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Spontaneous Activation of β_2- but Not β_1-Adrenoceptors Expressed in Cardiac Myocytes from $\beta_1\beta_2$ Double Knockout Mice

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ABSTRACT

Although ligand-free, constitutive β_2-adrenergic receptor (AR) signaling has been demonstrated in naive cell lines and in transgenic mice overexpressing cardiac β_2-AR, it is unclear whether the dominant cardiac β-AR subtype, β_1-AR, shares the ability of spontaneous activation. In the present study, we expressed human β_1- or β_2-AR via recombinant adenoviral infection in ventricular myocytes isolated from $\beta_1\beta_2$-AR double knockout mice, creating pure β_1-AR and β_2-AR systems with variable receptor densities. A contractile response to a nonselective β-AR agonist, isoproterenol, was absent in double knockout mouse myocytes but was fully restored after adenoviral β_1-AR or adenoviral β_2-AR infection. Increasing the titer of adenoviral vectors (multiplicity of infection 10–1000) led to a dose-dependent expression of β_1- or β_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 cellular distribution of β_1-AR and β_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, β_2-AR expression resulted in graded increases in baseline cAMP and contractility up to 428% and 233% of control, respectively, at the maximal β_2-AR density. These effects were specifically reversed by a β_2-AR inverse agonist, ICI 118,551 (10^{-7} M). In contrast, overexpression of β_1-AR, even at a greater density, failed to enhance either basal cAMP or contractility; the alleged β_1-AR inverse agonist, CGP 20712A (10^{-6} M), had no significant effect on basal contraction in these cells. Thus, we conclude that acute β_2-AR overexpression in cardiac myocytes elicits significant physiological responses due to spontaneous receptor activation; however, this property is β-AR subtype specific because β_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. β-Adrenergic receptor (β-AR) is a prototypical GPCR. At least two β-AR subtypes, β_1-AR and β_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 β-AR subtypes, recent studies have shown that β-AR subtypes play strikingly different functional roles via distinct signaling pathways in the heart. In particular, β_2-AR, but not β_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; β-AR, β-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, [^{125}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 β_2-AR has been elegantly demonstrated in several systems overexpressing wild-type (WT) β_2-AR (Chidiac et al., 1994; Milano et al., 1994; Bond et al., 1995) or expressing the constitutively active mutant of β_2-AR (Samama et al., 1993). For example, ligand-free cardiac β_2-AR activation is evidenced by significantly increased basal adenylyl cyclase activity, cAMP accumulation, or contractility in the absence of any ligand, and the β_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 β_1-AR shares the ability of spontaneous activation is still controversial. In transgenic mice overexpressing β_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 β_1- and β_2-AR antagonist, but not β_2-AR antagonists, induces a marked decrease in basal L-type Ca^{2+} current in the absence of ligand (Mewes et al., 1993). More recently, it has been shown that, in canine cardiac myocytes, a β_1-AR antagonist, CGP 20712A (CGP), inhibited the basal I_{Ca} by 27% (Nagykaldi et al., 1999). These results were interpreted to indicate spontaneous activation of β_1-AR.

The overall goal of the present study was to examine whether β-AR subtypes are different in terms of their propensity of spontaneous activation. To avoid complicated interactions between β-AR subtypes, because of the lack of absolutely selective β-AR subtype ligands, we took advantage of a recently developed β_1- and β_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 β-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 β-AR subtype inverse agonists. Our results indicate that overexpression of β_2-AR is associated with a robust increase in basal cAMP accumulation and contractility, which can be specifically reversed by the β_2-AR inverse agonist, ICI. Surprisingly, overexpression of β_1-AR to the same or even greater density has no effect on basal cAMP and contractility. These results indicate that β_1-AR, unlike β_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 Ca^{2+}, 2.5% fetal bovine serum (FBS), and 1% penicillin-streptomycin and then plated at 0.5 to $\sim 1 \times 10^4/cm^2$ with the same medium in the culture dishes precoated with 10 μg/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): $CaCl_2$ 1, NaCl 137, KCl 5.4, dextrose 15, $MgSO_4$ 1.3, NaH_2PO_4 1.2, and HEPES 20, pH 7.4 adjusted with 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°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_{peak} was measured as the time from stimulation to peak shortening; T_{50} was measured as the time from the peak to 50% relaxation.

