

# Spontaneous Activation of $\beta_2$ - but Not $\beta_1$ -Adrenoceptors Expressed in Cardiac Myocytes from $\beta_1\beta_2$ Double Knockout Mice

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## ABSTRACT

Although ligand-free, constitutive  $\beta_2$ -adrenergic receptor (AR) signaling has been demonstrated in naive cell lines and in transgenic mice overexpressing cardiac  $\beta_2$ -AR, it is unclear whether the dominant cardiac  $\beta$ -AR subtype,  $\beta_1$ -AR, shares the ability of spontaneous activation. In the present study, we expressed human  $\beta_1$ - or  $\beta_2$ -AR via recombinant adenoviral infection in ventricular myocytes isolated from  $\beta_1\beta_2$ -AR double knockout mice, creating pure  $\beta_1$ -AR and  $\beta_2$ -AR systems with variable receptor densities. A contractile response to a nonselective  $\beta$ -AR agonist, isoproterenol, was absent in double knockout mouse myocytes but was fully restored after adenoviral  $\beta_1$ -AR or adenoviral  $\beta_2$ -AR infection. Increasing the titer of adenoviral vectors (multiplicity of infection 10–1000) led to a dose-dependent expression of  $\beta_1$ - or  $\beta_2$ -AR with a maximal density of  $1207 \pm 173$  (36-fold over the wild-type control value) and  $821 \pm 38$  fmol/mg protein (69-fold), respectively. Using confocal immunohistochemistry, we directly visualized the cel-

lular distribution of  $\beta_1$ -AR and  $\beta_2$ -AR and found that both subtypes were distributed on the cell surface membrane and transverse tubules, resulting in a striated pattern. In the absence of ligand,  $\beta_2$ -AR expression resulted in graded increases in baseline cAMP and contractility up to 428% and 233% of control, respectively, at the maximal  $\beta_2$ -AR density. These effects were specifically reversed by a  $\beta_2$ -AR inverse agonist, ICI 118,551 ( $10^{-7}$  M). In contrast, overexpression of  $\beta_1$ -AR, even at a greater density, failed to enhance either basal cAMP or contractility; the alleged  $\beta_1$ -AR inverse agonist, CGP 20712A ( $10^{-6}$  M), had no significant effect on basal contraction in these cells. Thus, we conclude that acute  $\beta_2$ -AR overexpression in cardiac myocytes elicits significant physiological responses due to spontaneous receptor activation; however, this property is  $\beta$ -AR subtype specific because  $\beta_1$ -AR does not exhibit agonist-independent spontaneous activation.

G protein-coupled receptors (GPCRs) constitute the largest class of cell surface-signaling molecules, which are widely involved in regulating vital cellular processes.  $\beta$ -Adrenergic receptor ( $\beta$ -AR) is a prototypical GPCR. At least two  $\beta$ -AR subtypes,  $\beta_1$ -AR and  $\beta_2$ -AR, coexist in the heart of many mammalian species, including human (Xiao and Lakatta, 1993; Xiao et al., 1994; Altschuld et al., 1995; for review see Xiao et al., 1999b). Stimulation of these receptors by catecholamines increases cardiac contractility and heart rate

and accelerates cardiac relaxation via a  $G_s$ -adenylyl cyclase-cAMP-protein kinase A-signaling cascade. Although there is a high degree of structural and functional similarity between these  $\beta$ -AR subtypes, recent studies have shown that  $\beta$ -AR subtypes play strikingly different functional roles via distinct signaling pathways in the heart. In particular,  $\beta_2$ -AR, but not  $\beta_1$ -AR, couples to pertussis toxin-sensitive  $G_i$  proteins in addition to the well established  $G_s$ -signaling pathway (Xiao et al., 1995, 1999a; Kuschel et al., 1999).

A GPCR is proposed to exist in an equilibrium between two conformational states, an inactive form (R) and an active form (R\*), that can interact with G proteins (Samama et al., 1993; Bond et al., 1995; Neilan et al. 1999). In addition to ligand-induced activation, a small percentage of receptors

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**ABBREVIATIONS:** GPCR, G protein-coupled receptor;  $\beta$ -AR,  $\beta$ -adrenergic receptor; WT, wild type; ICI, ICI 118,551; CGP, CGP 20712A; DKO, double knockout; ISO, isoproterenol; FBS, fetal bovine serum; MEM, minimal essential medium;  $T_{peak}$ , the time from stimulation to peak shortening;  $T_{50}$ , the time from the peak to 50% relaxation; TA, cell twitch amplitude; ICYP, [<sup>125</sup>I]cyanopindolol; m.o.i., multiplicity of infection; PBS, phosphate-buffered saline; HA, hemagglutinin; IBMX, 3-isobutyl-1-methylxanthine; PTX, pertussis toxin.

are active in the absence of ligand. The ligand-independent activation of  $\beta_2$ -AR has been elegantly demonstrated in several systems overexpressing wild-type (WT)  $\beta_2$ -AR (Chidiac et al., 1994; Milano et al., 1994; Bond et al., 1995) or expressing the constitutively active mutant of  $\beta_2$ -AR (Samama et al., 1993). For example, ligand-free cardiac  $\beta_2$ -AR activation is evidenced by significantly increased basal adenylyl cyclase activity, cAMP accumulation, or contractility in the absence of any ligand, and the  $\beta_2$ -AR inverse agonist, ICI 118,511 (ICI), reverses these augmentations (Milano et al., 1994; Bond et al., 1995; Xiao et al., 1999a; Zhou et al., 1999a). However, whether  $\beta_1$ -AR shares the ability of spontaneous activation is still controversial. In transgenic mice overexpressing  $\beta_1$ -AR, the basal adenylyl cyclase activity and cardiac function remain unchanged compared with WT controls (Bertin et al., 1993; Zolk et al., 1998; Engelhardt et al., 1999). Nevertheless, in guinea pig and human cardiomyocytes, in the presence of forskolin, the mixed  $\beta_1$ - and  $\beta_2$ -AR antagonist, but not  $\beta_2$ -AR antagonists, induces a marked decrease in basal L-type  $\text{Ca}^{2+}$  current in the absence of ligand (Mewes et al., 1993). More recently, it has been shown that, in canine cardiac myocytes, a  $\beta_1$ -AR antagonist, CGP 20712A (CGP), inhibited the basal  $I_{\text{Ca}}$  by 27% (Nagykaldi et al., 1999). These results were interpreted to indicate spontaneous activation of  $\beta_1$ -AR.

