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Spontaneous β_2-Adrenergic Signaling Fails To Modulate L-Type Ca^{2+} Current in Mouse Ventricular Myocytes

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ABSTRACT

A receptor can be activated either by specific ligand-directed changes in conformation or by intrinsic, spontaneous conformational change. In the β_2-adrenergic receptor (AR) overexpression transgenic (TG4) murine heart, spontaneously activated β_2AR (β_2-R*) in the absence of ligands has been evidenced by elevated basal adenylyl cyclase activity and cardiac function. In the present study, we determined whether the signaling mediated by β_2-R* differs from that of a ligand-elicited β_2AR activation (β_2-**L**R*). In ventricular myocytes from TG4 mice, the properties of L-type Ca^{2+} current (I_{Ca}), a major effector of β_2-**L**R* signaling, was unaltered, despite a 2.5-fold increase in the basal cAMP level and a 1.9-fold increase in baseline contraction amplitude as compared with that of wild-type (WT) cells. Although the contractile response to β_2-R* in TG4 cells was abolished by a β_2AR inverse agonist, ICI118,551 (5×10^{-7} M), or an inhibitory cAMP analog, Rp-CPT-cAMPS (10^{-4} M), no change was detected in the simultaneously recorded I_{Ca}. These results suggest that the increase in basal cAMP due to β_2-R*, while increasing contraction amplitude, does not affect I_{Ca} characteristics. In contrast, the β_2AR agonist, zinterol elicited a substantial augmentation of I_{Ca} in both TG4 and WT cells (pertussis toxin-treated), indicating that L-type Ca^{2+} channel in these cells can respond to ligand-directed signaling. Furthermore, forskolin, an adenylyl cyclase activator, elicited similar dose-dependent increase in I_{Ca} amplitude in WT and TG4 cells, suggesting that the sensitivity of L-type Ca^{2+} channel to cAMP-dependent modulation remains intact in TG4 cells. Thus, we conclude that β_2-R* bypasses I_{Ca} to modulate contraction, and that β_2-**L**R* and β_2-R* exhibit different intracellular signaling and target protein specificity.

β-adrenergic receptor (AR) stimulation plays a prominent role in modulation of cardiac myocyte performance in response to an increased peripheral demand. Driven by sympathetic neurotransmitters and adrenal hormones, βAR activation regulates virtually all major steps of the cardiac cell excitation-contraction (E-C) coupling cascade, e.g., the sarcolemmal L-type Ca^{2+} current (I_{Ca}), sarcoplasmic reticulum (SR) Ca^{2+} release and reuptake, and the responsiveness of contractile myofilaments to cytosolic Ca^{2+}. Because I_{Ca} provides the trigger for SR Ca^{2+} release, and is a major determinant of intracellular calcium homeostasis, modulation of this current by βAR system has been extensively studied over the last two decades. It has been demonstrated that both β_1AR and β_2AR subtypes coexist in cardiac myocytes in many mammalian species, and that stimulation of each of these receptor subtypes increases cardiac I_{Ca} (Xiao and Lakatta, 1993; Cerbai et al., 1995) through the classic stimulatory G protein (G_s)-adenylyl cyclase-cAMP-protein kinase A (PKA) signaling cascade (Hartzell et al., 1991; Zhou et al., 1997; Skeberdis et al., 1997; Xiao et al., 1999). The existence and functional importance of a more rapid, direct interaction of the βAR-activated G_s and L-type Ca^{2+} channel remain controversial (Yatani and Brown, 1989; Hartzell et al., 1991; Zhou et al., 1997; Skeberdis et al., 1997).

A prevailing receptor theory (two-state model) states that a G protein-coupled receptor, such as β_1AR or β_2AR, exists in an equilibrium between two conformational states: an inactive (R) state and an active (R*) state, the latter having high affinity for G proteins (Bond et al., 1995). In the absence of a receptor agonist, spontaneous transition between the R* and R states results in a constitutive or intrinsic activation of only minority of receptors (Chidiac et al., 1994; Bond et al., 1995) and thus the functional significance of R* is not always evident. The presence of a large number of spontaneously activated β_2ARs (β_2-R*s), which alter basal function, has been experimentally demonstrated in a transgenic (TG) murine model, the TG4 mouse (Milano et al., 1994; Bond et al., 1995; Xiao et al., 1999), in which the human β_2AR is overex-

ABBREVIATIONS: βAR, β-adrenergic receptor; β-R*, spontaneously activated βAR; β-**L**R*, ligand activated βAR; CGP, CGP20712A; E-C, excitation-contraction; G_i and G_s, inhibitory and stimulatory G protein(s), respectively; I_{Ca}, L-type Ca^{2+} current; ICI, ICI118,551; NE, norepinephrine; PKA, cAMP-dependent protein kinase A; PLB, phospholamban; PTX, pertussis toxin; R and R*, inactive and active receptor conformational states, respectively; Rp-CPT-cAMPS, Rp diastereomers of 8-(4-chlorophenylthio)-cAMP; SR, sarcoplasmic reticulum; TG4 mice, transgenic mice overexpressing human β_2AR; WT mice, wild-type mice.

pressed by ~200-fold in a cardiac-specific manner. Hence, this transgenic model provides a unique opportunity to study the transmembrane signal transduction originating from unliganded β_2-R* in comparison with that from the ligand-activated β_2AR (β_2-**L**R*). According to the two-state receptor model, β_2-R* ought to be identical with β_2-**L**R*, because there is only a single active conformational state. However, there is no a priori reason that this has to be the case. By analogy to ionic channels and enzymes, it is more plausible that a receptor may possess multiple, distinct active conformations (Perez et al., 1996; Gurdal et al., 1997). If β_2-R* and β_2-**L**R* differ in their active conformational states, spontaneous and agonist-induced β_2-adrenergic signaling may not be functionally equivalent, e.g., in modulating their target proteins, such as L-type Ca^{2+} channels.