Riadioligand-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. Membranes were resuspended in binding buffer (75 mM Tris·HCl, pH 7.4, 12.5 mM $MgCl_2$, 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]cyanopindolol (ICYP). Nonspecific binding was determined in the presence of 10 μM propranolol. Reactions were conducted in 250 μl of binding buffer at 37°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_{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-β_1-AR tagged with hemagglutinin (HA) or adeno-β_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% NaN_3 in PBS, pH 7.4). Then, cells were incubated for 60 min at room temperature with primary antibodies for HA-tagged β_1-AR (anti-HA monoclonal antibody diluted by 1:500) or for β_2-AR (β_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 β_1-AR, whereas goat anti-rabbit IgG secondary antibodies were used for β_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 μm.

Measurement of cAMP Accumulation. After cells were treated with the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 1 mM), for 30 min at 37°C in a CO_2 incubator, they were incubated with either β-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 μl of membrane vesicles (20 μg of total protein) were added to a 40-μl reaction solution to make a final concentration of 4 mM Tris-EDTA and 1 mM IBMX. The reaction was performed at 37°C for 15

min, and 25 μl 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 Pharmaceutic 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 β_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 ± 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 β_1- and β_2-AR has little impact on resting cardiovascular function but completely abolishes the cardiac response to β-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 ± 8.3 pF, $n = 7$, for DKO cells, versus 158 ± 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 ± 0.52% of rest cell length, $n = 23$) and WT cells (9.19 ± 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 β_1- or β_2-receptors in DKO cells as assayed by radioligand-binding assay and confocal immunocytochemical staining (data not shown). The disruption of both β_1- and β_2-AR signaling was confirmed in single myocytes by the lack of a contractile response to the β-AR agonist, isoproterenol (ISO, 10^{-6} M), which activates all known β-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 β-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 β_1- and β_2-AR are the major functional β-AR subtypes in cardiac myocytes and that DKO cells provide a virtually null β-AR background with intact cAMP-signaling pathway.

Expression of β_1-AR or β_2-AR Subtype in DKO Myocytes. To investigate β_1- and β_2-AR subtype signaling individually, we expressed either β-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 β_1-AR or β_2-AR depended on the titer (m.o.i., 0–1000) of adeno-β_1-AR or adeno-β_2-AR. The average maximal receptor density was 1207 ± 173 fmol/mg protein for β_1-AR, and 821 ± 38 fmol/mg protein for β_2-AR at m.o.i. 1000. Because β_1-AR is the predominant β-AR subtype (β_1-AR: 33.5 ± 1.2; β_2-AR: 11.9 ± 1.7 fmol/mg, $n = 3$) and constitutes ~70% of the total β-ARs in WT cells, the relative increase in β_2-AR over WT control (~69-fold) was greater than that of β_1-AR (~36-fold). In addition, there was no significant change in β-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 β-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-β_1-AR- or adeno-β_2-AR-infected DKO mouse ventricular myocytes

	Rest Cell Length	TA	T_{peak}	T_{50}	*n*
	μm	*% cell length*	*ms*	*ms*	*cells/mice*
WT					
Fresh	127 ± 3.1	3.23 ± 0.21	90.5 ± 4.5	67.4 ± 3.9	31/15
Culture	108 ± 4.1[a]	3.50 ± 0.48	122 ± 3.5[a]	93.6 ± 7.8[a]	26/9
DKO					
Fresh	125 ± 3.6	3.17 ± 0.34	95.3 ± 3.3	77.3 ± 7.4	31/19
Culture	111 ± 4.7[b]	3.21 ± 0.28	129 ± 5.8[b]	100 ± 3.5[b]	37/12
β_1					
×10	119 ± 5.7	3.43 ± 0.86	133 ± 8.4	108 ± 19	10/3
×100	115 ± 3.9	3.24 ± 0.36	114 ± 6.7	88.8 ± 9.4	27/12
×1000	108 ± 4.2	3.74 ± 0.57	137 ± 10.7	89 ± 7.1	12/4
β_2					
×10	112 ± 5.5	3.65 ± 1.7	129 ± 2.4	118 ± 10	13/3
×100	114 ± 2.7	5.88 ± 0.6[c]	107.7 ± 3.2[c]	74.5 ± 4.5[c]	42/10
×1000	112 ± 4.5	7.87 ± 0.9[c]	109.8 ± 6.5[d]	64.6 ± 3.5[c]	14/3

