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The Third Intracellular Loop and the Carboxyl Terminus of β_2-Adrenergic Receptor Confer Spontaneous Activity of the Receptor

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ABSTRACT

It is well established that the β_2-adrenergic receptor (β_2-AR) exhibits a robust ligand-independent activity, whereas this property is considerably weaker in the closely related β_1-AR subtype. To identify the potential domain(s) of β_2-AR responsible for the spontaneous receptor activation, we created three chimeras in which the third intracellular loop ($\beta_1/\beta_{2\text{-Li3}}$) or the carboxyl terminus ($\beta_1/\beta_{2\text{-CT}}$) or both domains ($\beta_1/\beta_{2\text{-Li3CT}}$) of β_1-AR are replaced by the corresponding parts of the β_2-AR. Using adenoviral gene transfer, we individually expressed these β_1/β_2-AR chimeras in mouse cardiomyocytes lacking both native β_1-AR and β_2-AR (β_1/β_2 double knockout), and examined their possible spontaneous activities. Overexpression of these β_1/β_2-AR chimeras markedly elevated basal cAMP accumulation and myocyte contractility in the absence of agonist stimulation compared with those infected by a control adenovirus expressing β-galactosidase or an adenovirus expressing wild type β_1-AR. These effects were fully reversed by a β_2-AR inverse agonist, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol (ICI 118,551; 5×10^{-7} M), regardless of inhibition of G_i with pertussis toxin, but not by a panel of β_1-AR antagonists, including [2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluormethyl-2-imidazolyl)-phenoxy]-2-propanolmethanesulfonate (CGP20712A), betaxolol, bisoprolol, and metoprolol. Furthermore, we have shown that the C-terminal postsynaptic density 95/disc-large/ZO-1 (PDZ) motif of β_1-AR is not responsible for the lack of β_1-AR spontaneous activation, although it has been known that the β_1-AR PDZ motif prevents the receptor from undergoing agonist-induced trafficking and G_i coupling in cardiomyocytes. Taken together, the present results indicate that both the third intracellular loop and the C terminus are involved in β_2-AR spontaneous activation and that either domain seems to be sufficient to confer the receptor spontaneous activity.

β-ARs are prototypical members of G protein-coupled receptor (GPCR) superfamily, which shares a common overall structure feature: the seven hydrophobic transmembrane helical domains, an extracellular N terminus, and an intracellular C terminus. Stimulation of β-ARs by catecholamines activates the classic Gs-adenylyl cyclase-cAMP-protein kinase A (PKA) signaling pathway, which, in turn, regulates multiple cellular processes, including metabolic regulation, muscle contraction, cell growth, and cell death (Xiao, 2001). In the heart, β-AR stimulation enhances the force and rate of myocardial contraction and relaxation in response to stress or exercise, allowing the heart to increase its output by severalfold within seconds.

According to the ternary complex model and the cubic ternary complex model, GPCRs, including β-ARs, exist in an equilibrium between two functionally and conformationally distinct states: an inactive conformation (R) and an active conformation capable of activating G proteins (R*) (Samama et al., 1993; Bond et al., 1995; Neilan et al., 1999). In the absence of a receptor ligand, the receptor can undergo a spontaneous transition to the active state; the equilibrium between R and R* sets the level of basal receptor activation. Thus, an overexpression of a given receptor would be expected to proportionally increase the number of R* state receptors.

K.C. and Y.X. contributed equally to this study.

ABBREVIATIONS: β-AR, β-adrenergic receptor; GPCR, G protein-coupled receptor; PKA, protein kinase A; DKO, double knockout; WT, wild type; ICI 118,551, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol; CGP20712A, [2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluormethyl-2-imidazolyl)-phenoxy]-2-propanolmethanesulfonate; m.o.i., multiplicity of infection; MEM, minimal essential medium; FBS, fetal bovine serum; ICYP, iodocyanopindolol; PBS, phosphate-buffered saline; β-gal, β-galactosidase; ISO, isoproterenol; PTX, pertussis toxin; Adv, adenovirus.

It has been shown that both β_1- and β_2-AR antagonists elicit a negative inotropic effect in rat and human myocardium (Varma et al., 1999; Maack et al., 2000) and that transgenic mice with heart-specific overexpression of β_1-AR experiences increased heart rate despite unaltered basal cAMP and cardiac contractility (Engelhardt et al., 2001). These previous studies suggest that both β-AR subtypes might exhibit spontaneous activation. However, the interpretation of these studies might be complicated by the presence of the endogenous β_1-AR agonist norepinephrine, released from myocardial nervous endings.

Increasing evidence has shown that myocardial β_2-AR exhibits robust spontaneous activation in both in vivo and in vitro, whereas cardiac β_1-AR has little or considerably lower spontaneous activity in the same experimental settings. Specifically, in transgenic mice, cardiac-specific overexpression of the human β_2-AR overtly increases basal cardiac adenylyl cyclase activity, cAMP accumulation, and cardiac contractility in the absence of agonist stimulation (Milano et al., 1994; Bond et al., 1995; Xiao et al., 1999; Zhou et al., 1999a,b; Gong et al., 2000; Liggett et al., 2000; for review, see Rockman et al., 2002). Similar results have been reproduced with adenoviral gene transfer in cultured adult mouse cardiac myocytes (Zhang et al., 2000; Zhou et al., 2000b). In contrast, there is not detectable spontaneous β_1-AR activation under the same experimental conditions with respect to the same readouts (Engelhardt et al., 1999; Zhou et al., 2000b). Recent studies have also demonstrated that β_2-AR spontaneous activity is considerably greater (~5 times) than that of β_1-AR under the same experimental conditions (Engelhardt et al., 2001). Thus, compared with β_2-AR, β_1-AR exhibits either rather weak or no spontaneous activity depending on the endpoints examined in different studies.

Based on studies on chimeric or mutated GPCRs, it has been shown that the third intracellular loop that connects transmembrane domains V and VI of these receptors is an important determinant for G protein coupling (Kobilka et al., 1988; O'Dowd et al., 1988; Wong et al., 1990). The closely related dopamine receptor subtypes 1A and 1B exhibit strikingly different spontaneous activity, but a point mutation in the third intracellular loop completely abolishes this difference (Tiberi and Caron, 1994; Charpentier et al., 1996). This suggests that the third intracellular loop of certain GPCRs may play an important role not only in agonist-induced G protein coupling, but also in spontaneous receptor activation. Because the amino acid sequence of the third intracellular loop of β_1-AR markedly differs from that of β_2-AR (Green et al., 1992; Green and Liggett, 1994), we hypothesized that this difference might contribute to the distinctly different spontaneous activity of β_1-AR versus that of β_2-AR.

In addition to the third intracellular loop, the C terminus of β_2-AR seems to be critical in determining the efficiency and specificity of G protein coupling (O'Dowd et al., 1988, 1989). A β_2-AR mutant lacking this region exhibits intact ligand binding but impaired adenylyl cyclase response to agonist stimulation (O'Dowd et al., 1989). Furthermore, recent studies have shown that the C-terminal PDZ motif of β_1-AR contributes to the lack of the receptor trafficking and its G_i coupling in response to agonist stimulation (Xiang et al., 2002). Thus, we hypothesize that the difference between β_1-AR and β_2-AR in their spontaneous activation might be attributable to the differences in their third intracellular loop or in their C-termini, particularly the PDZ motif of β_1-AR.