In the present study, we examined the possible modulatory effects of β_2-R* on basal I_{Ca} and cell contraction in single ventricular myocytes and on basal cAMP in myocardium from TG4 mice and wild-type (WT) littermates. Surprisingly, we found no evidence that I_{Ca} was regulated by β_2-R* in TG4 heart cells. In contrast, both β_2-**L**R* signaling in the presence of pertussis toxin (PTX) and direct adenylyl cyclase activation by forskolin augmented I_{Ca} to an extent similar to that observed in WT cells. Our results support the idea that despite many similarities, β_2-R* and β_2-**L**R* may represent distinct functional conformation states of the receptor, eliciting different intracellular signaling patterns, and having differential effects on target proteins. These findings require an extension of the current model of β_2AR to encompass multiple active conformational states.

Experimental Procedures

Cell Isolation and Measurement of Contraction. Single murine cardiac myocytes were isolated from the hearts of 2- to 3-month-old mice via a standard enzymatic technique (Korzick et al., 1997). Briefly, hearts were retrogradely perfused with collagenase B and protease using the Langendorff method. Cells were shaken loose from the heart after this perfusion and then suspended in HEPES buffer 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 7.4, adjusted with NaOH. Ca^{2+} tolerant cells were kept at 37°C, with or without incubation with 1.5 μg/ml PTX for at least 3 h, as described previously (Xiao et al., 1995).

Cells were placed on the stage of an inverted microscope (Zeiss, model IM-35; Carl Zeiss, Thornwood, NY) and superfused with HEPES-buffered solution at a flow rate of 1.8 ml/min. Each cell was illuminated with red (650–750 nm) light through the normal brightfield path of the microscope and field stimulated at 0.5 Hz at 23°C. Cell length was monitored from the brightfield image by an optical edge tracking method using a photodiode array (model 1024 SAQ;, Reticon) with a 3-ms time resolution (Spurgeon et al., 1990).

Criteria for viable mouse myocytes have been described in a previous report (Korzick et al., 1997), i.e., 1) rod shape; 2) clearly defined sarcomeric striations; 3) a clear negative staircase after rest for a period of ~1 min; and 4) a stable steady-state contraction amplitude for at least 5 min before drug administration.

Ca^{2+} Current Measurement. I_{Ca} was measured via the whole-cell patch clamp technique using an Axopatch 1D amplifier (Axon Instruments Inc., Foster City, CA). Low-resistance (1–2 MΩ) micropipettes were pulled via a two-stage micropipette puller (model P-97; Sutter Instrument Co., Novato, CA). The average series resistance (R_s) in whole-cell configuration was 5.71 ± 0.28 MΩ for TG4 cells (n = 34) and 5.99 ± 0.39 MΩ for WT cells (n = 25), and routinely compensated ~70% in our experiments. To selectively examine I_{Ca}, cells were voltage-clamped at −40 mV to inactivate the sodium and T-type Ca^{2+} channels. Potassium currents were inhibited by appropriate blockers in the extracellular HEPES buffer solution (4 mM 4-aminopyridine, 5.4 mM CsCl substituted for KCl in standard HEPES buffer solution) and in the pipette solution containing: 100 mM CsCl, 10 mM NaCl, 20 mM tetraethylammonium chloride 20, 10 mM HEPES, 5 mM MgATP, and 5 mM EGTA; pH was adjusted to 7.2 with CsOH. In some experiments to simultaneously record I_{Ca} and cell contraction, EGTA was omitted from the pipette solution and normal HEPES buffer constituted the extracellular solution. I_{Ca} was elicited by 300-ms pulses from a holding potential of −40 mV to test potentials from −30 to +50 mV in 10-mV increments at 0.1 Hz at 23°C. To monitor drug effects, I_{Ca} elicited by a depolarization from −40 to 0 mV was continuously recorded. The amplitude of I_{Ca} was measured as the difference between the peak inward current and that at the end of 300-ms pulse. The decay of I_{Ca} was fitted to a biexponential function:

$$I_{Ca} = A_0 + A_f\exp(-t/\tau_f) + A_s\exp(-t/\tau_s)$$

Where τ_f and τ_s are the fast and slow inactivation time constants; A_0 is a constant; and A_f and A_s are amplitudes of fast and slow current components, respectively.

To determine whether there is a current-voltage (I-V) shift, the voltage-dependence of I_{Ca} steady-state activation was calculated from the equation:

$$g = I/(E_m - E_{rev})$$

where g is the membrane conductance, I is the peak current at a given test potential (E_m), and E_{rev} is the apparent reversal potential for I_{Ca} (+60 mV). The conductance at each test potential was then normalized to peak conductance. The data were fit by a Boltzmann equation:

$$d_\infty = \{1 + \exp[-(V_m - V_{1/2})/k]\}^{-1}$$

where d_∞ is the steady-state activation, and $V_{1/2}$ represents the half-maximal activation voltage. k is the slope factor of the steady-state activation curve.

Measurement of cAMP Accumulation. Cardiac membranes were prepared as previously described (Xiao et al., 1998). cAMP levels were assayed by the radioimmunoassay. Briefly, 10 μl of membrane vesicles (20 μg total protein) was added to a 40-μl reaction mixture to make a final concentration of 4 mM Tris-EDTA and 10 μM Ro 20–1724 (an inhibitor of phosphodiesterase IV) with or without 0.5 μM ICI 118,551 (ICI is a β_2AR inverse agonist). The reaction was performed for 15 min at 37°C and 25 μl of supernatant was assayed using a cAMP ^{3}H assay kit obtained from Amersham (Arlington Heights, IL). Protein was measured using the Bradford method (Bio-Rad, Richmond, CA) with BSA as the standard.

Materials. PTX, tetrodotoxin, forskolin, isoproterenol hydrochloride, norepinephrine (NE), prazosin, and Ro 20–1724 were purchased from Sigma Chemical Co. (St. Louis, MO). Rp diastereomers of 8-(4-chlorophenylthio)-cAMP (Rp-CPT-cAMPS) was purchased from Biolog Life Science Institute (La Jolla, CA). cAMP assay kits were purchased from Amersham. Zinterol was kindly supplied by Bristol-Myers (Evansville, IN); ICI was kindly supplied by Imperial Chemical Industry (London, United Kingdom). CGP20712A (CGP) was kindly supplied by Ciba-Geigy Corp. (Basel, Switzerland).

Data Analysis. Data are reported as mean ± S.E.M. Student's t test was used to test for differences between TG4 and WT groups and for PTX-treated and nontreated groups; a paired t test was used for assessing the significance of drug effects. A value of $P < .05$ was considered to be statistically significant.