[a] $P < .01$ versus fresh-isolated WT.
[b] $P < .01$ versus fresh-isolated DKO.
[c] $P < .01$ versus DKO-culture.
[d] $P < .05$ versus DKO-culture.

ther β-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 β_1-AR or β_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 β-AR agonist, ISO, on cellular cAMP accumulation and contraction. ISO (10^{-6} M) significantly elevated cAMP accumulation in DKO cells infected with either β_1-AR (at m.o.i. 100, from 10.1 ± 2.4 to 33.8 ± 6.3 pmol/mg protein, $n = 4$, $P < .05$) or β_2-AR (at m.o.i. 100, from 23.2 ± 7.4 to 37.89 ± 8.42 pmol/mg protein, $n = 4$, $P < .05$). Concomitantly, stimulation of either β-AR subtype by ISO (10^{-6} M) markedly augmented TA with a comparable maximal response (11.8 ± 1.1% of rest cell length for adeno-β_1-AR at m.o.i. 100, $n = 10$, $P < .01$; 11.9 ± 1.0% of rest cell length for Adeno-β_2-AR at m.o.i. 100, $n = 11$, $P < .01$), which was selectively blocked by specific β-AR antagonists, CGP or ICI, respectively (Fig. 4). These results indicate that expression of β_1-AR or β_2-AR in DKO myocytes fully restores the functionality of β-AR, thus providing pure β_1-AR and β_2-AR experimental systems.

Receptor Density and Spontaneous β-AR Subtype Signaling. Next, we examined spontaneous activation of β-AR subtypes by expressing β_1-AR or β_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 β_2-AR at m.o.i. ≥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 β_2-AR overexpression transgenic model (Milano et al., 1994; Xiao et al., 1999a). In sharp contrast, overexpression β_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 β_1-AR, but both were abbreviated in cells infected with adeno-β_2-AR at m.o.i. 100 or 1000 (Table 1). Taken together, these results indicate that ligand-free β-AR signaling is subtype specific, i.e., it is evident for β_2-AR but not for β_1-AR.

Effects of β-AR Inverse Agonists. To further characterize ligand-independent β-AR signaling, β-AR inverse agonists were used to inhibit spontaneous receptor activation. A number of β-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 β-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 β_2-AR inverse ag-