In the present study, we created three $\beta_{1\beta2}$-AR chimeras to examine the potential roles of the third intracellular loop and the C terminus of β_2-AR in the receptor spontaneous activation. In addition, we created a β_1-AR–PDZ mutant in which the PDZ motif Glu-Ser-Lys-Val was mutated into Glu-Ala-Ala-Ala (Xiang et al., 2002) to explore the possibility that the lack of β_1-AR spontaneous activity might be caused by the C-terminal PDZ motif of the receptor. To avoid complicated interactions of native β-ARs with the β_1-AR–PDZ mutant or with the chimeras, we individually expressed the β_1/β_2 chimeras or the β_1-AR–PDZ mutant in myocytes from β_1/β_2-AR double knockout (DKO) mice (Rohrer et al., 1999), using adult mouse myocyte culture and adenoviral gene transfer techniques (Zhou et al., 2000a). We found that overexpression of those chimeras, similar to overexpression of wild-type (WT) β_2-AR, enhances spontaneous receptor activation, which is fully reversed by a β_2-AR inverse agonist, ICI 118,551 (5×10^{-7} M), but not by β_1-AR antagonists such as CGP20712A (3×10^{-7} M), betaxolol (10^{-6} M), bisoprolol (10^{-6} M), and metoprolol (10^{-6} M). In contrast, the β_1-AR–PDZ mutant does not exhibit spontaneous activity, although the mutant receptor undergoes internalization and G_i coupling, as is the case for WT β_2-AR (Xiang et al., 2002). The present results suggest that either the third intracellular loop or the C terminus of β_2-AR is sufficient to induce the receptor spontaneous activation, whereas the C-terminal PDZ motif of β_1-AR is not responsible for the relative lack of β_1-AR spontaneous activity.

Experimental Procedures

Adenoviral Constructs. Replication-defective adenoviruses encoding β_1/β_2-AR chimeras or a β_1-AR–PDZ domain mutant where the β_1-AR carboxyl-terminal PDZ motif Glu-Ser-Lys-Val was mutated into Glu-Ala-Ala-Ala (Xiang et al., 2002) or WT β-AR subtypes were constructed. Briefly, the cDNA encoding β_1/β_2-AR chimeras (Fig. 1), including β_1/β_{2Li3} (in which the third intracellular loop of β_1-AR is replaced by that of β_2-AR), β_1/β_{2CT} (in which the C terminus of β_1-AR is replaced by the counterpart of β_2-AR), $\beta1/\beta_{2Li3CT}$ (in which both the third loop and the C-terminal domain of β_1-AR are replaced by those of β_2-AR), the β_1-AR–PDZ mutant, and WT mouse β_1- and β_2-AR were inserted into the E1 region of the adenoviral genome by homologous recombination. Standard viral amplification and CsCl purification methods were used to amplify and purify adenoviruses encoding β_1/β_2-AR chimeras or the WT β_1-AR or β_2-AR. The multiplicity of viral infection (m.o.i.) for each virus was determined by dilution assay in human embryonic kidney 293 cells.

Myocyte Isolation, Culture, and Adenoviral Infection. Single mouse cardiac myocytes were isolated from 2~3-month-old $\beta_1\beta_2$-AR DKO mice with an enzymatic technique (Zhou et al., 2000a). Cells were then cultured and infected with adenoviral vectors, as described previously (Zhou et al., 2000a). Before culture, myocytes were washed three times with minimal essential medium (MEM) containing 1.2 mM Ca^{2+}, 2.5% fetal bovine serum (FBS), and 1% penicillin-streptomycin and then plated with the same medium in the culture dishes precoated with 10 μg/ml mouse laminin. Adenovirus-mediated gene transfer was implemented by adding a minimal volume of the FBS-free MEM containing an appropriate titer of gene-carrying adenovirus. The full volume of FBS-free MEM was supplied after culture for another 1 to 2 h. All experiments were performed after 24 h of adenoviral infection.

Measurement of Cell Contraction. Cells were placed on the stage of an inverted microscope (model IM-35; Zeiss, Thornwood,

NY), electrically stimulated at 0.5 Hz at 23°C, and perfused with HEPES-buffered solution consisting of 1 mM $CaCl_2$, 137 mM NaCl, 5.4 mM KCl, 15 mM dextrose, 1.3 mM $MgSO_4$, 1.2 mM NaH_2PO_4, and 20 mM HEPES, pH adjusted to 7.4 with NaOH. Cell length was monitored from the bright-field image by an optical edge-tracking method using a photodiode array (model 1024 SAQ; Reticon, Boston, MA) with a 3-ms time resolution (Spurgeon et al., 1990). In a subset of experiments, cells were incubated with PTX (1.5 μg/ml at 37°C for at least 3 h). PTX-treated cells were compared with nontreated myocytes from the same heart that had been kept at 37°C in the absence of PTX for an equal time. Both PTX-treated and nontreated cells were then kept at room temperature for the rest of the experimental day (for 6~8 h).

Radioligand-Binding Assay. Twenty-four hours after adenoviral infection, cardiac myocytes were harvested in lysis buffer (5 mM Tris-HCl, pH 7.4, with 5 mM EGTA) and homogenized with 15 strokes on ice. Samples were centrifuged at 30,000*g* for 15 min to pellet membranes. The membrane protein was then resuspended in binding buffer (75 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, and 2 mM EDTA) and stored in aliquots at −80°C. Binding assays were performed on 25 μg of membrane protein using saturating amounts of the β-AR–specific ligand [^{125}I]iodocyanopindolol (ICYP), as described previously (Zhou et al., 2000b). Saturation experiments were performed with [^{125}I]ICYP concentrations ranging from 1 to 300 pM. Competition experiments were carried out at 50 pM [^{125}I]ICYP. Nonspecific binding was determined in the presence of 10 μM propranolol and was usually 10 to 30% of total binding of [^{125}I]ICYP (50 pM). All assays were performed in duplicate, and receptor density was normalized to milligrams of membrane protein. K_d and the maximal number of binding sites (B_{max}) for [^{125}I]ICYP were determined by Scatchard analysis of saturation binding isotherms.

Immunocytochemical Staining and Confocal Imaging. β_1/β_2-AR DKO cells were infected by either Adv-β_1-AR, Adv-β_2-AR, or Adv-β_1/β_2-chimera in culture for 24 h. Cells were washed twice with phosphate-buffered saline (PBS) and fixed with ice-cold methanol plus acetone (7:3) for 10 min and rinsed twice with PBS containing 0.2% Triton X-100. Nonspecific binding was reduced by a 30-min incubation with washing solution (5% bovine serum albumin, 2% horse serum, 0.2% Triton X-100, and 0.01% NaN_3 in PBS, pH 7.4). Then, cells infected with Adv-β_1-AR or Adv-$\beta_1/\beta_{2\text{-Li3}}$ were incubated