Results

In the absence of exogenous β_2AR agonists, the basal cAMP level was increased by 2.5-fold in TG4 relative to WT

hearts (Fig. 1A). Concomitantly, basal contraction amplitude was enhanced by 1.9-fold in single ventricular myocytes isolated from TG4 mice (Fig. 1B). A β_2AR inverse agonist, ICI (5×10^{-7} M), which had no significant effect on either basal cAMP or contractility in WT mice, reduced the baseline cAMP (Fig. 1A) and contractility of TG4 cells (Fig. 1B) to levels similar to those of WT littermates. These data are in agreement with previous observations that ICI depresses the elevated basal adenylyl cyclase activity, heart rate and cardiac contractility in vivo and in isolated atria (Milano et al., 1994; Bond et al., 1995; Du et al., 1996). Therefore, the results so far support the notion of spontaneous β_2AR activation in the absence of an agonist (Chidiac et al., 1994; Milano et al., 1994; Bond et al., 1995; Xiao et al., 1999) and indicate that β_2-R* augments cAMP production and cardiac contractility, as is the case for ligand-induced β_2AR stimulation (Xiao and Lakatta, 1993; Xiao et al., 1994, 1995; Altschuld et al., 1995; Zhou et al., 1997). If β_2-R* and β_2-**L**R* were functionally equivalent, as predicted by the two-state model, the L-type Ca^{2+} channel, a key target effector of β_2-**L**R* signaling, would be modulated by β_2-R* in a similar fashion, i.e., baseline I_{Ca} in TG4 cells would be expected to be tonically elevated and sensitive to ICI. To our surprise, basal I_{Ca} was not elevated in TG4 cells (see below). Furthermore, although ICI (5×10^{-7} M) rapidly and reversibly attenuated the augmented baseline contraction amplitude in TG4 ventricular myocytes (Fig. 2A), it had virtually no effect on the amplitude (Fig. 2B; 97.2 ± 3.4% of control, $n = 9$) and time course (Fig. 2C) of I_{Ca} in TG4 cells. This result was further confirmed by the simultaneous recording of I_{Ca} and contraction using the EGTA-free pipette solution. As shown in Fig. 3, ICI induced a marked decrease in cell contraction amplitude without any change of I_{Ca} in the same TG4 cell.

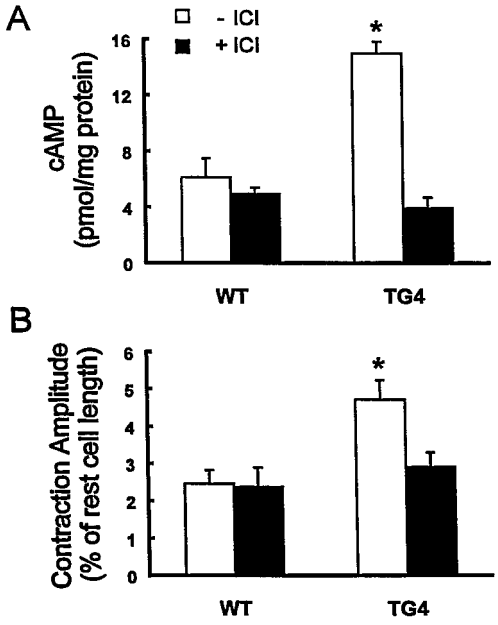

Fig. 1. Comparison of the basal cAMP (A) and contractility (B) in the β_2AR TG4 mice and in WT mice. Both basal cAMP and contraction amplitude are significantly increased in TG4 as compared with that of WT mice, and both increases can be reversed by a β_2AR inverse agonist, ICI 118,551 (ICI, 5×10^{-7} M); $n = 3$ for cAMP measurements; $n = 12$ and 9 for contraction measurements in WT and TG4 cells, respectively. *$P <$ .01 for TG4 without ICI group compared with other groups.

The differential effects of β_2-R* on I_{Ca} and contractility are in sharp contrast to the traditional views that the L-type Ca^{2+} channel is an obligatory effector of β_2AR signaling (Xiao and Lakatta, 1993; Cerbai et al., 1995; Altschuld et al., 1995; Zhou et al., 1997). The results also raise doubts as to whether the β_2-R* effect to augment contractility in TG4 myocytes even requires the classical cyclase-cAMP-PKA signaling. To directly address this issue, we used an inhibitory cAMP analog, Rp-CPT-cAMPS, to specifically block PKA activation. As shown in Fig. 3, similar to the effect of the inverse agonist ICI, Rp-CPT-cAMPS reversed the β_2-R* effect on contraction without affecting the simultaneously recorded I_{Ca}. This observation indicates that the β_2-R*-stimulated inotropic effect in TG4 cells depends largely on β_2-R*-elicited cAMP signaling, as does β_2-**L**R* (Zhou et al., 1997; Skeberdis et al., 1997; Xiao et al., 1999). Thus, the inability of β_2-R* to modulate L-type Ca^{2+} channels may be attributed to either a qualitative difference between β_2-R* and β_2-**L**R*, or to an alteration in L-type Ca^{2+} channels of TG4 cells (see below).

To further characterize the L-type Ca^{2+} channel properties in TG4 cells, whole-cell I_{Ca} amplitude, current-voltage relation, and inactivation kinetics were systematically examined in both TG4 and WT ventricular myocytes. Figure 4A shows typical traces of I_{Ca} elicited by a depolarization from −40 to 0 mV in a WT and a TG4 myocyte in the absence of any β_2AR

