treated TG4 and WT cells was accompanied by an increase in the [Ca^{2+}]i transient as indexed by the increase in the fluorescence signal of the Ca^{2+}-sensitive probe Fluo-3. Thus, the full efficacy of β2AR stimulation is revealed in TG4 as well as in WT mouse cardiac cells only if cells were pretreated with PTX to eliminate the Gs-mediated inhibitory signaling.

Figure 5. Dose response of contraction amplitude to the β2AR agonist zinterol in WT (A) and TG4 (B) mouse ventricular myocytes with and without PTX treatment. Each cell was superfused with a single concentration of the β2AR agonist zinterol. All measurements were obtained under steady-state conditions after 10 minutes of exposure to zinterol and are presented as mean±SEM (at each concentration, n=5 to 6). The overall drug effect (in both PTX-treated WT and TG4 cells) is significant at P<0.001 (by 2-way ANOVA). There is a significant difference between +PTX and −PTX groups (by Bonferroni, P<0.001 in both WT and TG4 groups).

Rp-cAMPS Reversed the PTX-Rescued I_{Ca} Response to β2AR Stimulation

Because I_{Ca} is the key factor of the β2AR-mediated positive inotropic effect in rat and canine myocytes,10–12,14 we next measured the I_{Ca} response of mouse cardiomocytes to β2AR stimulation. Similar to the contractile response, in the absence of PTX treatment, the β2AR agonist zinterol (10^{-5} mol/L) could not increase I_{Ca} in WT or TG4 myocytes (data not shown). However, in PTX-treated cells, zinterol significantly enhanced the current amplitude in both WT and TG4 mice (Figure 7, left panels). The PTX-restored stimulatory effect of zinterol on I_{Ca} was completely abolished by a specific cAMP-dependent protein kinase A (PKA) inhibitor, an inhibitory cAMP analog Rp-cAMPS (Figure 7, right panels), consistent with previous observations in other species.11 Similar results were obtained from the other 4 WT and TG myocytes. These results suggest that the PTX-rescued murine cardiac β2AR function is mediated by a cAMP-dependent signaling pathway.

β2AR Stimulation Selectively Increases Gs Activation

The PTX sensitivity of the β2AR effect in murine (Figure 5) and rat hearts10,11,24 suggest that cardiac β2AR couples to Gs proteins. However, these results neither prove a direct interaction of β2AR and Gs proteins nor identify which specific G proteins couple to β2AR. Theoretically, the effect of PTX could be the consequence of disruption of tonic inhibitory actions of G proteins. To determine the interaction of β2AR and Gs proteins directly and to identify which specific PTX-sensitive G proteins are involved, we measured the G protein activation by photoaffinity labeling α subunits of G proteins with the photoreactive GTP analog [γ-32P]GTP-AzA.17 As the binding of an agonist to G protein-coupled receptors increases the rate of exchange of GTP for GDP on G protein α subunits (see Reference 25 for a review), the magnitude to which [γ-32P]GTP-AzA incorporates into α subunits of G proteins affords a direct assessment of G protein activation in response to receptor stimulation. Subse-
The absence (con, thin line) and presence (thick line) of the $\beta_2$ muscarinic acetylcholine receptor agonist carbachol (10$^{-8}$ mol/L) increased the incorporation of $[\gamma^32P]GTP$ into subunits of $G_{i2}$ and $G_{i3}$ to 146.3 $\pm$ 8.8% of control ($P<0.01$, n = 12) and 148.9 $\pm$ 5.6% of control ($P<0.01$, n = 13), respectively, in TG4 cardiomyocytes (Figure 9A). Similar results were obtained from WT mouse myocardium (Figure 9B). The magnitude of the $\beta_2$AR-induced increases in the $\alpha$ subunits of $G_{i2}$ and $G_{i3}$ photolabeling is similar to that induced by the muscarinic acetylcholine receptor agonist carbachol (10$^{-3}$ mol/L) (Figures 8A and 9B). The stimulatory effect of the $\beta_2$AR agonist was specifically and significantly abolished by the $\beta_2$AR antagonist ICI (Figures 8C, 8D, and 9A). Furthermore, the nonselective $\beta_2$AR agonist ISO also clearly enhanced the incorporation of $[\gamma^32P]GTP$ into $\alpha$ subunits of both $G_{i2}$ and $G_{i3}$ (Figures 8E and 9A), and this activation was specifically abolished by the $\beta_2$AR antagonist ICI but not by the selective $\beta_2$AR antagonist CGP (Figures 8E and 9A). Similarly, the $\beta_2$AR agonist NE (10$^{-6}$ mol/L) had no significant effects on $G_i$ activation (Figure 9B). Thus, the $G_i$ coupling is specific for $\beta_2$AR in both WT and TG4 myocardium. Finally, the $\beta_2$AR-stimulated $G_i$ activation was prevented by PTX treatment in both TG4 and WT mice (Figures 8F and 9B). Taken together, the present biochemical data, in conjunction with the physiological data described above, provide direct and compelling evidence that $\beta_2$ARs but not $\beta_1$ARs in native myocardium couple to PTX-sensitive $G$ proteins $G_{i2}$ and $G_{i3}$.