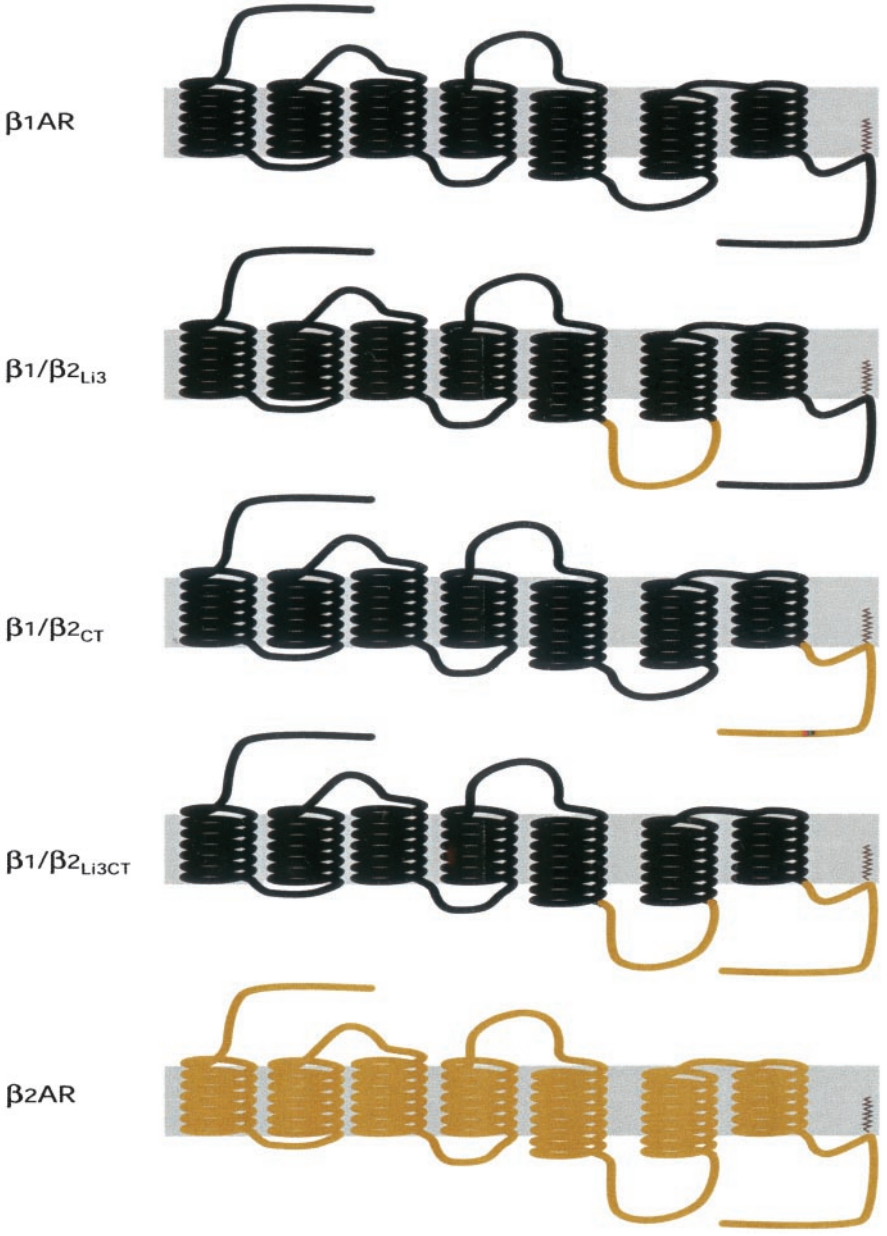

Fig. 1. Topology of β_1-AR, β_2-AR, and β_1/β_2 chimeras. The β_1-AR construct is shown in black, and the β_2-AR construct is shown in gold. The β_1/β_2-AR chimeras include β_1/β_{2Li3} (in which the third intracellular loop of β_1-AR is replaced by that of β_2-AR), β_1/β_{2CT} (in which the C terminus of β_1-AR is replaced by the counterpart of β_2-AR), and $\beta1/\beta_{2Li3CT}$ (in which both the third loop and C terminus of β_1-AR are replaced by those of β_2-AR)

with a primary antibody reacting with β_1-AR (diluted by 1:500), whereas cells infected by Adv-β_2-AR or Adv-$\beta_1/\beta_{2\text{-CT}}$ or Adv-$\beta_1/\beta_{2\text{-Li3CT}}$ were incubated with an antibody reacting with β_2-AR C terminus (diluted by 1:100) for 60 min at room temperature. After being rinsed four times with PBS, including 0.2% Triton X-100, cells were stained with Cy3-conjugated goat anti-rabbit IgG secondary antibodies (1:500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for another 60 min in the dark. As a negative control, cells were incubated with secondary antibodies in the absence of primary antibodies (data not shown). As an additional negative control, another subset of DKO cells infected with an adenovirus expressing β-galactosidase (Adv-β-gal) was treated with the same protocol. Immunofluorescence was then detected by a laser scanning confocal microscope (LSM-510; Zeiss) with optical section thickness of 1.0 μm, as described previously (Zhou et al., 2000b).

Measurement of cAMP Accumulation. Intracellular cAMP was measured as described previously (Zhou et al., 2000b). Briefly, after adenoviral infection for 24 h, cells were treated with a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (1 mM), for 30 min at 37°C, then were incubated with a β-AR agonist, isoproterenol (ISO; 10^{-6} M), or a β_2-AR inverse agonist, ICI 118,551 (5×10^{-7}), for 10 min. The treated cells were washed with ice-cold PBS, pH 7.5, and homogenized and centrifuged with a buffer containing 4 mM EDTA. cAMP level was determined using a cAMP ^{3}H assay kit obtained from Amersham Biosciences (Piscataway, NJ). Protein content was measured using the Bradford method (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard.

Statistical Analysis. Data are reported as mean ± S.E. Student's *t* test, paired *t* test, or analysis of variance were used, when appropriate, to test for differences among the means. A value of $P < 0.05$ was considered to be statistically significant.

Materials. Isoproterenol, (±)-metoprolol, (±)-propranolol, 3-isobutyl-1-methylxanthine, CGP20712A, and minimal essential medium were purchased from Sigma (St. Louis, MO). (±)-Bisoprolol hemifumarate and betaxolol hydrochloride were purchased from Tocris Cookson Inc. (Ellisville, MO). ICI 118,551 was kindly supplied by ICI Pharmaceutic Group (Wilmington, DE). Fetal bovine serum, penicillin-streptomycin, and mouse laminin were purchased from Invitrogen (Carlsbad, CA). The cAMP assay kit was purchased from Amersham Biosciences. [^{125}I]Iodocyanopindolol was purchased from PerkinElmer Life Sciences (Boston, MA). β_1-AR and β_2-AR polyclonal antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The secondary antibodies were purchased from Vector Laboratories (Burlingame, CA)

Results

Expression and Subcellular Distribution of β_1/β_2-AR Chimeras in β_1/β_2-AR DKO Mouse Cardiac Myocytes. To determine whether the β_1/β_2-AR chimeras undergo spontaneous activation, we first individually expressed the β_1/β_2-AR chimeras or WT β-AR subtypes at a comparable level in mouse $\beta_1\beta_2$ DKO myocytes with the use of adenoviral gene transfer. The exact receptor density was measured by radioligand binding assay with [^{125}I]ICYP. The average receptor density was 373.5 ± 53.3, 444.4 ± 14.4, and 372.8 ± 27.4 fmol/mg of protein in cells-infected by Adv-$\beta_1/\beta_{2\text{-Li3}}$, Adv-$b_1/\beta_{2\text{-CT}}$, and Adv-$\beta_1/\beta_{2\text{-Li3-CT}}$, respectively, all at m.o.i. of 100 (Fig. 2A). The densities of the chimeras were similar to that of β_1-AR (450.0 ± 46.0 fmol/mg of protein) or β_2-AR (401.0 ± 43.0 fmol/mg protein) expressed in DKO myocytes by adenoviral gene transfer at the same m.o.i. (Fig. 2A). Furthermore, there was no significant difference among the β_1/β_2-AR chimeras and WT β-ARs in their affinities for [^{125}I]ICYP binding (data not shown).

Using confocal imaging in conjunction with immunocytochemical staining, we found that, in the absence of agonists, the expressed β_1/β_2-AR chimeras were largely concentrated on cell surface membranes, including transverse tubules, with little staining of the cytosol, resulting in a clear striated appearance (Fig. 2B). In addition, the perinuclear region was also enriched with immunostaining. This is similar to intracellular distribution pattern of WT β_1-AR or β_2-AR (Zhou et al., 2000b).

Affinities of ICI 118,55 and CGP20712A for β_1/β_2-AR Chimeras. Next, we examined the binding properties of WT β_1-AR, β_2-AR and the chimeras for a well characterized β_1-AR selective antagonist, CGP20712A, and a β_2-AR selective antagonist, ICI 118,551. As shown in Table 1 and Fig. 3, there was no detectable difference among the chimeras or between WT β_1-AR and those chimeras. This result indicates that the third intracellular loop and the C terminus do not play a major role in determining β-AR subtype-selective binding of ICI 118,551 or CGP20712A, suggesting that ligand binding sites of β-ARs are not located in those domains.