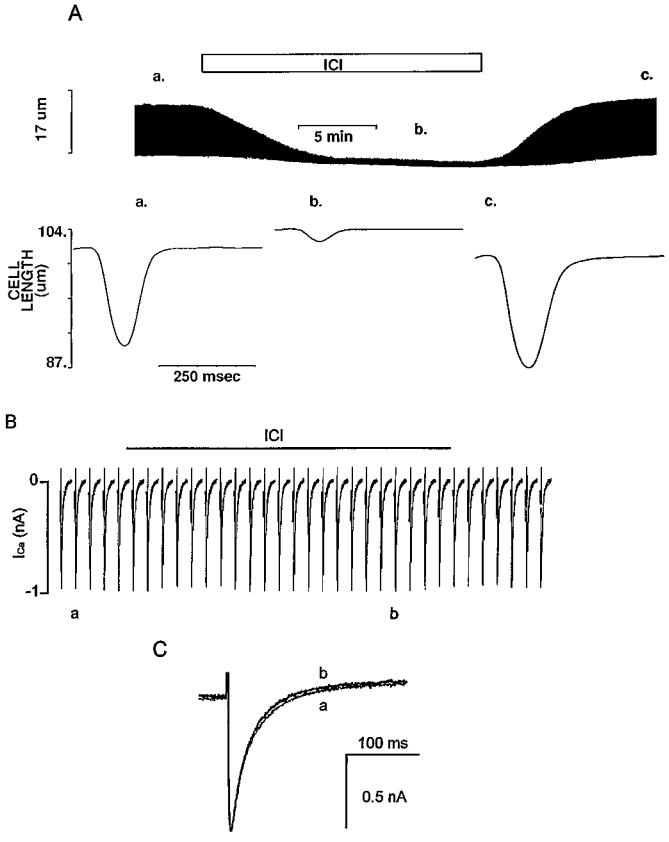

Fig. 2. A β_2AR inverse agonist, ICI (5×10^{-7} M), depresses the basal contraction but not I_{Ca} in TG4 cardiomyocytes. A, an example of the effect of ICI on basal contraction amplitude. Top, a continuous chart recording of cell length. An upward deflection indicates cell shortening. Bottom, the twitch is displayed at higher resolution at times indicated in top panel. A downward deflection indicates cell shortening. B, typical continuous recording of I_{Ca} in response to ICI. I_{Ca} is elicited every 30 s by 300-ms pulses from −40 to 0 mV. C, superimposed traces of I_{Ca} recorded before and after exposure to ICI at times indicated in B.

ligands. The baseline I_{Ca} in TG4 and WT cells are virtually indistinguishable in amplitude and time course (Fig. 4A), consistent with the absence of ICI-sensitive (β_2-R*) component of I_{Ca} described above. The average amplitude of I_{Ca} at 0 mV was 1.01 ± 0.05 nA in TG4 (n = 34) and 1.03 ± 0.07 nA in WT cells (n = 38). Rundown of I_{Ca} was not significantly different between these two groups (12.4 ± 4.9 and 14.1 ± 6.2% at 10 min for TG4 and WT cells, respectively; n = 3 for both groups). Because there was no significant difference in cell membrane capacitance (166 ± 10 pF, n = 34, in TG4 cells versus 161 ± 12 pF, n = 38, in WT cells), the density of I_{Ca} (i.e., I_{Ca} normalized by capacitance) was also similar in TG4 and WT groups (6.73 ± 0.43 pA/pF, n = 34 and 6.86 ± 0.49 pA/pF, n = 38, respectively). The similarity in membrane capacitance between TG4 and WT cells is consistent with a previous report that no cellular hypertrophy occurs in 2- to 4-month-old TG4 hearts (Milano et al., 1994; Xiao et al., 1999).

Next, we determined the current-voltage relation of I_{Ca} in both TG4 and WT myocytes. Cells were depolarized from a holding potential of −40 mV to various test potentials from −30 to +50 mV in 10-mV increments. Over the entire voltage range examined, the I_{Ca} density-voltage relations in TG4 and WT cells overlapped (Fig. 4B), indicating that voltage-dependent activation of L-type Ca^{2+} channel in TG4 cells was unchanged as compared with WT controls. Furthermore, I_{Ca} inactivation time constants (τ_f and τ_s) and the voltage-dependence of τ_f or τ_s of WT cells were similar to those of TG4 cells (Fig. 4C); likewise, there is no difference in the amplitude proportion of the two exponential components between these two groups (A_f/A_s = 1.24 ± 0.08 at 0 mV, n = 20, in TG4 versus 1.19 ± 0.16, n = 19, in WT). Therefore, no measured parameters of I_{Ca}, including amplitude, voltage-dependence, and inactivation kinetics were altered by spontaneous β_2AR activation in TG4 cardiac myocytes.

If L-type Ca^{2+} channels in TG4 cells were somehow modified via compensatory mechanisms so that I_{Ca} could no longer respond to β_2-R*-mediated cAMP signaling, the I_{Ca} response to any other cAMP signaling should be similarly blunted. However, forskolin, an activator of adenylyl cyclase, induced a robust increase in the Cd^{2+}-sensitive I_{Ca} in TG4 cells (Fig. 5, A and B). More importantly, the dose-response

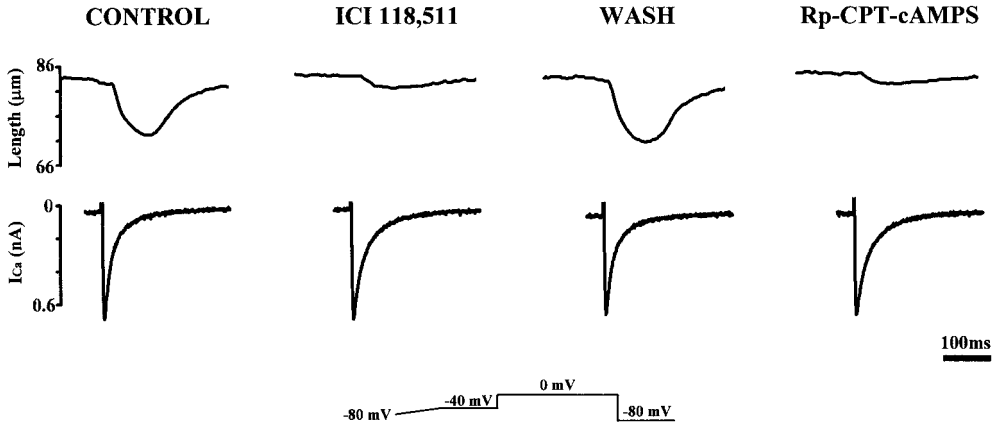

Fig. 3. A typical example of simultaneous recording of TG4 cell contraction and I_{Ca} in response to the β_2AR inverse agonist, ICI (5×10^{-7} M) or a PKA inhibitor, Rp-CPT-cAMPS (10^{-4} M) under the whole-cell voltage clamp condition without EGTA in the pipette. The voltage clamp protocol is shown as the inset. Shortening of cell length is shown in the upper panel and I_{Ca} in the lower panel. Similar results were obtained in three other cells.