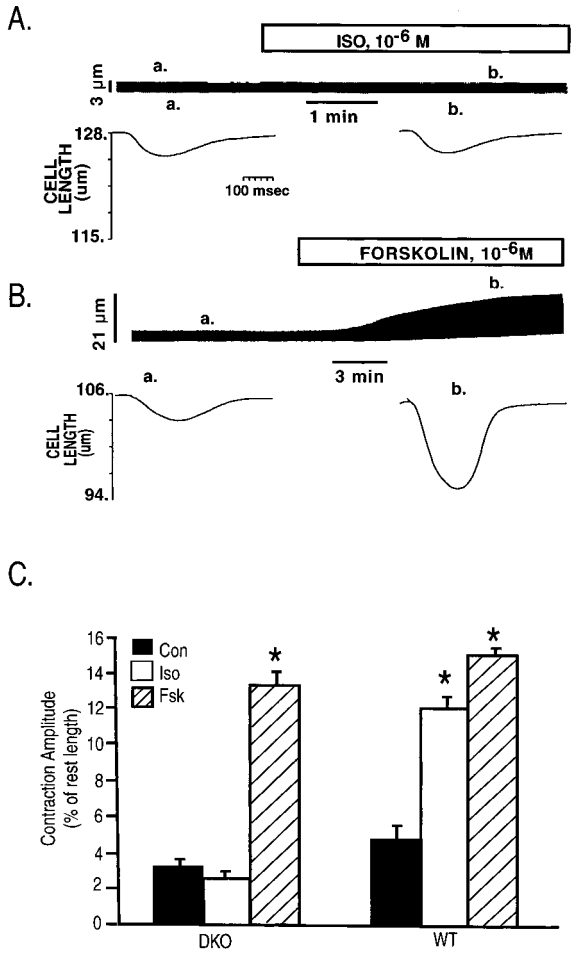

Fig. 1. Contractile response to a nonselective β-AR agonist, ISO (10^{-6} M), or to an adenylyl cyclase activator, forskolin (Fsk, 10^{-6} M), in adult WT and $\beta_1\beta_2$-AR DKO mouse ventricular myocytes. Forskolin (B), but not ISO (A), increases the contraction amplitude in DKO myocytes. Top panels, 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 ± 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).

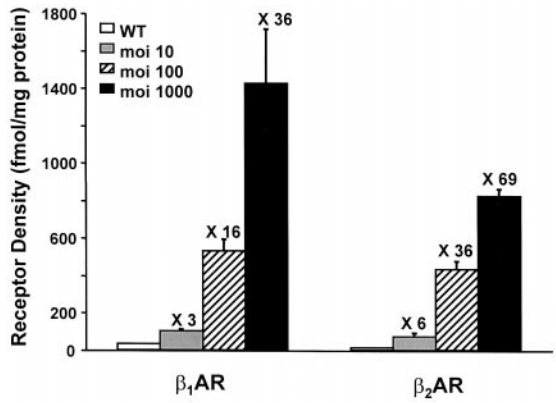

Fig. 2. Receptor density in crude membranes from cultured WT adult mouse ventricular myocytes or from DKO myocytes with either adeno-β_1-AR or adeno-β_2-AR (m.o.i. 1–1000) infection. The densities of β-AR subtypes were assayed using ICYP (see *Experimental Procedures*). K_d values of ICYP are 71.6 ± 17.6, 85.0 ± 23.1, and 61.0 ± 16.6 pmol for adeno-β_1-AR at m.o.i. 10, 100, and 1000, respectively. K_d values of ICYP are 40.9 ± 9.6, 59.8 ± 9.2, and 46.1 ± 16.9 pmol for adeno-β_2-AR at m.o.i. 10, 100 and 1000, respectively. Data presented are means ± S.E.M. of three independent experiments (nine hearts), each performed in duplicate.

onist, ICI, a β_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 ± 4.5, 9.4 ± 6.6, and 12.6 ± 6.4% of control, respectively, $n = 7$ for ICI and CGP, $n = 12$ for propranolol).

In DKO cells infected with adeno-β_2-AR (m.o.i. 100), ICI significantly decreased the baseline contraction amplitude to 58.4 ± 2.7% of control ($n = 7$, $P < .01$, Fig. 6), confirming the existence of spontaneous β_2-AR activation. Furthermore, ICI fully reversed the enhanced basal cAMP level in DKO cells overexpressing β_2-AR at m.o.i. 100 (Fig. 6C). However, CGP failed to reveal any spontaneous β_1-AR activity, because it had no discernible effect on the baseline contraction amplitude (Fig. 6) in DKO cells infected with adeno-β_1-AR at m.o.i. 100. The nonselective β-AR antagonist, propranolol (10^{-6} M), fully reversed the enhancement of contraction amplitude induced by β_2-AR spontaneous activation without altering the baseline contractility of cells expressing β_1-AR (Fig. 6B). These results reinforce the conclusion that β-AR subtypes exhibit distinctly different spontaneous activity.