Stimulation of β_1/β_2-AR Chimeras by Isoproterenol Increases Intracellular cAMP and Myocyte Contractility. To define the functionality of the chimerical receptors, we examined the maximal contractile and relaxant effects in response to a β-AR agonist, ISO (10^{-6} M), stimulation. ISO markedly enhanced the contraction amplitude (Fig. 4A), abbreviated the 50% relaxation time (T_{50}) (Fig. 4B) in all groups tested, including three β_1/β_2-AR chimeras, the β_1-AR–PDZ, and two WT β-AR subtypes. ISO-induced positive inotropic effect and acceleration of relaxation were associated with a marked elevation of intracellular cAMP accumulation (Fig. 4C). These results indicate that agonist-induced stimulation

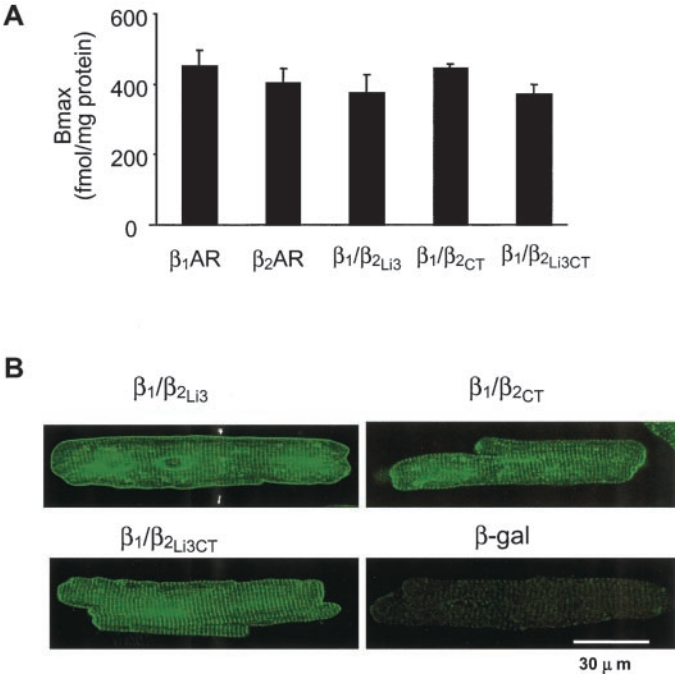

Fig. 2. Expression and intracellular distribution of β_1/β_2-AR chimeras in adult β_1/β_2 DKO mouse cardiac myocytes. A, receptor density was assayed using [^{125}I]ICYP binding (see *Materials and Methods*). Data presented are mean ± S.E. of three independent experiments (cells from nine hearts), each performed in duplicate. B, the immunofluorescent signal of β_1/β_2-AR chimeras is mainly localized to the sarcolemmal membranes, including transverse tubules, rendering a striated distribution pattern. The negative control shows cells infected with Adv-β-gal.

of these chimeras effectively activates cAMP/PKA signaling cascade and leads to positive inotropic and lusitropic effects in adult mouse cardiac myocytes.

Expression of β_1/β_2-AR Chimeras Increases Baseline Contractility and Basal cAMP Accumulation. To examine the possible spontaneous activity of the chimeras, we measured basal myocyte contractile properties and cAMP accumulation in the absence of β-AR agonist. The contraction amplitude of cells expressing any of the chimeras was at a markedly elevated level that is comparable with the baseline of cells infected by Adv-β_2-AR (Table 2). On average, baseline contractility was augmented by 50~60%, compared with that in myocytes expressing β_1-AR or β-gal (Table 2). In addition, the kinetics of cell contraction, including time to peak (T_{peak}), time to 50% relaxation (T_{50}), and time to 90% relaxation (T_{90}), were significantly accelerated in myocytes expressing these chimeras relative to those infected by Adv-β_1-AR or Adv-β-gal (Table 2).

Basal cAMP level was concomitantly elevated in cells expressing β_1/β_2-AR chimeras relative to the baseline in cells expressing β_1-AR, whereas it was not significantly different from that in cells expressing β_2-AR (Fig. 6B). These data further confirm that, like WT β_2-AR, the β_1/β_2-AR chimeras are able to undergo spontaneous activation in the absence of agonist stimulation.

Because our previous studies have demonstrated that association of β_1-AR with postsynaptic density 95 or a related protein via its C-terminal PDZ motif dictates signaling specificity by retaining the receptor at the cell surface and preventing interaction with Gi proteins (Xiang et al., 2002), we next examined whether β_1-AR C-terminal PDZ motif is responsible for the lack of the receptor spontaneous activation. We found that the contractile parameters in myocytes expressing the β_1-AR–PDZ mutant were not different from those in cells expressing WT β_1-AR or β-gal (Table 2), indicating that functional disruption of the β_1-AR PDZ motif is unable to restore β_1-AR spontaneous activity.

Reversal of Enhanced Basal Contractility and cAMP Accumulation by the β_2-AR Inverse Agonist, ICI 118,551. To further evaluate spontaneous activity of the β-AR chimeras, we examined the possible effects of the β2-AR inverse agonist, ICI 118,551 (5×10^{-7} M), on myocyte contractility and total cellular cAMP accumulation. Figure 5 shows the typical examples of contractile response to ICI 118,551 in cells expressing WT β_1-AR, WT β_2-AR, or one of the β_1/β_2-AR chimeras. Remarkably, in cells infected with an adenoviral vector encoding β_1/β_2-AR chimera, ICI 118,551 rapidly and reversibly reduced the enhanced basal contraction amplitude, as was the case for the cell expressing WT β_2-AR (Fig. 5, B–E). On average, the baseline contractility was decreased by 50~60% (Fig. 6A). In sharp contrast, in cells infected by Adv-β_1-AR, the baseline contractility was insensitive to ICI 118,551 (Figs. 5A and 6A), in agreement with our previous observation (Zhou et al., 2000b). Consistent with the results on cell contraction, ICI 118,55 treatment decreased the basal cAMP level by 60~65% in cells expressing WT β_2-AR or β_1/β_2-AR chimeras, but not in cells expressing WT β_1-AR (Fig. 6B). In contrast, β_1-AR antagonists, including CGP20712A (3×10^{-7} M), betaxolol (10^{-6} M), bisoprolol (10^{-6} M), and metoprolol (10^{-6} M), had no significant effect on basal cell contractility in any group tested (Fig. 7 and Table 3). These results suggest that the replacement of either the third intracellular loop, the C terminus, or both domains of β_1-AR with that of β_2-AR confers spontaneous receptor activation.

It has been demonstrated that the inverse agonist ICI 118,551 can activate the β_2-AR/G_i pathway, resulting in a negative inotropic effect in cardiomyocytes from failing human heart or transgenic mice overexpressing β_2-AR (TGβ2) in a PTX-sensitive manner (Gong et al., 2002). To determine whether the inhibitory effects of ICI 118,551 on basal contraction is mediated by activation of the β_2-AR/G_i pathway, we compared the effects of ICI 118,551 in cells expressing β_2-AR or the chimeras in the presence and absence of PTX treatment. We found that PTX had no significant effect on ICI 118,551-mediated reduction in basal myocyte contractility (Table 4), indicating that ICI 118,551 acts as an inverse agonist rather than an agonist of the G_i pathway under our experimental conditions. Moreover, PTX treatment exerted no significant effect on the elevated basal contraction because of spontaneous receptor activation (Table 4). This indicates that, in contrast to ligand-stimulated β_2-AR, the R* state WT β_2-ARs and R* state β_1/β_2-AR chimeras are not coupled to G_i signaling pathway, supporting the existence of more than one active receptor conformational states (Zhou et al., 1999a,b).