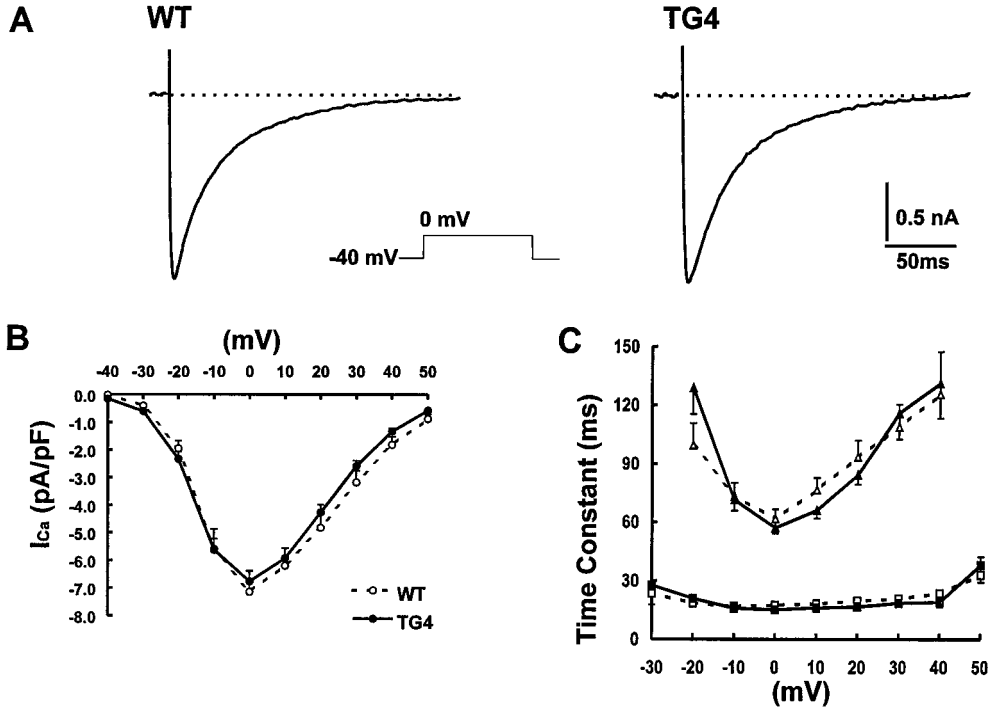

Fig. 4. Properties of basal L-type Ca^{2+} current (I_{Ca}) recorded in single ventricular myocytes isolated from TG4 and WT mice. A, representative traces of I_{Ca} recorded from TG4 and WT cells. Inset, voltage clamp protocol to elicit I_{Ca}. B, current density-voltage curves obtained from TG4 and WT cells. C, relationship between voltage and inactivation time constants of I_{Ca} in TG4 and WT cells. The decay of I_{Ca} is fitted to the sum of two exponentials (see *Experimental Procedures*); n = 19 to 20 for data presented in B and C.

curves of I_{Ca} to forskolin in TG4 and WT cells virtually overlapped, with no significant difference in EC_{50} (3.97×10^{-7} M for WT and 5.96×10^{-7} M for TG4; $P > .05$, Fig. 5C). Thus, the sensitivity of cardiac L-type Ca^{2+} channel to cAMP-PKA modulation remains intact in TG4 mice.

Our recent studies have shown that cardiac β_2AR couples to the PTX-sensitive inhibition proteins, (G_i) G_{i2} and G_{i3} (Xiao et al., 1995, 1999), and that this coupling partially offsets the β_2AR agonist-mediated contractile response in rat myocytes (Xiao et al., 1995) and completely negates the β_2AR agonist-mediated contractile (Xiao et al., 1999) and I_{Ca} responses (Fig. 5, A and B) in TG4 and WT murine ventricular myocytes. Therefore, it is reasonable to assume that an excessive G_i coupling to β_2-R* could be involved in the inability of β_2-R* to modulate I_{Ca}. To test this hypothesis, baseline I_{Ca} was re-examined in PTX-treated cells and compared with that in PTX-untreated cells. Figure 6B shows that in TG4 cells, PTX treatment had no significant effect on the baseline I_{Ca} amplitude or its current-voltage relation. Similar results were also obtained in WT cells (Fig. 6A). Moreover, even in PTX-treated TG4 cells, neither the amplitude nor the kinetics of the basal I_{Ca} were affected by ICI (data not shown). These results suggest that G_i proteins are not involved in the unresponsiveness of I_{Ca} to β_2-R*.