The overall goal of the present study was to examine whether  $\beta$ -AR subtypes are different in terms of their propensity of spontaneous activation. To avoid complicated interactions between  $\beta$ -AR subtypes, because of the lack of absolutely selective  $\beta$ -AR subtype ligands, we took advantage of a recently developed  $\beta_1$ - and  $\beta_2$ -AR double knockout (DKO) mouse model (Rohrer et al., 1999) and recent advances in methods to culture adult mouse ventricular myocytes (Zhou et al., 2000). We expressed either  $\beta$ -AR subtype over a wide range of receptor density in cultured  $\beta_1\beta_2$ -AR DKO ventricular myocytes and investigated the physiological or biochemical responses in the absence or presence of  $\beta$ -AR subtype inverse agonists. Our results indicate that overexpression of  $\beta_2$ -AR is associated with a robust increase in basal cAMP accumulation and contractility, which can be specifically reversed by the  $\beta_2$ -AR inverse agonist, ICI. Surprisingly, overexpression of  $\beta_1$ -AR to the same or even greater density has no effect on basal cAMP and contractility. These results indicate that  $\beta_1$ -AR, unlike  $\beta_2$ -AR, is not able to undergo ligand-independent constitutive activation in intact mouse cardiac myocytes.

## Experimental Procedures

**Myocyte Isolation, Culture, and Adenoviral Infection.** The investigation conforms to National Institutes of Health guiding principles in the care and use of animals. Single mouse cardiac myocytes were isolated from the hearts of 2- to 3-month-old mice with an enzymatic technique and then were cultured and infected with adenoviral vectors, as described previously (Zhou et al., 2000). Before culture, myocytes were washed three times with minimal essential medium (MEM) containing 1.2 mM  $\text{Ca}^{2+}$ , 2.5% fetal bovine serum (FBS), and 1% penicillin-streptomycin and then plated at  $0.5$  to  $1 \times 10^4/\text{cm}^2$  with the same medium in the culture dishes precoated with  $10 \mu\text{g}/\text{ml}$  mouse laminin. After 1 h of culture (to achieve attachment), the culture medium was aspirated along with unattached cells. 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 (Zeiss, model IM-35, Zeiss, Thornwood, NY) and superfused with HEPES-buffered solution consisting of (in mM):  $\text{CaCl}_2$  1,  $\text{NaCl}$  137,  $\text{KCl}$  5.4, dextrose 15,  $\text{MgSO}_4$  1.3,  $\text{NaH}_2\text{PO}_4$  1.2, and HEPES 20, pH 7.4 adjusted with  $\text{NaOH}$ . Each cell was illuminated with red (650–750 nm) light through the normal bright-field path of the microscope and electrically stimulated at 0.5 Hz at  $23^\circ\text{C}$ . 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).  $T_{\text{peak}}$  was measured as the time from stimulation to peak shortening;  $T_{50}$  was measured as the time from the peak to 50% relaxation.

**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  $30,000g$  for 15 min to pellet membranes. Membranes were resuspended in binding buffer (75 mM Tris HCl, pH 7.4, 12.5 mM  $\text{MgCl}_2$ , 2 mM EDTA) and stored in aliquots at  $-80^\circ\text{C}$ . Binding assays were performed on  $25 \mu\text{g}$  of membrane protein using saturating amounts of the  $\beta$ -AR-specific ligand [ $^{125}\text{I}$ ]cyanopindolol (ICYP). Nonspecific binding was determined in the presence of  $10 \mu\text{M}$  propranolol. Reactions were conducted in  $250 \mu\text{l}$  of binding buffer at  $37^\circ\text{C}$  for 1 h. The binding reaction was terminated by addition of ice-cold 10 mM Tris-HCl (pH 7.4) to the membrane suspension, followed by rapid vacuum filtration through glass-fiber filters (Whatman GF/C). Each filter was washed three times with an additional 7 ml of ice-cold 10 mM Tris-HCl. The radioactivity of the wet filters was determined in a gamma counter. 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 ( $B_{\text{max}}$ ) for ICYP were determined by Scatchard analysis of saturation binding isotherms.

**Immunocytochemical Staining and Confocal Imaging.**  $\beta_1\beta_2$ -DKO cells were infected by either adeno- $\beta_1$ -AR tagged with hemagglutinin (HA) or adeno- $\beta_2$ -AR [multiplicity of infection (m.o.i.) 100] for 24 h. Cells were washed twice with phosphate-buffered saline (PBS) and fixed with cold methanol plus acetone (7:3) for 10 min and rinsed twice with PBS containing 0.2% Triton. Nonspecific binding was reduced by a 30-min incubation with Blotto solution (5% BSA, 2% horse serum, 0.2% Triton, and 0.01%  $\text{NaN}_3$  in PBS, pH 7.4). Then, cells were incubated for 60 min at room temperature with primary antibodies for HA-tagged  $\beta_1$ -AR (anti-HA monoclonal antibody diluted by 1:500) or for  $\beta_2$ -AR ( $\beta_2$ -AR polyclonal antibodies diluted by 1:100). After the cells were rinsed four times with PBS, including 0.2% Triton, they were stained with Texas Red-conjugated secondary antibodies (1:100, Vector Laboratories, Burlingame, CA) for another 60 min in the dark: horse anti-mouse IgG secondary antibodies were used for  $\beta_1$ -AR, whereas goat anti-rabbit IgG secondary antibodies were used for  $\beta_2$ -AR staining. As a negative control, cells were incubated with secondary antibodies in the absence of primary antibodies. As an additional negative control, another subset of DKO cells cultured without viral infection was treated with the same protocol. Immunofluorescence was then detected by a laser scanning confocal microscope (LSM-410, Zeiss) with optical section thickness of  $1.0 \mu\text{m}$ .

**Measurement of cAMP Accumulation.** After cells were treated with the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 1 mM), for 30 min at  $37^\circ\text{C}$  in a  $\text{CO}_2$  incubator, they were incubated with either  $\beta$ -AR agonists or inverse agonists for 10 min. Cells were then harvested, and cAMP levels were assayed as previously described (Xiao et al., 1999a) with minor modifications. Briefly,  $10 \mu\text{l}$  of membrane vesicles ( $20 \mu\text{g}$  of total protein) were added to a  $40\text{-}\mu\text{l}$  reaction solution to make a final concentration of 4 mM Tris-EDTA and 1 mM IBMX. The reaction was performed at  $37^\circ\text{C}$  for 15

min, and 25  $\mu$ l of supernatant were assayed using a cAMP  $^3$ H assay kit obtained from Amersham (Arlington Heights, IL). Protein content was measured using the Bradford method (Bio-Rad, Richmond, CA) with bovine serum albumin as the standard.