Discussion

Difference between β-AR Subtypes in Their Spontaneous Activation. In addition to ligand-induced activation, some ligand-free GPCRs manifest spontaneous activity. Such spontaneous activity has been demonstrated for opioid peptide receptor (Neilan et al., 1999), gonadotropin receptors (Schulz et al., 1999), luteinizing hormone receptor (Shenker et al., 1993; Kudo et al., 1996), melanocyte-stimulating hormone receptor (Robbins et al., 1993), and glucagon receptors (Hjorth et al., 1998). However, this feature is not universally shared by all GPCRs. The diversity is exemplified by the differential ability of β-AR subtypes to undergo spontaneous activation in myocardium or single isolated cardiac myocytes. Although β_2-AR shows a robust spontaneous activity in a variety of model systems (Samama et al., 1993; Chidiac et al., 1994, 1996; Milano et al., 1994; Bond et al., 1995; Xiao et al., 1999; Zhou et al., 1999a,b; Zhang et al., 2000), many studies failed to demonstrate the ability of β_1-AR to undergo a spontaneous activation, by virtue of regulating myocyte contractility and cAMP formation (Engelhardt et al., 1999; Zhou et

TABLE 1
Binding affinity of ICI 118,551 and CGP20712A for WT β_1-AR, β_2-AR, or β_1/β_2-AR chimeras

	β_1-AR	β_2-AR	$\beta_1/\beta_{2\text{-Li3}}$	$\beta_1/\beta_{2\text{-CT}}$	$\beta_1/\beta_{2\text{-Li3CT}}$
K_i (CGP)	7.71 ± 0.12	4.61 ± 0.07*	7.80 ± 0.02	7.95 ± 0.14	7.81 ± 0.02
K_i (ICI)	5.80 ± 0.12	8.16 ± 0.01*	5.92 ± 0.04	6.16 ± 0.05	5.88 ± 0.11

* $P < 0.05$ versus β_1-AR and chimeras.
ICI, ICI 118,551; CGP, CGP20712A.

al., 2000b). More recent studies, however, have identified a moderate spontaneous activity of β_1-AR in terms of heart rate modulation (Engelhardt et al., 2001).

The disparities of these studies might be explained by different model systems or readouts (Port and Bristow, 2001). For instance, there might be regional differences in the behavior of β_1-AR with respect to its spontaneous activity in the atrium versus ventricle (Zhou et al., 2000b; Engelhardt, 2001). In this regard, it has been shown that the inverse agonist activity of a variety of β-AR antagonists are significantly greater in rat right atria than that in other cardiac preparations (left atria, right ventricles, papillary muscles) (Varma et al., 1999), supporting the proposal that the spontaneous activity of β_1-AR is endpoint- and organ region-dependent. Furthermore, concentrations of β-AR antagonists used in different studies may also influence the experimental outcomes, because some antagonists can act as inverse agonists or neutral antagonists depending on their concentrations (Chidiac et al., 1994; Bond et al., 1995).

It is noteworthy that the inhomogeneous feature of spontaneous activity has been also reported for other closely related GPCRs. For example, the dopamine receptor subtypes 1A and 1B show markedly different spontaneous activities. Similarly, the highly conserved gonadotropin receptors, luteinizing hormone receptor and follicle-stimulating hormone receptor, manifest a variable propensity to undergo spontaneous activation (Kudo et al., 1996; Schulz et al., 1999).

The Third Intracellular Loop or the C Terminus of β_2-AR Is Sufficient for the Receptor Spontaneous Activity. The most important finding of the present study is that expression of the β_1/β_2-AR chimeras (β_1/β_{2li3}, β_1/β_{2CT}, and β_1/β_{2li3CT}), similar to expression of WT β_2-AR, induces marked increases in basal cAMP accumulation and baseline contractility. Moreover, the β_2-AR inverse agonist, ICI 118,551, fully reverses these changes, whereas it has no effect on either parameter in cardiomyocytes expressing WT β_1-AR, the β_1-AR–PDZ mutant, or β-gal. In sharp contrast, a panel of β_1-AR antagonists neither enhances nor reduces

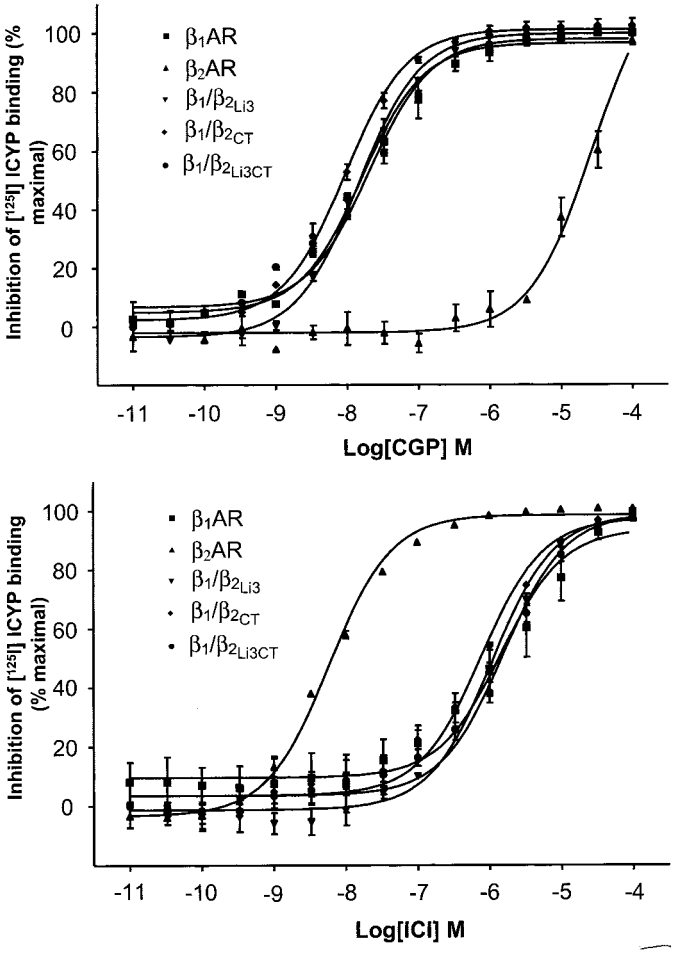

Fig. 3. Competition of [^{125}I]ICYP with the β_1-AR antagonist CGP20712A (CGP) (A) or the β_2-AR inverse agonist ICI 118,551 (ICI) (B) in membranes from DKO mouse cardiac myocytes infected with adenoviruses encoding either β_1-AR, β_2-AR, or one of the β_1/β_2-AR chimeras. Note that the chimeras behave as wild-type β_1-AR. The figures represent the mean ± S.E. of the displacement curves in cells from three mouse hearts.