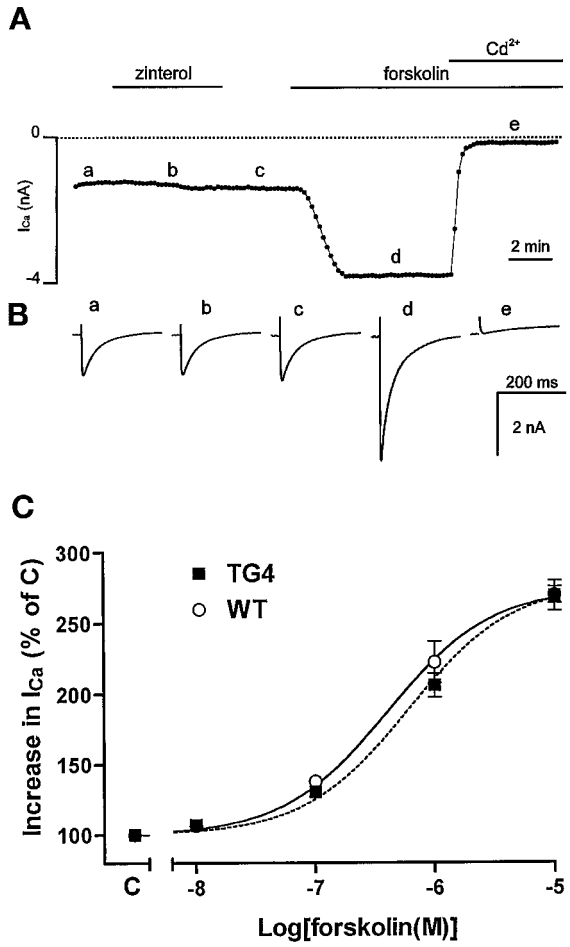

Fig. 5. Response of I_{Ca} to a β_2AR agonist, zinterol, or an adenylyl cyclase activator, forskolin, in PTX-untreated cardiomyocyte from TG4 mice. A, time course of changes in the peak amplitude of I_{Ca}. I_{Ca} is activated by 300-ms depolarization pulses from a holding potential of −40 to 0 mV at 0.1 Hz. Note that I_{Ca} is not affected by zinterol at 10^{-6} M, but is markedly increased by forskolin at 10^{-6} M, and that I_{Ca} is abolished by 5×10^{-5} M Cd^{2+}. B, selected current traces recorded before or after exposure to different drugs. Letters "a" to "e" correspond to those time points marked in A. C, dose-response curves of I_{Ca} to forskolin in myocytes from TG4 or WT hearts. The values of EC_{50} in WT (3.97×10^{-7} M) and TG4 (5.96×10^{-7} M) are not significantly different ($P > .05$). Each point represents mean ± S.E.M. of results from four to eight cells. Data are expressed as percentage of control value (C). Control values of I_{Ca} are 0.95 ± 0.09 nA for TG4 ($n = 17$) and 0.91 ± 0.07 nA for WT ($n = 20$).

Although G_i inhibition failed to rescue I_{Ca} response to β_2-R*, in the same TG4 cells, PTX permitted β_2-**L**R* induced by zinterol to significantly enhance I_{Ca} (Fig. 6, C and D). The PTX rescued I_{Ca} response to β_2-**L**R* in TG4 cells (149 ± 12% of control, at 0 mV, $n = 8$) was comparable with that of WT cells (153 ± 11% of control, at 0 mV, $n = 4$). In addition, the I_{Ca}-voltage relation was shifted leftward by zinterol ($V_{1/2}$ was −16.58 ± 1.33 and −23.03 ± 1.67 mV in the absence and presence of zinterol, respectively, $P = .01$, Fig. 6C), in agreement with previous observations in rat ventricular myocytes (Xiao and Lakatta, 1993). However, neither the inactivation kinetics (τ_f, 101 ± 8% of control, τ_s, 108 ± 3% of control), nor the ratio of A_f/A_s (95 ± 19% of control, $n = 5$) were significantly altered by zinterol in PTX-treated TG4 cells. Figure 6D shows that the I_{Ca} response to zinterol in a PTX-treated TG4 cell was completely blocked by the β_2AR-selective antagonist, ICI at 5×10^{-7} M (96.2 ± 6.2% of control, $n = 5$, $P > .05$ versus control). Thus, PTX treatment permits β_2-**L**R*, but not β_2-R*, to modulate L-type Ca^{2+} channel activity in TG4 heart.

Although in mouse cardiac myocytes β_1-AR is unable to couple to G_i proteins, as manifested by the G protein photoaffinity labeling profile (Xiao et al., 1999), previous studies in guinea pig (Hool and Harvey, 1997) raised doubt as to whether the PTX rescued effect of zinterol is related to the activation of β_1AR. We therefore examined the effect of β_1AR stimulation in the presence and absence of PTX treatment in TG4 myocytes. Interestingly, β_1AR agonist NE even at maximal concentration (NE 10^{-7} M) plus prazosin 10^{-6} M (Korzick et al., 1997) did not induce a discernible increase in I_{Ca} of TG4 cells, whereas it markedly increased I_{Ca} in WT myocytes (Fig. 7, A and B). The absence of I_{Ca} response to β_1AR stimulation is consistent with previous observations on the loss of contractile response to β_1AR stimulation by either NE plus prazosin or isoproterenol plus the β_2AR blocker, ICI (Bond et al., 1995; Du et al., 1996). Whereas PTX treatment fully rescued the contractile (Xiao et al., 1999, also see Fig. 7C) and I_{Ca} (Fig. 6) response to β_2AR agonist stimulation, it was unable to restore contractile and I_{Ca} response to β_1AR stimulation (Fig. 7). In addition, in TG4 cells, the PTX-restored contractile response to a mixed βAR agonist, isoproterenol 10^{-6} M, was specifically inhibited by a β_2AR antagonist, ICI 10^{-7} M, but not by a β_1AR antagonist, CGP 3×10^{-7} M (Fig. 7C). This further corroborates our previous notions that, unlike β_2AR, β_1AR does not couple to G_i protein(s) in mouse myocardium (Xiao et al., 1999).

Discussion

β_2-R* Does Not Regulate I_{Ca}. The presence of β_2-R* in the TG4 heart is evidenced by the elevated basal adenylyl cyclase activity (Milano et al., 1994) and cAMP production (Fig. 1A), the enhanced cardiac contractility (Milano et al., 1994; Bond et al., 1995; Du et al., 1996; Rockman et al., 1996; Xiao et al., 1999) (Fig. 1B), and the blockade of these aug-

mentations by the inverse β_2AR agonist, ICI (Milano et al., 1994; Bond et al., 1995; Du et al., 1996; Xiao et al., 1999) (Figs. 2 and 3). In the present study, we have provided direct evidence that β_2-R*-mediated modulation of cardiac contractility is largely cAMP-PKA-dependent, because it is sensitive to the PKA inhibitor Rp-CPT-cAMPS (Fig. 3). The most surprising and unexpected finding of this study is that baseline I_{Ca} in TG4 cardiac myocytes is not increased or altered by β_2-R* (Fig. 4). The simplest explanation for this observation would be that β_2-R*-directed signaling is totally diverted from the L-type Ca^{2+} channels. However, the interpretation for the results obtained from the transgenic model may not be so straightforward, because compensatory changes have been documented in TG4 hearts, e.g., down-regulation of the SR protein phospholamban (PLB) (Rockman et al., 1996) and up-regulation of G_i proteins (R-P.X., unpublished data). Several additional experiments have therefore been undertaken to explore alternative possibilities.