**Discussion**

**Concurrent Coupling of $\beta_2$AR to $G_i$ Proteins Negates the $\beta_2$AR-$G_i$-Mediated Contractile Response**

Using a photoaffinity labeling technique in conjunction with specific antibodies of different $G$ proteins, we found that $\beta_2$AR stimulation increases activation of $G_i$ proteins $G_{i2}$ and $G_{i3}$ (Figures 8 and 9), in a PTX- and ICI-sensitive manner, in addition to activation of $G_s$. These data provide the first direct biochemical evidence that $\beta_2$ARs in the native cellular environments can interact with PTX-sensitive $G$ proteins, specifically, $G_{i2}$ and $G_{i3}$. Physiological data showed that the concurrent coupling to $G_i$ proteins completely negates the $\beta_2$AR-$G_i$-mediated contractile, $[Ca^{2+}]_i$, and $I_{Na}$ responses. As a result, $\beta_2$ARs in murine hearts appear to be at an apparently dormant state in terms of these cardiac responses. More importantly, the cardiac $\beta_2$AR function was fully restored after inhibiting $G_i$ by PTX, as manifested by the robust effects of the $\beta_2$AR agonist zinterol to augment $[Ca^{2+}]_i$, $I_{Na}$, and contraction amplitudes in both PTX-treated TG4 and WT cells. An examination of the dose responses of contraction with and without PTX treatment in both TG4 and WT cells.
myocytes further reveals that the β2-AR-Gi coupling completely negates the Gi-mediated contractile response over a wide range of receptor densities and agonist concentrations. Thus, the concurrent coupling of β2-ARs to Gi proteins provides an explanation for the “mysterious” loss of agonist-induced β2-AR contractile response in TG4 and WT murine hearts. In addition, similar reasoning may also be applicable to explain the unresponsiveness of cardiac contractility to β2-AR stimulation in the β2-AR “knockout” mouse model as well as in myocardium of other mammalian species (e.g., guinea pig), in which β2-ARs are present but nonfunctional in terms of cardiac contractile modulation.

Figure 8. Effects of β2-AR stimulation by zinterol or isoproterenol plus the β2-AR blocker CGP 20712A on the incorporation of the photoreactive GTP analog [γ-32P]GTP-AzA into α subunits of Gi proteins. Membranes prepared from WT (A and B) or TG4 (C through F) mouse ventricles were photolabeled with [γ-32P]GTP-AzA. Photolabeled α subunits were subsequently incubated with Gα3 or Gα2 polyclonal antibodies (anti-rabbit) and immunoprecipitated. Precipitated proteins were subjected to SDS-PAGE. Activated G protein α subunits were visualized by autoradiography. A, Stimulatory effect of zinterol (Z) at 10−6 mol/L and the acetylcholine receptor agonist carbachol (cch, 10−6 mol/L) on the incorporation of [γ-32P]GTP-AzA into α subunits of Gα3. B, Photolabeling of α subunits of Gα3 in the absence and presence of zinterol (Z). The right 2 lanes show that control peptides of Gα3 antibody completely abolished the Gα3 antibody-induced protein precipitation. C and D, Photolabeling of α subunits of Gα3 and Gα2 in response to zinterol (Z) in the absence or presence of the β2-AR blocker ICI 118,551 (I) at 10−6 mol/L. E, Stimulatory effect of isoproterenol (IS) at 10−6 mol/L in the presence or absence of a selective β2-AR antagonist ICI (10−6 mol/L) or β2-AR blocker CGP (10−6 mol/L) on the photolabeling of α subunits of Gα3. F, PTX treatment prevents the stimulatory effect of zinterol (Z) on Gα2 activation. In addition, in all experiments, preimmune antisera did not precipitate any photolabeled proteins, indicating that precipitation with the antisera used in the present study was specific (data not shown). Molecular masses (kDa) of standard proteins are shown on the left.