**Materials.** Forskolin, isoproterenol hydrochloride, propranolol, alprenolol, IBMX, and minimal essential medium were purchased from Sigma (St. Louis, MO). ICI 118,551 was kindly supplied by ICI Pharmaceutical Group (Wilmington, DE). CGP 20712A was kindly supplied by CIBA-GEIGY Corp. (East Hanover, NJ). Fetal bovine serum, penicillin-streptomycin, and mouse laminin were purchased from Life Technologies (Gaithersburg, MD). The cAMP assay kit was purchased from Amersham. [ $^{125}$ I]Cyanopindolol was purchased from NEN Life Science Products, Inc. (Boston, MA). Anti-HA monoclonal antibody and  $\beta_2$ -AR polyclonal antibody were purchased from Berkeley Antibody Co. (Berkeley, CA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. The secondary antibodies were purchased from Vector Laboratories.

**Data Analysis.** Data are reported as means  $\pm$  S.E.M. Student's *t* test, paired *t* test, or ANOVA were used, when appropriate, to test for differences among the means. A value of *P* < .05 was considered to be statistically significant.

## Results

**Characterization of  $\beta_1\beta_2$ -AR DKO Mouse Ventricular Myocytes.** Previous *in vivo* studies in the  $\beta_1\beta_2$ -AR DKO mouse model have shown that elimination of both  $\beta_1$ - and  $\beta_2$ -AR has little impact on resting cardiovascular function but completely abolishes the cardiac response to  $\beta$ -AR agonist stimulation (Rohrer et al., 1999). In this study, we further characterized cardiac morphological and physiological properties of the DKO mouse at the single cell level. Ventricular myocytes from adult DKO mice were almost identical with those from WT mice with respect to cell length (Table 1) and membrane capacitance ( $153 \pm 8.3$  pF, *n* = 7, for DKO cells, versus  $158 \pm 15.3$  pF, *n* = 25, for WT controls; *P* > .05). All of the basal contractile properties, including twitch amplitude (TA), time to peak ( $T_{peak}$ ), and time to 50% relaxation ( $T_{50}$ ), were unaltered in DKO as compared with WT cells (Table 1). TA of the first beat after rest, which reflects the maximal contractile reserve, was also similar in DKO ( $9.47 \pm 0.52\%$  of rest cell length, *n* = 23) and WT cells ( $9.19 \pm 0.53\%$  of rest cell length, *n* = 28). After 24 h of culture, steady-state

TA in DKO cells was largely preserved, whereas the kinetics of contraction, including  $T_{50}$  and  $T_{peak}$ , were slowed by 30 to ~40%, as was the case with WT cells (Table 1; also see Zhou et al., 2000).

Consistent with the genotype, there were no detectable  $\beta_1$ - or  $\beta_2$ -receptors in DKO cells as assayed by radioligand-binding assay and confocal immunocytochemical staining (data not shown). The disruption of both  $\beta_1$ - and  $\beta_2$ -AR signaling was confirmed in single myocytes by the lack of a contractile response to the  $\beta$ -AR agonist, isoproterenol (ISO,  $10^{-6}$  M), which activates all known  $\beta$ -AR subtypes (Fig. 1A). This observation is in contrast to a robust inotropic effect of ISO in WT cells (Fig. 1C). To determine whether the downstream  $\beta$ -AR-signaling pathway is adaptively altered in DKO cells, adenylyl cyclase was directly activated by forskolin. As shown in Fig. 1, B and C, forskolin (Fsk,  $10^{-6}$  M) induced a marked increase in contraction amplitude in DKO cells, comparable with that in WT cells. These results indicate that  $\beta_1$ - and  $\beta_2$ -AR are the major functional  $\beta$ -AR subtypes in cardiac myocytes and that DKO cells provide a virtually null  $\beta$ -AR background with intact cAMP-signaling pathway.

**Expression of  $\beta_1$ -AR or  $\beta_2$ -AR Subtype in DKO Myocytes.** To investigate  $\beta_1$ - and  $\beta_2$ -AR subtype signaling individually, we expressed either  $\beta$ -AR subtype in cultured DKO mouse myocytes using adenovirus-mediated gene transfer. The exact level of receptor protein expression was measured by radioligand-binding assay using ICYP. Figure 2 shows that the expression level of either  $\beta_1$ -AR or  $\beta_2$ -AR depended on the titer (m.o.i., 0–1000) of adeno- $\beta_1$ -AR or adeno- $\beta_2$ -AR. The average maximal receptor density was  $1207 \pm 173$  fmol/mg protein for  $\beta_1$ -AR, and  $821 \pm 38$  fmol/mg protein for  $\beta_2$ -AR at m.o.i. 1000. Because  $\beta_1$ -AR is the predominant  $\beta$ -AR subtype ( $\beta_1$ -AR:  $33.5 \pm 1.2$ ;  $\beta_2$ -AR:  $11.9 \pm 1.7$  fmol/mg, *n* = 3) and constitutes ~70% of the total  $\beta$ -ARs in WT cells, the relative increase in  $\beta_2$ -AR over WT control (~69-fold) was greater than that of  $\beta_1$ -AR (~36-fold). In addition, there was no significant change in  $\beta$ -AR affinity for ICYP at different expression levels (Fig. 2). There was no detectable radioligand-binding signal in DKO cells infected with a marker transgene  $\beta$ -galactosidase, as expected.