If the L-type Ca^{2+} channel protein expression were reduced in TG4 heart cells so that I_{Ca} density in these cells was lower than normal in the absence of β_2-R*, it could mask a β_2-R*-mediated stimulatory effect on I_{Ca}. In other words, an adaptive "down-regulation" of I_{Ca} might offset an increase in this current induced by β_2-R*. This possibility was tested by using the inverse β_2AR agonist, ICI. Because ICI inactivates β_2-R* and prevents spontaneous β_2AR activation (Bond et al., 1995), the ICI-sensitive component would thus reflect the magnitude of the β_2-R* effect. We have found that ICI has no detectable effect on I_{Ca}, although it markedly reduces basal cell contractility and cAMP content (Figs. 1–3). Thus, our results do not support an adaptive reduction in L-type Ca^{2+} channel number in TG4 mice.

A second possible explanation for the absence of enhancement of I_{Ca} in TG4 cells is that L-type Ca^{2+} channels might be somehow modified, thereby losing their sensitivity to cAMP-dependent modulation. If this were the case, I_{Ca} should no longer respond to any other cAMP-dependent stimulation, or the responses should be markedly attenuated. This possibility, however, have also been excluded on the basis that agonist-elicited β_2AR stimulation enhances I_{Ca} (in PTX-treated TG4 myocytes) to an extent similar to that in (PTX-treated) WT cells; and that the I_{Ca} (in TG4 cells) dose-response curve to the adenylyl cyclase activator forskolin overlaps with that in WT cells (Fig. 5C), indicating that the responsiveness of L-type Ca^{2+} channels to cAMP-PKA-dependent regulation in TG4 cells is not significantly altered. Thus, the unresponsiveness of I_{Ca} to β_2-R* is not caused by the changes in the channel proteins.

In mammalian hearts, agonist-elicited β_2AR stimulation evokes bifurcated G_s- and G_i-mediated signaling cascades: the β_2AR-G_i pathway exerts a negative feedback control of the β_2AR-G_s effects (Xiao et al., 1995, 1999; Zhou et al., 1997). The G_i-mediated inhibition of G_s signaling could account for the apparent uncoupling of β_2-**L**R* to L-type Ca^{2+} channel in non-PTX-treated WT and TG4 cells, because PTX unmasks a de novo I_{Ca} response to β_2AR agonist zinterol (Fig. 6, C and D), and the β_2AR agonist zinterol enhances the photoaffinity labeling of the α subunits of the G_i proteins, G_{i2} and G_{i3} (Xiao et al., 1999). However, G_i-mediated inhibition cannot explain the inability of β_2-R* to augment I_{Ca} in TG4 cells, because PTX fails to potentiate basal I_{Ca}(Fig. 6B), and ICI has no effect on the baseline I_{Ca} regardless of PTX (Figs. 2 and 3). These functional results suggest that β_2-R* does not couple to G_i proteins as efficiently as does β_2-**L**R*. This is in good agreement with the fact that in transgenic mice with high or medium levels of β_2AR overexpression, β_2AR in the absence of an agonist, coprecipitates with G_s but barely with G_i/G_o (Gurdal et al., 1997). Taken together, we conclude that spontaneous β_2AR activation in TG4 cells, whereas increasing cell contractility, does not regulate I_{Ca}, a key effector of β_2-**L**R*.

Differences between β_2-R*- and β_2-LR*-Mediated Signaling. In contrast to the prediction of the two-state receptor model, the differential regulation of I_{Ca} by β_2-R* and

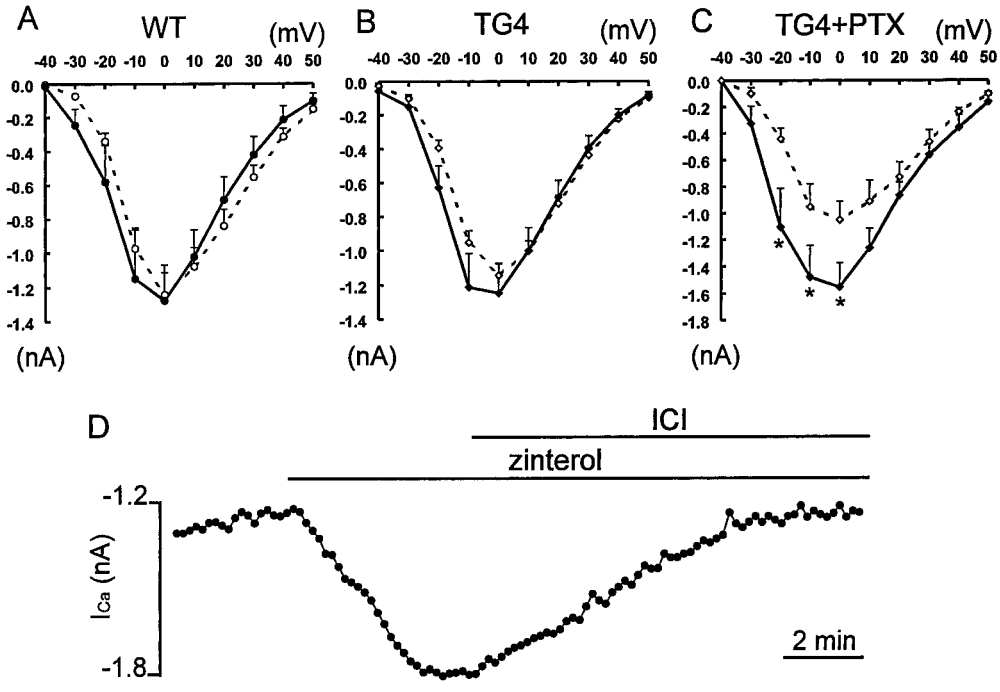

Fig. 6. Effect of PTX treatment on the baseline I_{Ca} and the response of I_{Ca} to zinterol in ventricular myocytes from WT and TG4 mice. A and B, current-voltage curves obtained in PTX-untreated (open symbols, dashed line) and PTX-treated cells (filled symbols, solid line) from WT (A) and TG4 (B) cells. C, current-voltage curves obtained before (open symbols, dashed lines) and 5 min after exposure to zinterol (10^{-6} M, filled symbols, solid lines) in PTX-treated TG4 cells. D, time course of changes in peak magnitude of I_{Ca} after exposure to zinterol (10^{-6} M) and the blockade of zinterol's effect by ICI (5×10^{-7} M) in a representative PTX-treated TG4 cell. In A–C, each symbol represents the means ± S.E.M. from 5 to 20 cells. *$P < .05$ compared with values before zinterol in PTX-treated TG4 cells.