Figure 9. Mean data of G protein photolabeling. Data are presented as percent of control (mean±SEM). A, Data obtained from TG4 mice. *P<0.01, †P<0.05 vs control; n=12 and 13 for Gi3 (ZINT) and Gi1 (ZINT), respectively; n=3 to 5 for all other groups. B, Data from WT mice. *P<0.01 vs control; n=8 and 16 for Gi3 (ZINT) and Gi1 (ZINT), respectively; n=3 to 5 for all other groups.

PTX-Rescued Murine Cardiac β2-AR Function Requires cAMP-Dependent PKA Activation

There is plenty of evidence indicating a coupling of β2-AR to adenyl cyclase to increase cAMP. In the present study and previous studies, the specific cAMP inhibitory analog Rp-cAMPS prevented the β2-AR-stimulated increase in Iκs in PTX-treated TG4 and WT myocytes (Figure 7) and in rat heart cells (with or without PTX), indicating that cAMP-dependent PKA activation is obligatory for mammalian β2-AR cardiac responses. Thus, the positive inotropic effects induced by both β2-AR and β3-AR subtypes are mediated by cAMP-PKA signaling pathways. However, β2-AR does not behave the same way as β3-AR with respect to coupling to Gi proteins under our experimental conditions (Figures 8 and 9). Similarly, our previous studies have shown that in rat ventricular myocytes, PTX pretreatment selectively enhances the positive inotropic effect of β2-AR stimulation, suggesting that PTX-sensitive Gi proteins specifically interact with β2-AR but not β3-AR. Therefore, the essential difference between β2-AR and β3-AR-mediated cardiac responses is largely due to the additional coupling of β2-AR to Gi proteins, which provides a negative feedback to the β2-AR-stimulated cAMP-dependent signaling.

Dual Coupling of β2-AR to Gi Proteins Mediates the Difference Between βAR Subtypes and the Species-Dependent Diversity in Cardiac β2-AR Responses

The coupling of β2-AR to Gi proteins is not unique to native murine β2-AR or to human β2-AR surrogated in mouse cardiac myocytes. Our previous studies have shown that although stimulation of both β2-AR and β3-AR increases the contraction amplitude in rat and canine ventricular myocytes, numerous
differences have been noted. Specifically, the \( \beta_2 \)-AR-stimulated positive inotropic effect and increase in cytosolic Ca\(^{2+} \) transient are dissociated from cAMP production and occur without increasing phosphorylation of cytoplasmic proteins, eg, the sarcoplasmic reticulum membrane protein phospholamban.\(^{1,13,14} \) Interestingly, in rat myocytes, PTX treatment not only potentiates the positive inotropic effect of \( \beta_2 \)-AR stimulation,\(^{15} \) it also largely reverses the differences between \( \beta_1 \)-AR and \( \beta_2 \)-AR.\(^{15} \) indicating \( \beta_2 \)-AR-activated G\(_i\) proteins play a key role in the differential cardiac response to \( \beta_2 \)-AR versus \( \beta_1 \)-AR subtype stimulation. A similar potentiating effect of PTX on \( \beta_2 \)-AR contractile response has also been observed in normal canine ventricular myocytes (Zhou et al, unpublished data, 1998). These results reinforce the idea that the concurrent coupling of \( \beta_2 \)-AR to functionally opposing G proteins is a universal phenomenon in mammalian hearts.

In murine cardiomyocytes, PTX permits a de novo contractile response (Figure 5). In contrast to mice, PTX pretreatment only augments an already extant positive \( \beta_2 \)-AR contractile response in other species examined.\(^{10,11,24} \) This diversity in cardiac \( \beta_2 \)-AR stimulation among species or within species under different circumstances may be largely accounted for on the basis of quantitative differences in the extent of \( \beta_2 \)-AR-G\(_i\) coupling. For example, the \( \beta_2 \)-AR-G\(_i\) coupling would be expected to be extremely robust in mouse heart, as manifested by the absence of a \( \beta_2 \)-AR-mediated positive inotropic effect without PTX pretreatment. In rat, an augmentation of the extent of \( \beta_2 \)-AR coupling to G\(_i\) proteins during development could explain the greater sensitivity of neonatal than that of adult heart cells to \( \beta_2 \)-AR activation in the absence of PTX.\(^{15} \) The difference between \( \beta_1 \)-AR and \( \beta_2 \)-AR in their G protein coupling profiles may also provide new insight for understanding the role of \( \beta \)AR subtypes in health and diseased mammalian heart (References 12 through 15; also see subsequent sections).