Furthermore, we examined intracellular distribution of ei-

TABLE 1

Basal cell length and contractile properties of freshly isolated or cultured WT, DKO, and adeno- $\beta_1$ -AR- or adeno- $\beta_2$ -AR-infected DKO mouse ventricular myocytes

	Rest Cell Length	TA	$T_{peak}$	$T_{50}$	<i>n</i>
	$\mu$ m	% cell length	ms	ms	cells/mice
WT					
Fresh	127 $\pm$ 3.1	3.23 $\pm$ 0.21	90.5 $\pm$ 4.5	67.4 $\pm$ 3.9	31/15
Culture	108 $\pm$ 4.1 <sup>a</sup>	3.50 $\pm$ 0.48	122 $\pm$ 3.5 <sup>a</sup>	93.6 $\pm$ 7.8 <sup>a</sup>	26/9
DKO					
Fresh	125 $\pm$ 3.6	3.17 $\pm$ 0.34	95.3 $\pm$ 3.3	77.3 $\pm$ 7.4	31/19
Culture	111 $\pm$ 4.7 <sup>b</sup>	3.21 $\pm$ 0.28	129 $\pm$ 5.8 <sup>b</sup>	100 $\pm$ 3.5 <sup>b</sup>	37/12
$\beta_1$					
$\times 10$	119 $\pm$ 5.7	3.43 $\pm$ 0.86	133 $\pm$ 8.4	108 $\pm$ 19	10/3
$\times 100$	115 $\pm$ 3.9	3.24 $\pm$ 0.36	114 $\pm$ 6.7	88.8 $\pm$ 9.4	27/12
$\times 1000$	108 $\pm$ 4.2	3.74 $\pm$ 0.57	137 $\pm$ 10.7	89 $\pm$ 7.1	12/4
$\beta_2$					
$\times 10$	112 $\pm$ 5.5	3.65 $\pm$ 1.7	129 $\pm$ 2.4	118 $\pm$ 10	13/3
$\times 100$	114 $\pm$ 2.7	5.88 $\pm$ 0.6 <sup>c</sup>	107.7 $\pm$ 3.2 <sup>c</sup>	74.5 $\pm$ 4.5 <sup>c</sup>	42/10
$\times 1000$	112 $\pm$ 4.5	7.87 $\pm$ 0.9 <sup>c</sup>	109.8 $\pm$ 6.5 <sup>d</sup>	64.6 $\pm$ 3.5 <sup>c</sup>	14/3

<sup>a</sup> *P* < .01 versus fresh-isolated WT.

<sup>b</sup> *P* < .01 versus fresh-isolated DKO.

<sup>c</sup> *P* < .01 versus DKO-culture.

<sup>d</sup> *P* < .05 versus DKO-culture.



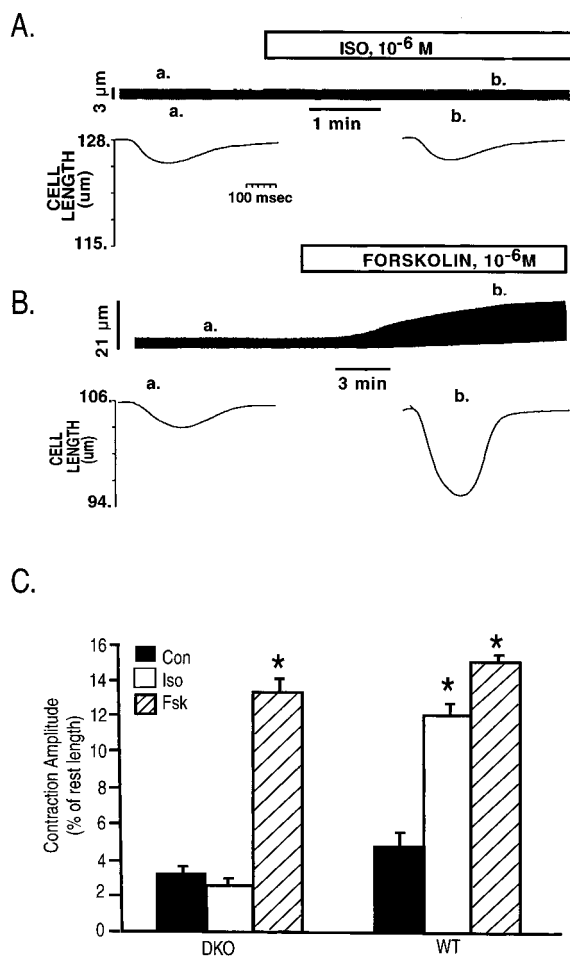
ther  $\beta$ -AR subtype expressed in the DKO mouse myocytes using adenoviral gene transfer (m.o.i. 100). Figure 3 shows that, in the absence of agonist stimulation, specific immunofluorescence for  $\beta_1$ -AR or  $\beta_2$ -AR is largely concentrated on cell surface membranes, including transverse tubules, with little staining of the cytosol, resulting in a clear striated pattern.

To determine whether the expressed receptors couple to downstream signaling pathway and modulate contractility, we examined effects of the nonselective  $\beta$ -AR agonist, ISO, on cellular cAMP accumulation and contraction. ISO ( $10^{-6}$  M) significantly elevated cAMP accumulation in DKO cells infected with either  $\beta_1$ -AR (at m.o.i. 100, from  $10.1 \pm 2.4$  to  $33.8 \pm 6.3$  pmol/mg protein,  $n = 4$ ,  $P < .05$ ) or  $\beta_2$ -AR (at m.o.i. 100, from  $23.2 \pm 7.4$  to  $37.89 \pm 8.42$  pmol/mg protein,  $n = 4$ ,  $P < .05$ ). Concomitantly, stimulation of either  $\beta$ -AR subtype by ISO ( $10^{-6}$  M) markedly augmented TA with a comparable maximal response ( $11.8 \pm 1.1\%$  of rest cell length for adeno- $\beta_1$ -AR at m.o.i. 100,  $n = 10$ ,  $P < .01$ ;  $11.9 \pm 1.0\%$  of rest cell

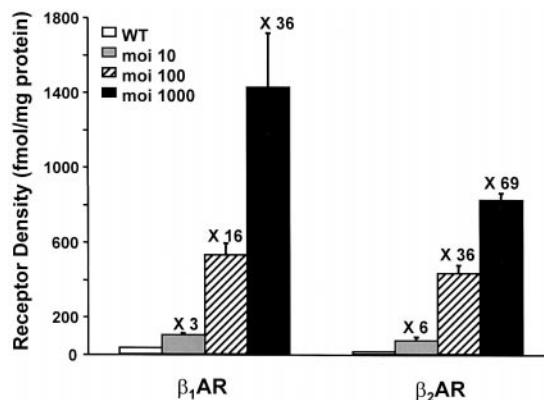
length for Adeno- $\beta_2$ -AR at m.o.i. 100,  $n = 11$ ,  $P < .01$ ), which was selectively blocked by specific  $\beta$ -AR antagonists, CGP or ICI, respectively (Fig. 4). These results indicate that expression of  $\beta_1$ -AR or  $\beta_2$ -AR in DKO myocytes fully restores the functionality of  $\beta$ -AR, thus providing pure  $\beta_1$ -AR and  $\beta_2$ -AR experimental systems.