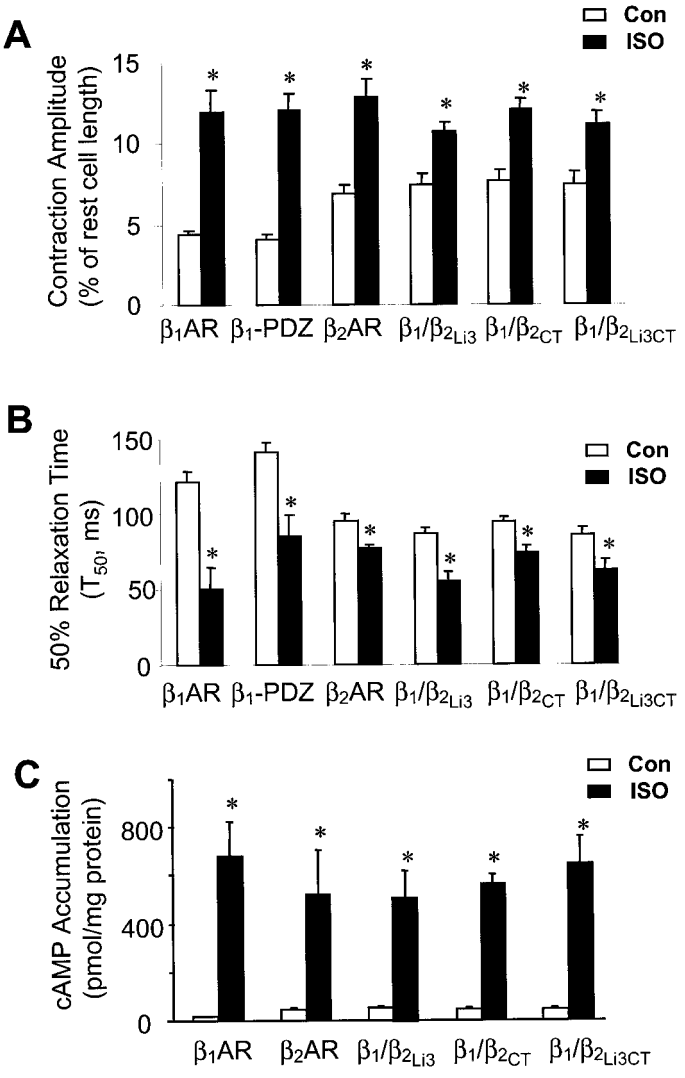

Fig. 4. Response of cell contraction and relaxation or cAMP accumulation to a nonselective β-AR agonist, ISO (10^{-6} M), in DKO mouse cardiac myocytes infected with adenoviruses encoding either β_1-AR, β_2-AR, the β_1-AR–PDZ mutant, or one of the β_1/β_2-AR chimeras. ISO (10^{-6} M) markedly increases cell contraction amplitude (A) and abbreviates 50% contractile relaxation time (T_{50}) (B). ISO-induced positive inotropic and lusitropic effects are accompanied by an increase in cAMP accumulation with a comparable maximal effect in all groups tested (C). *, $P < 0.01$ versus control (Con) (n = 6~14 cells from at least five hearts for each contraction and relaxation measurements; n = 3 independent experiments for cAMP assay).

basal contractility (Fig. 7 and Table 3) and cAMP level (data not shown) in cells expressing the β_1/β_2 chimeras. Similarly, expression of the β_1/β_2-AR chimeras (β_1/β_{2li3}, β_1/β_{2CT}, and β_1/β_{2li3CT}), but not WT β_1-AR, in rat cardiomyocytes also induces marked increases in baseline contractility in an ICI 118,551-sensitive manner.

It is noteworthy that expression of the β_1-AR–PDZ mutant cannot induce any spontaneous activity, although disruption of the function of the PDZ motif of β_1-AR converts the mutant receptor to β_2-AR in terms of receptor trafficking and G_i coupling in response to agonist stimulation (Xiang et al., 2002). Here, we have demonstrated that the disruption of the PDZ domain cannot shift the receptor conformation into an active one, indicating that the relative lack of constitutive activity of the β_1-AR is not related to its anchoring to specific proteins via its PDZ domain.

These aforementioned observations not only reinforce the previous notion that β_2-AR, but not β_1-AR, exhibits spontaneous activity in terms of its ability to increase myocyte cAMP formation and contractility (Milano et al., 1994; Bond et al., 1995; Du et al., 1996; Engelhardt et al., 1999; Xiao et al., 1999; Gong et al., 2000; Zhou et al., 2000b), but also reveal that the third intracellular loop and the C terminus of β_2-AR are critically involved in the receptor spontaneous activity. Several lines of evidence suggest that the third loop and the C-terminal domain of β_2-AR seem to be equally important in promoting spontaneous activity. The three chimeras behave in a manner similar to that of WT β_2-AR, as manifested by a comparable increase in baseline contraction and basal cAMP accumulation. The β_2-AR inverse agonist, ICI 118,551, is able to completely reverse the spontaneous activities of the β_1/β_2-AR chimeras, as is the case for WT β_2-AR. These results indicate that the spontaneously activated conformations induced by the replacement of the third loop or C terminus or both domains of β_1-AR with the counterpart(s) of β_2-AR are similar to the active conformational state (R*) of WT β_2-AR. Because constitutively active β_2-AR mutants are characterized by a remarkable structural instability and enhanced conformational flexibility (Gether et al., 1995; Gether et al., 1997a, 1997b; Rasmussen et al., 1999), the third intracellular loop and the C terminus might be involved in determining the flexibility of the receptor or the transition from inactive to active conformations. Further study is required to pinpoint the specific amino acids of those domains responsible for the receptor spontaneous activity.

The Third Intracellular Loop and the C Terminus Are Not Involved in β-AR Subtype-Selective Binding of Antagonists. ICI 118,551, but not CGP20712A, effectively reverses the chimera-mediated augmentations in basal myocyte cAMP accumulation and contractility; this raised the question of whether the third intracellular loop and the C terminus of β-ARs affect the binding affinity of selective antagonists. To shed light on this particular issue, we measured the affinity of either CGP20712A or ICI 118,551 for each chimera and WT β_1-AR or β_2-AR using [^{125}I]iodocyanopindolol competitive binding assay. The present results reveal that none of the chimeras differs from WT β_1-AR in terms of binding affinity for selective antagonists. Thus, the third intracellular loop and the C terminus of β-ARs are not essentially involved in ligand binding, although those domains play important roles in interacting with G proteins and adenylyl cyclase (O'Dowd et al., 1988; Green et al., 1992). This is consistent with previous reports that the binding domain of β-ARs is mainly located in a pocket in the transmembrane domains 3, 5, and 6 (Dixon et al., 1987; Dohlman et al., 1988; Wong et al., 1988; Hockerman et al., 1996) and that other transmembrane domains, such as 2, 6, and 7, may also play a role in determining β-AR subtype-selectivity for antagonists (Marullo et al., 1990; Kurose et al., 1998; Isogaya et al., 1998, 1999; Kikkawa et al., 1998). Thus, the inhibitory effects of ICI 118,551 on the chimera-induced elevations of myocyte contraction and cAMP production is mediated by its inverse agonist functionality, rather than by enhanced binding affinity of ICI 118,551 to those chimeras.

It is also noteworthy that the affinity values reported in Table 1 should be taken as the outcome with R state receptors (inactive form). The binding affinity of ICI 118,551 for R* state receptors (active form) is presently unknown, because R* state receptors are thought to constitute only a minor fraction of the total receptor population. For WT β_2-AR, its high ICI 118,551 affinity ensures a nearly 100% binding of ICI 118,551 (5×10^{-7}M) to the R state receptors. The action of ICI 118,551 as an inverse agonist can be achieved, therefore, by stabilizing the R state and preventing R-to-R* transition. As for β_1/β_2-AR chimeras, however, only 20 to 30% of the receptors are expected to be occupied by ICI 118,551 at 5×10^{-7} M. Because ICI 118,551 can effectively block the physiological and biochemical consequences of spontaneous receptor activation, the R* states of β_1/β_2-AR chimeras should be conformationally similar to R*-state WT β_2-AR,

TABLE 2
Basal contractile properties of cultured β_1/β_2-AR DKO mouse ventricular myocytes infected with adenoviral vectors encoding target genes
Cells used in each group are from at least 10 hearts. Values are presented as mean ± S.E.