### Peculiar Features of TG4 Myocytes

The results of the present study show that TG4 mouse ventricular myocytes overexpressing human \( \beta_2 \)-AR exhibit a markedly enhanced baseline contractility, which can be reversed by the inverse \( \beta_2 \)-AR agonist ICI (Figure 1). Because our experiments were conducted in superfused single, isolated ventricular myocytes, possible endogenous catecholamine contamination, which might complicate the interpretation of observations of previous studies in vivo and in isolated atria,\(^{1,7,21} \) can be completely ruled out. Thus, the results of the present study confirm and extend previous studies and provide evidence at the single cell level for the functional existence of spontaneous active \( \beta_2 \)ARs in TG4 mice. Conceptually, a small fraction of receptors undergoes spontaneous transition to an active state (R*) at any time, even in the absence of agonist.\(^{7} \) The \( \sim200\)-fold overexpression of the \( \beta_2 \)-AR in TG4 hearts results in more receptors in the R* state, which constitutively increase basal adenyl cyclase activity\(^{1,7} \) and baseline cellular contractility (Figure 1).

The results of the present study also show that the inability of \( \beta_1 \)-AR agonists to augment the contractility of TG4 cardiomyocytes cannot be explained by a saturation of contractility at baseline. The reason for this is that the adenyl cyclase activator forskolin can further increase contraction amplitude over the enhanced basal contraction, indicating that the cAMP-PKA signaling is still capable of modulating the contractility of TG4 heart cells. More importantly, the lack of \( \beta_2 \)-AR positive inotropic effect was also observed in WT mouse myocytes, indicating that the null contractile response to \( \beta_2 \)-AR agonists has nothing to do with the receptor overexpression or chronic spontaneous \( \beta_2 \)-AR activation in the transgenic model, but it is a fundamental property of \( \beta_2 \)-AR signaling in murine heart. In addition, the results of the present study indicate that the \( \beta_2 \)-AR-G\(_i\) coupling is retained over a wide range of \( \beta_2 \)-AR densities and agonist concentrations.

Another peculiar feature of TG4 hearts is an absence of contractile response to \( \beta_1 \)-AR stimulation, as manifested by the inability of \( \beta_1 \)-AR stimulation by NE or ISO in vivo\(^{7,21} \) or the inability of ISO plus the \( \beta_2 \)-AR blocker ICI to increase cardiac contractility in single isolated myocytes (data not shown). Although PTX treatment fully rescued the \( \beta_2 \)-AR responsiveness in TG4 cardiomyocytes, it was not able to rescue the lost cardiac response to \( \beta_2 \)-AR stimulation in these transgenic mice (data not shown). This is consistent with the observation that \( \beta_2 \)-AR does not couple to G\(_i\) proteins (Figures 8 and 9). These results suggest that a different mechanism might be involved in the subsensitivity of TG4 hearts to \( \beta_2 \)-AR stimulation (eg, desensitization of the receptor via an enhanced basal PKA-dependent receptor phosphorylation or by the \( \beta \)-adrenergic receptor kinase \( \beta \)ARK\(^{2,51,52} \)).

#### Do Spontaneous Active \( \beta_2 \)ARs Differ From Ligand-Stimulated \( \beta_2 \)ARs?

According to the current “two-state” model of receptor theory,\(^{7} \) receptors exist in equilibrium of an inactive state (R) and an active state (R*) in terms of the ability to interact with G proteins. This model predicts that spontaneous active receptors should be identical to ligand-stimulated active receptor species (LR*), given that there is a sole active state. The results of the present study, however, provide several lines of evidence to suggest that spontaneously activated \( \beta_2 \)-AR may differ from the ligand-stimulated \( \beta_2 \)-AR. First, whereas spontaneous active \( \beta_2 \)ARs in TG4 heart, presumably only a small fraction of total receptor population,\(^{1} \) increased the cell contractility by about 3-fold, \( \beta_2 \)-AR agonists, at maximal concentrations that would be expected to occupy a large quantity of the excessive \( \beta_2 \)ARs in TG4 cells, were unable to further increase contraction amplitude, even though the cell contractility and \( \beta_2 \)-AR-cAMP signaling are not saturated. Second, PTX treatment only slightly potentiated the basal contractility (in TG4 cells only) but had a disproportionally large potentiating effect on the agonist-stimulated contractile response in both TG4 and WT heart cells (Figure 5), suggesting that the spontaneously activated \( \beta_2 \)-AR, unlike the agonist activated \( \beta_2 \)-AR, only weakly couples or does not couple to G\(_i\) proteins. In this respect, recent studies in transgenic mice with high or medium overexpression of cardiac \( \beta_2 \)-AR have demonstrated that spontaneously activated \( \beta_2 \)ARs coprecipitate with G\(_i\), but not G\(_j\), proteins in the absence of agonist.\(^{33} \) Our preliminary data have also consis-
requently shown that the β2AR inverse agonist ICI (5×10⁻⁷ mol/L) reduced the basal incorporation of [γ⁻³²P]GTP-Aza into α subunits of G, but not G, proteins in TG4 mice (Avdonin et al, unpublished data, 1998). Taken together, we suggest that spontaneous active β2ARs are predominantly coupled to Gi with little or no coupling to G, proteins, whereas the ligand-activated β2ARs couple to both G, and G, proteins. The distinct difference between the spontaneous and ligand-induced active β2ARs demonstrated in the present study and in previous studies of murine myocardium requires a reformulation of the current model⁷ to describe receptor–G protein coupling in the physiological context.