**Receptor Density and Spontaneous  $\beta$ -AR Subtype Signaling.** Next, we examined spontaneous activation of  $\beta$ -AR subtypes by expressing  $\beta_1$ -AR or  $\beta_2$ -AR over a wide range of receptor density in the null background of DKO myocytes. The possible spontaneous receptor activation was determined using cellular cAMP and contractility as the biochemical and physiological readouts. In DKO cells expressing  $\beta_2$ -AR at m.o.i.  $\geq 100$ , the baseline cAMP was significantly elevated in the absence of ligand (Fig. 5A). Similarly, baseline contraction amplitude was also markedly increased in these cells (Fig. 5B and Table 1). These results are consistent with previous observations in a  $\beta_2$ -AR overexpression transgenic model (Milano et al., 1994; Xiao et al., 1999a). In sharp contrast, overexpression  $\beta_1$ -AR to the same or even higher levels had no effect on either baseline cAMP or contractility (Fig. 5 and Table 1). Furthermore, neither  $T_{50}$  nor  $T_{peak}$  of contraction was altered in cells overexpressing  $\beta_1$ -AR, but both were abbreviated in cells infected with adeno- $\beta_2$ -AR at m.o.i. 100 or 1000 (Table 1). Taken together, these results indicate that ligand-free  $\beta$ -AR signaling is subtype specific, i.e., it is evident for  $\beta_2$ -AR but not for  $\beta_1$ -AR.

**Effects of  $\beta$ -AR Inverse Agonists.** To further characterize ligand-independent  $\beta$ -AR signaling,  $\beta$ -AR inverse agonists were used to inhibit spontaneous receptor activation. A number of  $\beta$ -AR ligands, including ICI (Milano et al., 1994; Bond et al., 1995), CGP (Nagykaldi et al., 1999), and propranolol (Mewes et al., 1993), have been reported as  $\beta$ -AR inverse agonists based on their effects on unliganded receptors. However, the effect and potency of any given ligand are variable depending on the experimental conditions (Chidiac et al., 1996; Guerrero and Minneman, 1999). Taking advantage of the null background of uninfected DKO cells, we first examined possible nonspecific effects of a  $\beta_2$ -AR inverse ag-



**Fig. 1.** Contractile response to a nonselective  $\beta$ -AR agonist, ISO ( $10^{-6}$  M), or to an adenylyl cyclase activator, forskolin (Fsk,  $10^{-6}$  M), in adult WT and  $\beta_1\beta_2$ -AR DKO mouse ventricular myocytes. Forskolin (B), but not ISO (A), increases the contraction amplitude in DKO myocytes. Top panels, continuous chart recordings of the change in cell length. An upward deflection indicates cell shortening. Bottom panels, expanded time scale of the contractile traces at time points indicated in the respective top panel. A downward deflection indicates cell shortening. C, the average data presented as means  $\pm$  S.E.M. Note that the baseline contraction amplitudes were not significantly different in the freshly isolated WT and DKO myocytes (see Table 1). \* $P < .01$  versus control (Con).



**Fig. 2.** Receptor density in crude membranes from cultured WT adult mouse ventricular myocytes or from DKO myocytes with either adeno- $\beta_1$ -AR or adeno- $\beta_2$ -AR (m.o.i. 1–1000) infection. The densities of  $\beta$ -AR subtypes were assayed using ICYP (see *Experimental Procedures*).  $K_d$  values of ICYP are  $71.6 \pm 17.6$ ,  $85.0 \pm 23.1$ , and  $61.0 \pm 16.6$  pmol for adeno- $\beta_1$ -AR at m.o.i. 10, 100, and 1000, respectively.  $K_d$  values of ICYP are  $40.9 \pm 9.6$ ,  $59.8 \pm 9.2$ , and  $46.1 \pm 16.9$  pmol for adeno- $\beta_2$ -AR at m.o.i. 10, 100 and 1000, respectively. Data presented are means  $\pm$  S.E.M. of three independent experiments (nine hearts), each performed in duplicate.

onist, ICI, a  $\beta_1$ -AR inverse agonist, CGP, and the nonselective antagonist, propranolol. Neither the selective antagonists, ICI ( $10^{-7}$  M) and CGP ( $10^{-6}$  M), nor the nonselective antagonist, propranolol, had any significant effect on baseline contraction amplitude in the absence of adenoviral infection ( $-5.6 \pm 4.5$ ,  $9.4 \pm 6.6$ , and  $12.6 \pm 6.4\%$  of control, respectively,  $n = 7$  for ICI and CGP,  $n = 12$  for propranolol).