	Lrest	T_{peak}	T_{50}	T_{90}	Contraction Amplitude	*n*
	μm	*ms*	*ms*	*ms*	*% of Lrest*	*cells*
β-gal	106.8 ± 7.6	175.2 ± 5.5	169.1 ± 8.0	446.7 ± 22.1	4.0 ± 0.3	56
β_1-AR	111.5 ± 2.1	174.2 ± 4.4	124.0 ± 6.5†	284.7 ± 17.5†	4.5 ± 0.2	69
β_1-AR–PDZ mutant	113.2 ± 1.8	166.6 ± 3.8	141.4 ± 6.0*†	372.2 ± 21.7*†	4.1 ± 0.3	59
β_2-AR	112.2 ± 2.6	141.4 ± 5.0*	95.2 ± 5.0*	212.9 ± 15.1*	6.9 ± 0.6*	37
$\beta_1/\beta_{2\text{-Li3}}$	102.6 ± 2.6	150.0 ± 5.6*	100.5 ± 5.7*	190.0 ± 15.2*	7.7 ± 0.4*	52
$\beta_1/\beta_{2\text{-CT}}$	112.2 ± 2.4	160.8 ± 6.3*	107.7 ± 4.6*	223.2 ± 10.0*	7.5 ± 0.4*	68
$\beta_1/\beta_{2\text{-Li3CT}}$	102.6 ± 3.0	150.4 ± 5.1*	94.5 ± 4.8*	198.0 ± 16.0*	7.5 ± 0.6*	48

Lrest, rest cell length; T_{peak}, the time from electrical stimulation to peak shortening; T_{50}, the time from peak shortening to 50% relaxation; T_{90}, the time from peak shortening to 90% relaxation; contraction amplitude, cell shortening presented as percentage of rest cell length.
* $P < 0.05$ versus β_1-AR and β-gal.
† $P < 0.05$ versus β-gal.

manifesting much higher ICI 118,551 affinity than the R-state chimeras. If this is the case, inhibition of spontaneous activation of the chimeras is achieved by a different mechanism (i.e., mainly by quenching the receptor from the R* state).

Possible Impact of Receptor Overexpression on the Receptor Signaling. The present results indicate that the chimera-mediated increases in myocyte contraction amplitude and cAMP accumulation are comparable with that induced by either WT β_1- or β_2-AR stimulation with the same agonist (ISO, 10^{-6} M) (Fig. 4), indicating agonist-induced receptor activation remains unaltered in these chimeras. Interestingly, the present data show a similar increase in cAMP formation in response to β_1-AR and β_2-AR subtype stimulation. This is in contrast to the previous notion that these β-AR subtypes are differentially coupled to cAMP production, with the β_2-AR severalfold more tightly coupled. Because it has been proposed that the greater coupling of β_2-AR to cAMP production compared with β_1-AR is attribut-

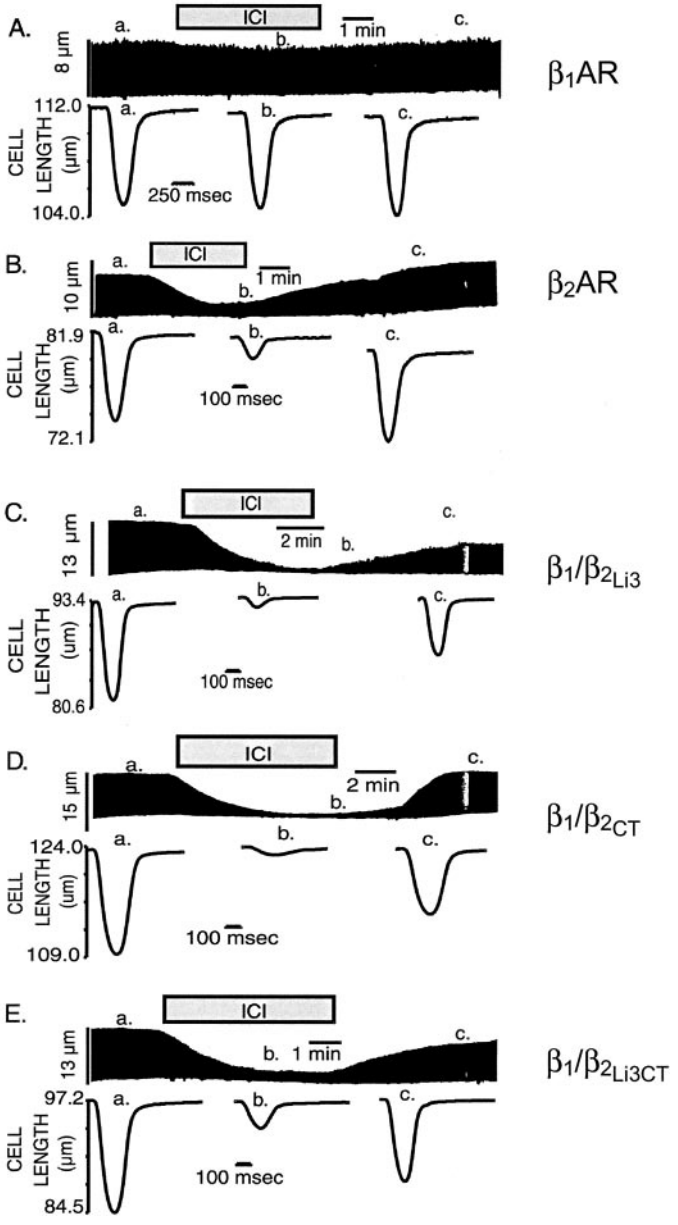

Fig. 5. Typical examples of the inhibitory effect of the β_2-AR inverse agonist, ICI 118,551 (ICI, 5×10^{-7} M), on basal contraction amplitude in DKO myocytes expressing either β_1-AR, β_2-AR, or a β_1/β_2-AR chimera. In each example, the top shows a continuous chart recording of the change of cell length. An upward deflection indicates cell shortening. The bottom shows contraction traces displayed at a higher resolution at time points as indicated. A downward deflection indicates cell shortening.

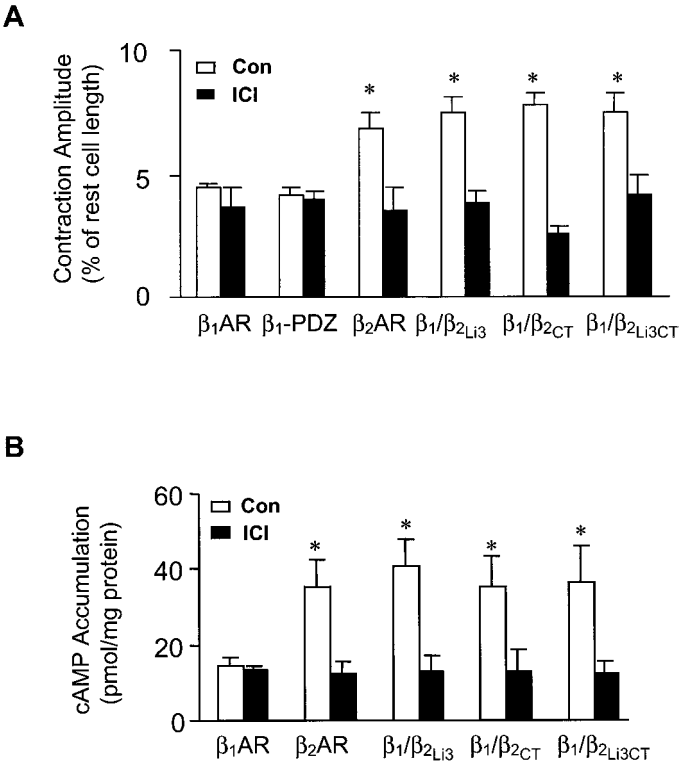

Fig. 6. Average effects of the β_2-AR inverse agonist ICI 118,551 on baseline contractility (A) and cAMP accumulation (B) in DKO myocytes expressing either β_1-AR, β_2-AR, the β_1-AR–PDZ mutant, or one of the β_1/β_2-AR chimeras. Note that both baseline contraction amplitude and basal cAMP accumulation are significantly increased in cells expressing β_2- or β_1/β_2-AR chimeras but not in those expressing the β_1-AR–PDZ mutant, compared with that in cells expressing β_1-AR. The increases in both parameters are fully reversed by the β_2AR inverse agonist ICI 118,551 (ICI; 5×10^{-7} M), *, $P < 0.01$ versus control (Con) (n = 37~69 cells from at least 10 hearts for cell contraction measurements; n = 3~5 independent experiments for cAMP measurements).