Discussion

Subtype-Specific β-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 β-AR subtypes in mouse cardiac myocytes over a wide range of receptor density. The primary finding of this study is that overexpression of β_2-AR increases the basal cAMP accumulation and contraction amplitude in a receptor density-dependent manner and that the β_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 β_2-AR are, therefore, in agreement with previous findings in the transgenic model chronically overexpressing β_2-AR (Milano et al., 1994; Xiao et al., 1999a). Thus, both in vivo and in vitro, acute or chronic overexpression of β_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 β_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 β_1-AR, the dominant β-AR subtype in the heart. Overexpression of β_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 β_1-AR inverse agonist, CGP, does not affect the basal cAMP level or contractility in myocytes overexpressing β_1-AR (Fig. 6). These observations are consistent with the results from transgenic mice overexpressing β_1-AR, although the β_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 β-AR agonists or antagonists, the complicated interactions between β_1- and β_2-AR subtypes, and the possible compensatory changes in transgenic models, the present results provide evidence that the β_1-AR, unlike β_2-AR, does not undergo

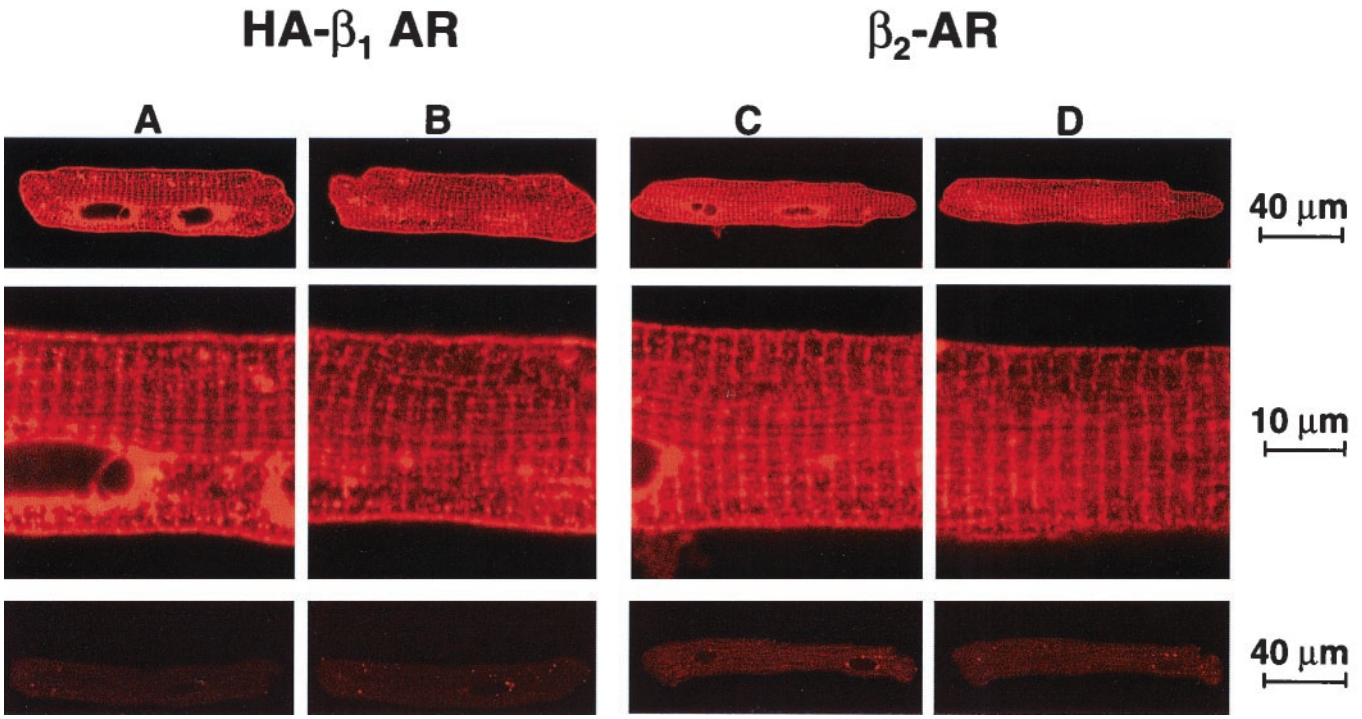

Fig. 3. Intracellular distribution of β_1-AR (A and B) or β_2-AR (C and D) in adult mouse cardiac myocytes infected by either adeno-β_1-AR or adeno-β_2-AR (m.o.i. 100). A and C, images taken from medium optical sections; B and D, images taken from cortical sections. The top panel shows whole cell images. The immunofluorescent signal of either β_1-AR or β_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 β-AR subtype intracellular distribution pattern. The bottom panel shows negative controls obtained in the absence of any primary antibody.