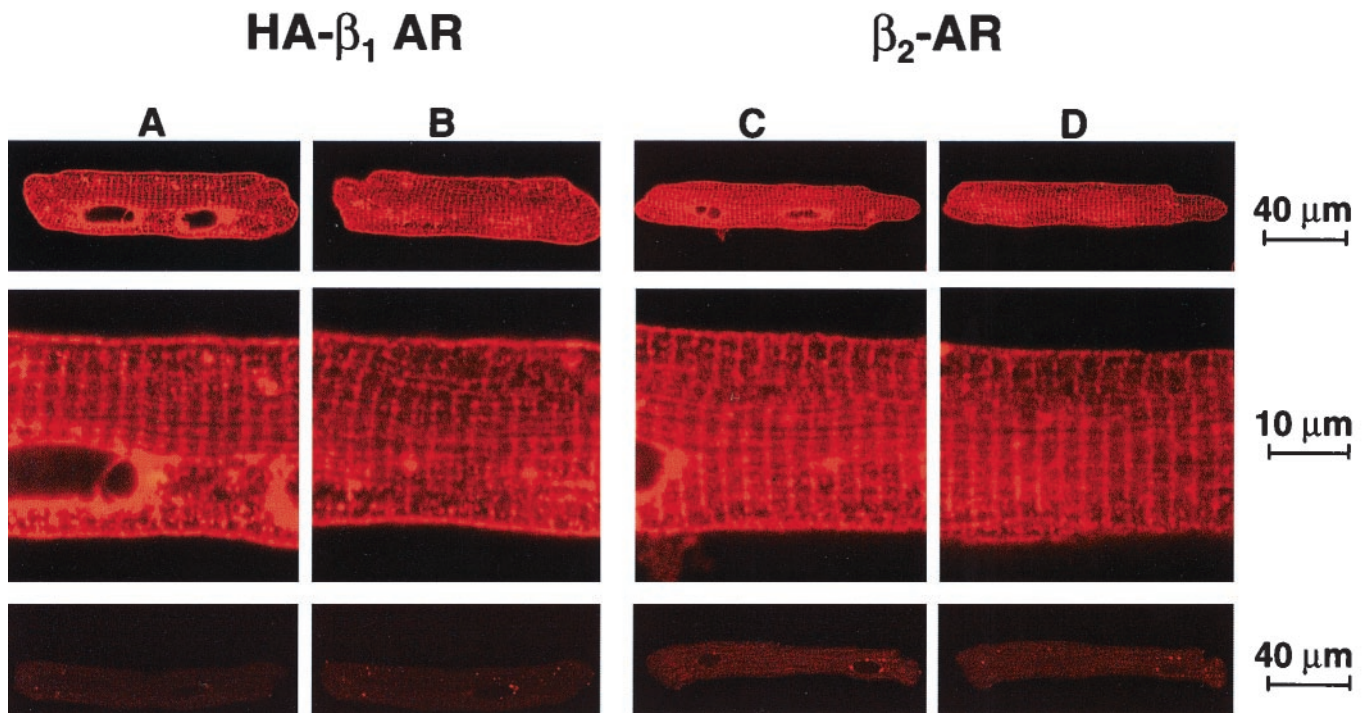
In DKO cells infected with adeno- $\beta_2$ -AR (m.o.i. 100), ICI significantly decreased the baseline contraction amplitude to  $58.4 \pm 2.7\%$  of control ( $n = 7$ ,  $P < .01$ , Fig. 6), confirming the existence of spontaneous  $\beta_2$ -AR activation. Furthermore, ICI fully reversed the enhanced basal cAMP level in DKO cells overexpressing  $\beta_2$ -AR at m.o.i. 100 (Fig. 6C). However, CGP failed to reveal any spontaneous  $\beta_1$ -AR activity, because it had no discernible effect on the baseline contraction amplitude (Fig. 6) in DKO cells infected with adeno- $\beta_1$ -AR at m.o.i. 100. The nonselective  $\beta$ -AR antagonist, propranolol ( $10^{-6}$  M), fully reversed the enhancement of contraction amplitude induced by  $\beta_2$ -AR spontaneous activation without altering the baseline contractility of cells expressing  $\beta_1$ -AR (Fig. 6B). These results reinforce the conclusion that  $\beta$ -AR subtypes exhibit distinctly different spontaneous activity.

## Discussion

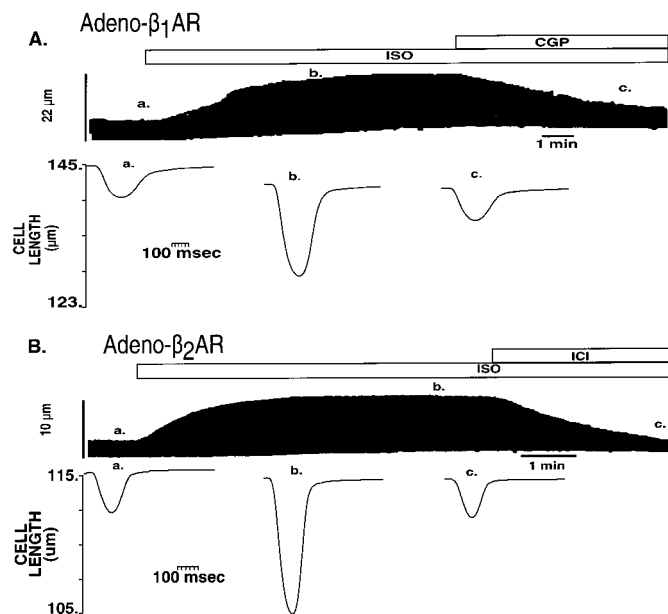
**Subtype-Specific  $\beta$ -AR Spontaneous Activation.** In the present study, we combined the  $\beta_1\beta_2$  DKO mouse model with the adenoviral gene expression technique to create a pure experimental setting to individually study  $\beta$ -AR subtypes in mouse cardiac myocytes over a wide range of receptor density. The primary finding of this study is that overexpression of  $\beta_2$ -AR increases the basal cAMP accumulation

and contraction amplitude in a receptor density-dependent manner and that the  $\beta_2$ -AR inverse agonist, ICI, can fully reverse these changes, with little nonspecific effect in the uninfected DKO myocytes. The observations in cardiac myocytes acutely overexpressing  $\beta_2$ -AR are, therefore, in agreement with previous findings in the transgenic model chronically overexpressing  $\beta_2$ -AR (Milano et al., 1994; Xiao et al., 1999a). Thus, both in vivo and in vitro, acute or chronic overexpression of  $\beta_2$ -AR reveal constitutive activity of the receptor in mouse ventricular myocytes. Because basal cAMP level and contractility are concomitantly enhanced, the positive inotropic effect induced by spontaneous  $\beta_2$ -AR activation is likely mediated by the cAMP-signaling pathway, as is the case in TG4 mice (Xiao et al., 1999a).