Implications of β2AR-Gi Coupling in the Heart

In addition to modulating the β2AR-G,-mediated enhancement in cardiac contractility, the β2AR-stimulated Gi activation might have chronic effects, eg, cellular metabolism or excitability or cell growth, which requires additional investigations. In this regard, it is intriguing that β2AR overexpression in TG4 mice is not associated with a cardiac or cellular hypertrophy¹ and exhibits no change in the size of single isolated cardiomyocytes (as shown in the present study), whereas a genetic manipulation of G,-cAMP signaling system³ or chronic Gi stimulation by agonists³⁴,³⁵ is often associated with cardiac hypertrophy or heart failure. Thus, we speculate that Gi subtypes may differentially regulate cell growth as a result of the additional β2AR-Gi coupling. In addition, it has been shown that inhibition of Gi function by PTX treatment increases the occurrence of spontaneous cell contractions in rat ventricular myocytes¹⁰ and arrhythmia in intact rats (Eschenhagen et al, unpublished data, 1998) during Gi agonist stimulation. Thus, an activation of the β2AR-coupled Gi proteins may have some cardiac protective functions.

The demonstration that β2AR couples to Gi, and Gi, also provides new insights for the pathogenesis of heart failure. It is generally acknowledged that heart failure in human and animal models is characterized by a deterioration in cardiac contractility and a reduced catecholamine responsiveness, which are associated with an increase in Gi mRNA levels,³⁶ Gi activity as indicated by PTX-induced ribosylation³⁷ or Gi, protein amount in human⁸ or in animal models⁹ and an increase in the ratio of β2AR to β2AR as a result of a selective downregulation of β2ARs.⁴⁰–⁴² It has been proposed that the upregulation of Gi proteins may contribute to the suppressed β2AR, particularly β2AR, contractile response in the failing hearts.³⁶–³⁹ However, this hypothesis has not been directly examined, because most previous studies failed to determine whether the increased Gi activity differentially affected β2AR subtype signaling. On the basis of biochemical and physiological evidence for a coupling of β2AR to Gi, proteins (References 10 and 11 and the present study), it is possible that, on one hand, the upregulation of Gi, proteins could protect the diseased heart from Ca²⁺ overloading and arrhythmia; and on the other hand, the upregulated Gi, signaling in failing hearts could offset or mask the β2AR-stimulated positive inotropic effect, resulting in an attenuation or loss of the overall β2AR-mediated inotropic response. Additional studies are required to test these provocative hypotheses.

In summary, we demonstrate that β2AR stimulation cannot augment contractile function in isolated single WT or receptor overexpression transgenic (TG4) cardiac myocytes, although spontaneous β2AR activation enhances the baseline contractility of TG4 myocytes. We also provide the first biochemical evidence that in murine cardiac myocytes, β2AR is dually coupled to inhibitory G proteins Gi₂ and Gi₃ in addition to Gi. PTX treatment permits β2AR stimulation to induce a robust augmentation in contraction, associated with an increase in IC₅₀ and [Ca²⁺], transient. The PTX-restored cardiac β2AR response can be reversed by the inhibitory cAMP analog Rp-cAMPS. Thus, the β2AR-coupled Gi pathway exerts a strong negative feedback to the β2AR-mediated, cAMP-dependent cardiac contractile and [Ca²⁺] transient and IC₅₀ responses. These findings may have important implications not only for understanding signaling mechanisms and functionality of cardiac βAR subtypes but also for devising future strategies for the treatment of human heart failure via genetic therapy.

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