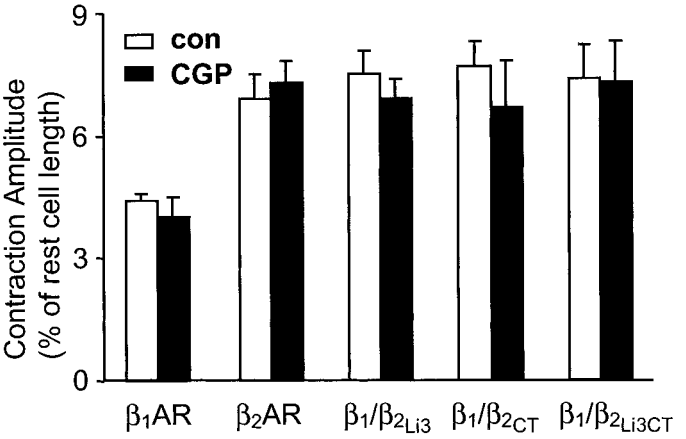

Fig. 7. Response of cell contraction to the β_1-AR antagonist CGP20712A (CGP) in DKO mouse cardiomyocytes expressing WT β-AR subtypes or a β_1/β_2-AR chimera. Note that CGP20712A (3×10^{-7} M) did not affect basal contraction amplitude in any group tested (n = 10~22 cells from eight hearts).

TABLE 3

Effects of β_1-AR antagonists on basal contraction amplitude of cultured WT rat ventricular myocytes expressing either one of the β_1/β_2-AR chimeras or WT β_1-AR

Contraction amplitude is presented as percentage of rest cell length ± S.E. (*n* refers to the number of cells from at least five hearts for each group). All β_1-AR antagonists were at 1 μM.

	Baseline (*n*)	Betaxolol (*n*)	Bisoprolol (*n*)	Metoprolol (*n*)
β_1-AR	7.5 ± 0.3 (58)	6.6 ± 0.7 (14)	6.8 ± 0.7 (11)	7.5 ± 0.5 (15)
$\beta_1/\beta_{2\text{-Li3}}$	14.1 ± 0.6 (51)*	15.2 ± 0.7 (9)*	13.3 ± 1.7 (10)*	14.2 ± 0.6 (7)*
$\beta_1/\beta_{2\text{-CT}}$	12.2 ± 0.5 (62)*	12.7 ± 0.9 (8)*	13.5 ± 0.2 (8)*	11.5 ± 1.6 (11)*
$\beta_1/\beta_{2\text{-Li3CT}}$	14.7 ± 0.5 (53)*	16.7 ± 0.8 (8)*	14.7 ± 1.1 (7)*	15.4 ± 0.9 (7)*

* $P < 0.01$ versus β_1-AR.

TABLE 4

Effects of PTX on ICI118.551-induced negative contractile responses in rat cardiomyocytes overexpressing β_2-AR or the β_1/β_2-AR chimeras

Contraction amplitude is presented as percentage of rest cell length ± S.E. Cells were from at least three hearts in each group; *n* refers to the number of cells as indicated.

	β_2-AR (n)	$\beta_1/\beta_{2\text{-Li3}}$ (n)	$\beta_1/\beta_{2\text{-CT}}$ (n)	$\beta_1/\beta_{2\text{-Li3CT}}$ (n)
Non-PTX				
Baseline	12.1 ± 1 (10)	14.1 ± 0.6 (51)	12.2 ± 0.5 (62)	14.7 ± 0.5 (53)
ICI	8.2 ± 0.6 (10)*	6.3 ± 0.4 (17)*	7.5 ± 0.5 (25)*	8.5 ± 0.5 (18)*
PTX				
Baseline	12.5 ± 1.0 (13)	13.4 ± 0.9 (9)	12.4 ± 0.5 (16)	13.8 ± 0.6 (7)
ICI	6.6 ± 1.1 (13)*	7.7 ± 0.9 (9)*	6.3 ± 0.5 (16)*	9.3 ± 0.3 (7)*

* $P < 0.01$ versus baseline.
ICI, ICI 118,551 (5×10^{-7} M).

able, at least in part, to the distinct intracellular localization of these β-AR subtypes in cardiac myocytes with β_2-AR enriched in caveolae and β_1-AR uniformly distributed on cell surface membranes (Rybin et al., 2000; Ostrom et al., 2001), overexpression of β_1-or β_2-AR might alter the intracellular distribution pattern of these receptors, thereby abolishing the difference in their functional compartmentalization.

Possible Physiological and Pathological Relevance. It has been shown that although the efficacy of pharmacological stimulation of β-AR may be limited by receptor desensitization and proarrhythmic effects, overexpression of β_2-AR in the heart of transgenic mice or of dominant-negative inhibitor of βARK (βARKct) leads to enhanced cardiac contractility because of the receptor spontaneous activation or reduced receptor desensitization, which have beneficial effects in the normal, dilated cardiomyopathic and failing hearts by providing contractile support without significant cardiotoxic consequence (Milano et al., 1994; Koch et al., 1995; Rockman et al., 1998; Dorn et al., 1999). Indeed, crossing transgenic mice overexpressing cardiac β_2-AR at appropriate levels (e.g., 30-fold) with transgenic mice overexpressing $G_{\alpha q}$ not only improves the cardiac performance but also reverses hypertrophy in the $G_{\alpha q}$ overexpression heart failure model (Dorn et al., 1999), although high-level overexpression of β_2-AR results in heart failure later (Freeman et al., 2001). Because extremely high levels of β_2-AR overexpression (e.g., 350~1000 fold) fail to rescue the genetic mouse heart failure model and can be detrimental at early time points (Dorn et al., 1999; Liggett et al., 2000) and β_2-AR overexpression higher than 60-fold over the density of native cardiac β_2-ARs is highly toxic to cardiac tissue over the long term (Freeman et al., 2001; Liggett, 2000, 2001), caution must be exercised when designing therapies to enhance β_2-AR signaling so that the beneficial level of spontaneous receptor activation is not exceeded.

Notably, cardiac transgenic overexpression of β_1-AR by 5- to 45-fold in mice leads to marked myocyte hypertrophy and fibrosis within a few weeks after birth and heart failure within several months (Engelhardt et al., 1999; Bisognano et al., 2000). This suggests that enhanced β_1-AR stimulation could be a risk factor aggravating certain cardiac diseases (Lattion et al., 1999; Mewes et al., 1993; Bristow, 2000). This hypothesis is supported by the fact that in cultured rat or mouse cardiac myocytes, sustained β_1-AR stimulation overtly increases myocyte apoptosis (Bisognano et al., 2000; Zaugg et al., 2000). In addition, our recent studies have revealed that the β_1-AR apoptotic effect is mediated by cAMP/PKA-independent activation of Ca^{2+}/calmodulin dependent protein kinase II (Zhu et al., 2003). Moreover, in humans, the Arg389Gly naturally occurring polymorphism of β_1-AR leads to enhanced receptor response to agonist-induced stimulation (Mason et al., 1999), which is associated with enhanced risk of chronic heart failure (Small et al., 2002; Wagoner et al., 2002). Taken together, selective inhibition of β_1-AR in combination with concurrent activation of β_2-AR could be more effective than nonselective β_1-AR blockade in improving the function of the failing heart.

In summary, the present results provide evidence that replacement of the third intracellular loop (β_1/β_{2Li3}) or the C terminus (β_1/β_{2CT}) or both domains (β_1/β_{2Li3CT}) of β_1-AR with the counterpart(s) of β_2-AR converts β_1- to β_2-AR in terms of receptor spontaneous activation. The chimera-induced increases in myocyte baseline contractility and basal cAMP accumulation are fully reversed by the β_2-AR inverse agonist, ICI 118,551, but not by β_1-AR antagonists. Furthermore, disruption of the β_1-AR PDZ motif cannot restore spontaneous activity of the receptor. These results indicate that either the third intracellular loop or the C-terminal domain of β_2-AR is sufficient to confer β_2-AR spontaneous activity and that the β_1-AR PDZ motif is not responsible for the lack of β_1-AR spontaneous activity.

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