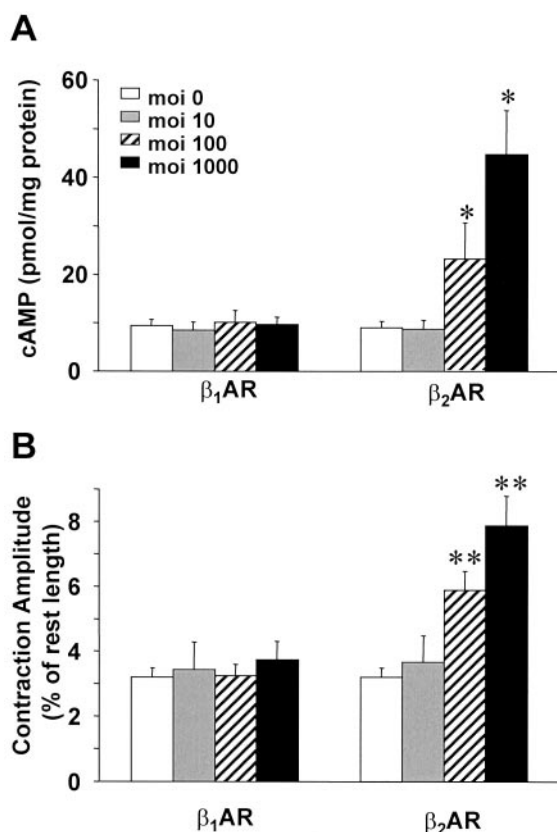
To our surprise, the property of spontaneous activation is not shared by  $\beta_1$ -AR, the dominant  $\beta$ -AR subtype in the heart. Overexpression of  $\beta_1$ -AR to similar or even greater levels has virtually no effect on basal cAMP, contraction amplitude, or contractile kinetics (Table 1). Furthermore, the alleged  $\beta_1$ -AR inverse agonist, CGP, does not affect the basal cAMP level or contractility in myocytes overexpressing  $\beta_1$ -AR (Fig. 6). These observations are consistent with the results from transgenic mice overexpressing  $\beta_1$ -AR, although the  $\beta_1$ -AR level (5–15 fold over WT) in the transgenic model (Engelhardt et al., 1999) was considerably lower compared with that in the present study. Because the present experimental setting avoids the potential nonspecificity of  $\beta$ -AR agonists or antagonists, the complicated interactions between  $\beta_1$ - and  $\beta_2$ -AR subtypes, and the possible compensatory changes in transgenic models, the present results provide evidence that the  $\beta_1$ -AR, unlike  $\beta_2$ -AR, does not undergo



**Fig. 3.** Intracellular distribution of  $\beta_1$ -AR (A and B) or  $\beta_2$ -AR (C and D) in adult mouse cardiac myocytes infected by either adeno- $\beta_1$ -AR or adeno- $\beta_2$ -AR (m.o.i. 100). A and C, images taken from medium optical sections; B and D, images taken from cortical sections. The top panel shows whole cell images. The immunofluorescent signal of either  $\beta_1$ -AR or  $\beta_2$ -AR is mostly localized to the sarcolemmal membrane including transverse tubules, giving rise to a striated appearance. The middle panel shows enlarged views to better illustrate  $\beta$ -AR subtype intracellular distribution pattern. The bottom panel shows negative controls obtained in the absence of any primary antibody.



**Fig. 4.** Contractile response to a mixed  $\beta$ -AR agonist, ISO ( $10^{-6}$  M), and a specific antagonist for  $\beta_1$ -AR (CGP,  $10^{-6}$  M) or for  $\beta_2$ -AR (ICI,  $10^{-7}$  M) in DKO myocytes infected with adeno- $\beta_1$ -AR (A) or adeno- $\beta_2$ -AR at m.o.i. 100 (B). Cell shortening is indicated by the upward deflection in continuous chart recordings (top) or the downward deflection in the contractile traces obtained at the indicated time points.

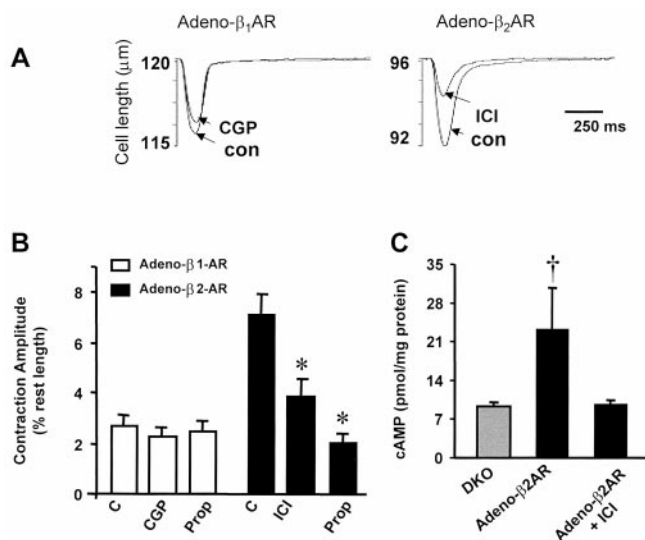


**Fig. 5.** Average baseline cAMP accumulation (A) and contraction amplitude (B) in DKO myocytes infected with adeno- $\beta_1$ -AR or  $\beta_2$ -AR at various multiplicities of infection (m.o.i.) ( $n = 4-11$  for cAMP assay; and  $n = 10-43$  for each contraction amplitude measurements). \* $P < .05$  versus control (m.o.i. 0); \*\* $P < .01$  versus control (m.o.i. 0).

spontaneous activation in mouse cardiac myocytes. Similar findings were observed in cultured (wild-type) rat ventricular myocytes overexpressing  $\beta_1$ -AR or  $\beta_2$ -AR (R.-P. Xiao and S.-J. Zhang, unpublished observations).

However, it might be argued that  $\beta_1$ -AR desensitization due to spontaneous activation might account for the lack of changes in basal cAMP and contractile parameters in myocytes overexpressing  $\beta_1$ -AR and the apparent absence of  $\beta_1$ -AR spontaneous activity. However, several lines of evidence argue against this hypothesis. First,  $\beta_1$ -AR desensitization is not evident under our experimental conditions, because ISO stimulation augmented contractility (Fig. 4), with an  $EC_{50}$  in the nanomolar range (data not shown). Second, the ligand-binding results indicate that the  $K_d$  of ICYP for  $\beta_1$ -AR is similar to that for  $\beta_2$ -AR (Fig. 2, legend), further suggesting that the affinity of  $\beta_1$ -AR to ligand remains intact in our experimental system. Because receptor internalization is an important desensitization mechanism, we also examined whether overexpressing  $\beta_1$ -AR causes receptor internalization. The confocal imaging data further exclude the possibility of receptor desensitization, because most of  $\beta_1$ -ARs as well as  $\beta_2$ -ARs are retained on the sarcolemmal and transverse tubule membranes in the absence of agonist stimulation (Fig. 3).