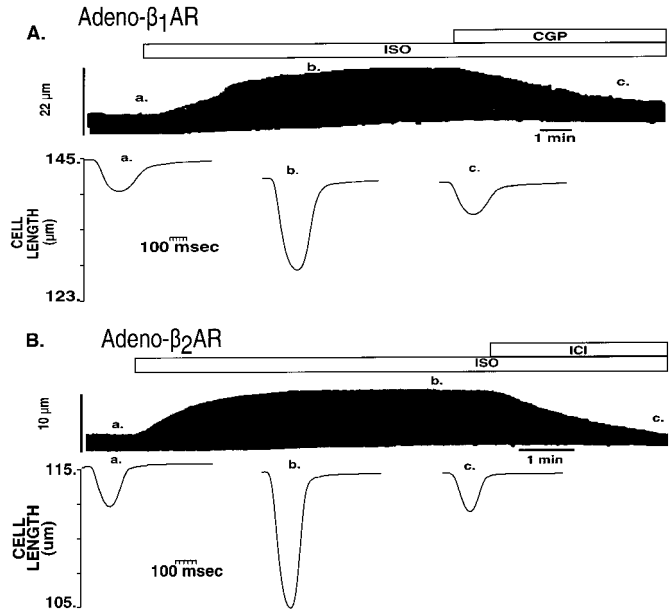

Fig. 4. Contractile response to a mixed β-AR agonist, ISO (10^{-6} M), and a specific antagonist for β_1-AR (CGP, 10^{-6} M) or for β_2-AR (ICI, 10^{-7} M) in DKO myocytes infected with adeno-β_1-AR (A) or adeno-β_2-AR at m.o.i. 100 (B). Cell shortening is indicated by the upward deflection in continuous chart recordings (top) or the downward deflection in the contractile traces obtained at the indicated time points.

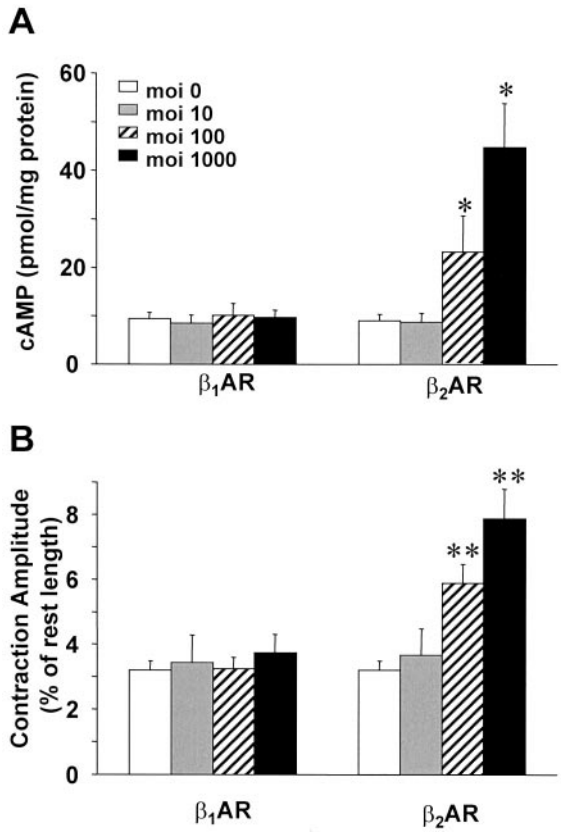

Fig. 5. Average baseline cAMP accumulation (A) and contraction amplitude (B) in DKO myocytes infected with adeno-β_1-AR or β_2-AR at various multiplicities of infection (m.o.i.) (n = 4–11 for cAMP assay; and n = 10–43 for each contraction amplitude measurements). *$P < .05$ versus control (m.o.i. 0); **$P < .01$ versus control (m.o.i. 0).

spontaneous activation in mouse cardiac myocytes. Similar findings were observed in cultured (wild-type) rat ventricular myocytes overexpressing β_1-AR or β_2-AR (R.-P. Xiao and S.-J. Zhang, unpublished observations).