Thus, the capacity to manifest spontaneous activity may not exist in all GPCRs. In agreement with our findings, the highly conserved gonadotropin receptors, luteinizing hormone receptor, and follicle-stimulating hormone receptor, have markedly different constitutive activity (Kudo et al., 1996; Schulz et al., 1999). In addition, dopamine receptor subtypes 1A and 1B also exhibit strikingly different constitutive activity, and a point mutation in the third intracellular loop completely abolishes this difference (Tiberi and Caron, 1994; Charpentier et al., 1996). In fact, the amino acid sequence of the third intracellular loop of  $\beta_1$ -AR also markedly



**Fig. 6.** Effect of  $\beta$ -AR inverse agonists on baseline contractility and cAMP in DKO myocytes expressing  $\beta_1$ -AR or  $\beta_2$ -AR. A, representative contractile traces recorded in adeno- $\beta_1$ -AR (left) or adeno- $\beta_2$ -AR (right) infected myocytes, before (con) and 5 min after application of inverse agonists, CGP ( $10^{-6}$  M) or ICI ( $10^{-7}$  M), respectively. A downward deflection indicates cell shortening. B, average data on basal contraction amplitude, \* $P < .01$  versus control (con;  $n = 4-15$ ). C, average data on cAMP accumulation. † $P < .05$  versus other groups ( $n = 4-11$ ).



differs from that of  $\beta_2$ -AR (Green and Liggett, 1994). The importance of this difference in determining the constitutive activation of  $\beta$ -AR subtypes merits further investigations. It is interestingly to note that the propensity of a receptor to undergo spontaneous activation is not necessarily related to the capacity to create a constitutively active mutant of the receptor. For example, both  $\beta_1$ -AR and  $\beta_2$ -AR can be readily mutated to generate constitutive activity (Samama et al., 1993; Lattion et al., 1999), even though they exhibit markedly different magnitudes of spontaneous activity, as evidenced by the present results.

**Physiological Relevance of Spontaneous  $\beta_2$ -AR Signaling.** As shown in Table 1 and Fig. 5, the baseline contractility was proportionally enhanced with increasing  $\beta_2$ -AR density. For example, at the maximal receptor density (adeno- $\beta_2$ -AR, 1000), the basal contraction amplitude is  $\sim 66\%$  of ISO-induced maximal contraction amplitude in WT cells (Fig. 1C; Fig. 5B). Because a relatively high receptor density of  $\beta_2$ -AR (69-fold over WT) is required in this case, it is inferred that only a small fraction of  $\beta_2$ -ARs exhibits spontaneous activity at any given time. Alternatively, the spontaneously activated receptors may have a lower intrinsic efficacy than the ligand-stimulated receptors. Although ligand-free  $\beta_2$ -AR signal exerts little effect on cardiac contractility under physiological conditions, an overexpression of  $\beta_2$ -AR may still provide a potential therapeutic strategy to provide contractile support for the failing heart. Recent studies have demonstrated that cardiac-specific overexpression of  $\beta_2$ -AR markedly augments cardiac function in an agonist-independent manner, without causing cellular or cardiac hypertrophy (Milano et al., 1994; Bond et al., 1995; Xiao et al., 1999a). Moreover, a 30-fold overexpression of  $\beta_2$ -AR not only rescues cardiac function but also reverses cardiac hypertrophy induced by  $G_{\alpha_q}$  overexpression (Dorn et al., 1999). In contrast, increasing evidence has demonstrated that there is an inverse relationship between plasma norepinephrine levels and survival in patients with chronic heart failure (Cohn et al., 1984) and that  $\beta$ -AR blockade provides salutary effects on morbidity and mortality in patients with heart failure (Eichhorn and Bristow, 1996). In addition, cardiac transgenic overexpression of  $\beta_1$ -AR by 5- to 15-fold in mice leads to marked myocyte hypertrophy, accompanied by fibrosis within a few weeks after birth and heart failure within a few months (Engelhardt et al., 1999). These studies support the idea that spontaneous  $\beta_2$ -AR activity may have important implications in gene therapy of chronic heart failure (Drazner et al., 1997; Maurice et al., 1999). In fact, our recent studies have shown that spontaneous  $\beta_2$ -AR activation provides the heart contractile support via an  $I_{Ca}$ -independent signaling pathway (Zhou et al., 1999b). This unique property may also contribute to the normal phenotype of  $\beta_2$ -AR overexpression transgenic models (Milano et al., 1994; Bond et al., 1995; Xiao et al., 1999a).

**Diversity of  $\beta$ -AR Subtype Signaling.** The present finding that  $\beta_1$ -AR and  $\beta_2$ -AR differ in their ability to undergo spontaneous activation provides another line of evidence for the diversity of  $\beta$ -AR subtype signaling. Our previous studies have shown that ligand-activated  $\beta_2$ -AR, but not  $\beta_1$ -AR, stimulates  $G_i$  proteins in addition to  $G_s$  (Xiao et al., 1995, 1999a), resulting in opposing effects on cardiac contractility. The efficiency of the coupling of  $\beta_2$ -AR to  $G_i$ , to a large extent, appears to underlie the species-dependent differences in car-

diac  $\beta_2$ -AR functional roles (for review see Xiao et al., 1999b). For example, in WT mouse cardiac myocytes,  $\beta_2$ -AR stimulation elicits a robust contractile response only after  $G_i$  function is inhibited via pertussis toxin (PTX)-mediated rebosylation, whereas in rat and canine cardiac myocytes,  $\beta_2$ -AR contractile responses are present but can be further enhanced by PTX treatment. In this regard, it is surprising to find that, unlike native WT mouse  $\beta_2$ -AR or the chronically overexpressed human  $\beta_2$ -AR in transgenic mice (TG4 mice) (Xiao et al., 1999b), stimulation of the acutely expressed  $\beta_2$ -AR in mouse ventricular myocytes with ISO elicits a robust positive inotropic response in the absence of PTX treatment (Fig. 4). It is also noteworthy that  $\beta_2$ -AR in noninnervated rat neonatal cardiomyocytes exhibits a much lower PTX sensitivity compared with that in innervated adult rat cardiomyocytes (Steinberg et al. 1999). The reduced  $\beta_2$ -AR sensitivity to PTX treatment in both acutely expressed receptor in vitro and the noninnervated neonatal myocytes suggests that innervation or chronic receptor stimulation may play an essential role in promoting the receptor- $G_i$  coupling. Additionally, the acutely expressed and chronically expressed  $\beta_2$ -ARs may have different intracellular distributions with different accessibility to other signaling components, such as G proteins. Thus, the diversity of receptor signaling not only depends on the receptor subtypes and conformational states but also relates to cell types and cell conditions.

In summary, the present results reveal another facet of the intrinsic difference between  $\beta_1$ - and  $\beta_2$ -AR, in addition to their distinct ligand-induced activation: the  $\beta_2$ -AR, but not  $\beta_1$ -AR, exhibits spontaneous activation in a receptor density-dependent manner. This finding provides new insights into the signaling diversity of these closely related receptor subtypes in the physiological context of a cardiac myocyte.

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