However, it might be argued that β_1-AR desensitization due to spontaneous activation might account for the lack of changes in basal cAMP and contractile parameters in myocytes overexpressing β_1-AR and the apparent absence of β_1-AR spontaneous activity. However, several lines of evidence argue against this hypothesis. First, β_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 β_1-AR is similar to that for β_2-AR (Fig. 2, legend), further suggesting that the affinity of β_1-AR to ligand remains intact in our experimental system. Because receptor internalization is an important desensitization mechanism, we also examined whether overexpressing β_1-AR causes receptor internalization. The confocal imaging data further exclude the possibility of receptor desensitization, because most of β_1-ARs as well as β_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 β_1-AR also markedly

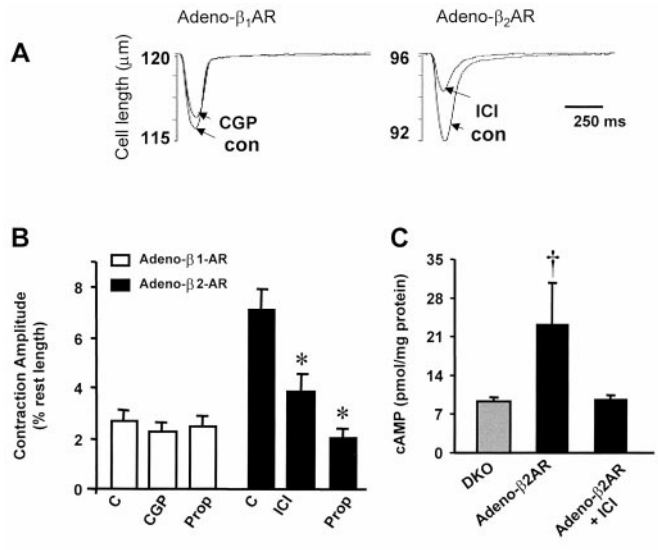

Fig. 6. Effect of β-AR inverse agonists on baseline contractility and cAMP in DKO myocytes expressing β_1-AR or β_2-AR. A, representative contractile traces recorded in adeno-β_1-AR (left) or adeno-β_2-AR (right) infected myocytes, before (con) and 5 min after application of inverse agonists, CGP (10^{-6} 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 β_2-AR (Green and Liggett, 1994). The importance of this difference in determining the constitutive activation of β-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 β_1-AR and β_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 β_2-AR Signaling. As shown in Table 1 and Fig. 5, the baseline contractility was proportionally enhanced with increasing β_2-AR density. For example, at the maximal receptor density (adeno-β_2-AR, 1000), the basal contraction amplitude is ~66% of ISO-induced maximal contraction amplitude in WT cells (Fig. 1C; Fig. 5B). Because a relatively high receptor density of β_2-AR (69-fold over WT) is required in this case, it is inferred that only a small fraction of β_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 β_2-AR signal exerts little effect on cardiac contractility under physiological conditions, an overexpression of β_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 β_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 β_2-AR not only rescues cardiac function but also reverses cardiac hypertrophy induced by $G_{q\alpha}$ 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 β-AR blockade provides salutary effects on morbidity and mortality in patients with heart failure (Eichhorn and Bristow, 1996). In addition, cardiac transgenic overexpression of β_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 β_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 β_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 β_2-AR overexpression transgenic models (Milano et al., 1994; Bond et al., 1995; Xiao et al., 1999a).

Diversity of β-AR Subtype Signaling. The present finding that β_1-AR and β_2-AR differ in their ability to undergo spontaneous activation provides another line of evidence for the diversity of β-AR subtype signaling. Our previous studies have shown that ligand-activated β_2-AR, but not β_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 β_2-AR to G_i, to a large extent, appears to underlie the species-dependent differences in cardiac β_2-AR functional roles (for review see Xiao et al., 1999b). For example, in WT mouse cardiac myocytes, β_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, β_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 β_2-AR or the chronically overexpressed human β_2-AR in transgenic mice (TG4 mice) (Xiao et al., 1999b), stimulation of the acutely expressed β_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 β_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 β_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 β_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 β_1- and β_2-AR, in addition to their distinct ligand-induced activation: the β_2-AR, but not β_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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