β_2-**LR*** suggests that the liganded and unliganded active β_2ARs are different active receptor species, likely having different conformations and initiating distinct postreceptor signaling pathways. Several lines of additional evidence support this hypothesis. First of all, whereas β_2-R* in TG4 heart significantly increases the baseline contractility, β_2-**LR*** induced by zinterol or isoproterenol at maximal concentrations are unable to further increase contraction amplitude (Milano et al., 1994; Du et al., 1996; Xiao et al., 1999), even though the basal contractility is not at the maximum contractile state yet (Du et al., 1996; Xiao et al., 1999). Secondly, β_2-R*, unlike β_2-**LR***, does not couple to G_i proteins, as reflected by the lack of a PTX effect on the basal I_{Ca} (Fig. 6) and by immunoprecipitation data on receptor-G protein interaction (Gurdal et al., 1997). Finally, it has recently been shown that in rat and mouse cardiac myocytes, multiple active conformational states of β_2AR can be induced by different β_2AR ligands (R-P.X., unpublished data). Similar observations have been reported previously for β_2AR and other G protein-coupled receptors in transfected cells (e.g., Eason et al., 1994) or artificial lipid vesicles (Gether et al., 1997). The present finding that β_2-R* differs from β_2-**LR*** is in general agreement with the emerging concept of multiple active receptor states for a given receptor.

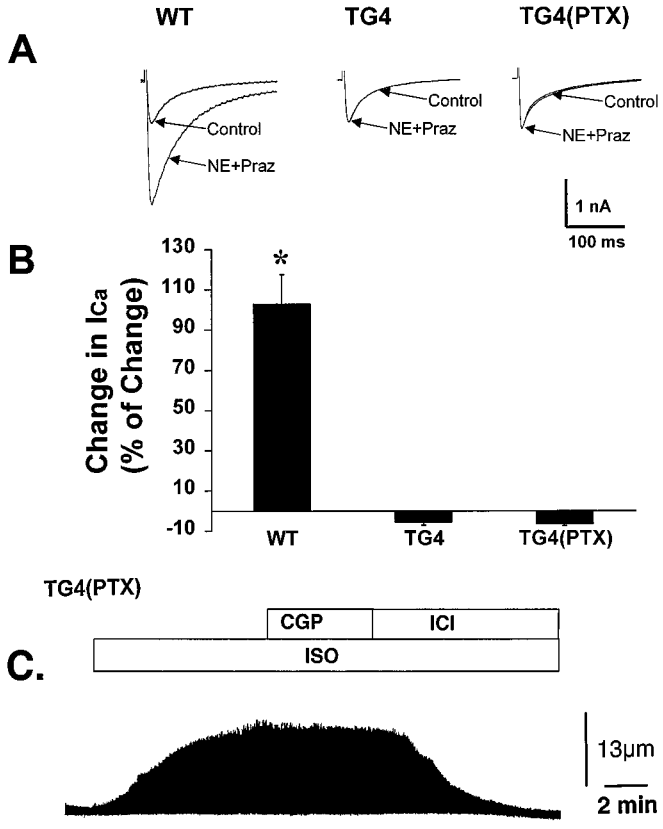

Fig. 7. I_{Ca} and contractile response to βAR stimulation in mouse ventricular myocyte. A, typical current traces recorded before or after β_1AR stimulation by norepinephrine (NE, 10^{-7} M) plus prazosin (Praz, 10^{-6} M) in representative WT, TG4, and PTX-treated TG4 myocytes. B, average increase in I_{Ca} elicited by a depolarization pulse from −40 to 0 mV in response to β_1AR stimulation. Control values of I_{Ca} are 1.03 ± 0.14 nA for WT (n = 7), 1.09 ± 0.06 nA for TG4 (n = 5), and 1.21 ± 0.15 nA for PTX-treated TG4 myocytes (n = 3). *P < .01 versus control. C, a typical example of continuous chart recording of cell length following βAR stimulation by isoproterenol (ISO, 10^{-6} M) in a PTX-treated TG4 cell. An upward deflection indicates cell shortening. The β_2AR antagonist, ICI118511 (ICI, 10^{-7} M), but not the β_1AR antagonist, CGP20712A (CGP, 3×10^{-7} M), specifically inhibited the PTX-rescued contractile response to ISO. Similar results are observed in other 10 cells.

Another intriguing difference between β_2-R* and β_2-**LR*** is manifested by their chronic noncontractile effect. Agonist-induced, chronic, mixed βAR or β_2AR stimulation has been shown to enhance cardiac cell growth in vitro (Boluyt et al., 1995; Zhou et al., 1996) and cause cardiac hypertrophy in vivo (Kudej et al., 1997). Cardiac hypertrophy also occurs in other transgenic murine models in which G_s or the cAMP signaling cascade has been genetically up-regulated (Iwase et al., 1996). In contrast, the TG4 model is exceptional in that it has tonically elevated cardiac contractile function and cAMP signaling without evident cardiac and cellular hypertrophy as shown in the present and previous studies (Milano et al., 1994; Xiao et al., 1999, Heubach et al., 1999). Given the central role of sarcolemmal I_{Ca} in intracellular Ca^{2+} homeostasis, and given the role of Ca^{2+} signaling in cell hypertrophy in vivo and in vitro (Molkentin et al., 1998), it is tempting to speculate that the lack of L-type Ca^{2+} current response to β_2-R*, as demonstrated here, may be of particular relevance to the lack of cardiac hypertrophy and cardiomyopathy in the TG4 model.

The present results also illustrate that, although both β_2-**LR*** (Xiao et al., 1999) and β_2-R* (Fig. 3) couple to cAMP-dependent signal transduction pathway, their cAMP signaling may be differentially compartmentalized. Specifically, the cyclase activity or cAMP-PKA signal due to β_2-R* must be somehow shielded from L-type Ca^{2+} channels, but is readily accessible to other E-C coupling machineries. In contrast to β_2-R*, previous studies in many species (rat, mouse, and dog) have shown that, L-type Ca^{2+} channel is the major target protein of β_2-**LR***, whereas the SR and other cytosolic proteins do not always respond to β_2-**LR***-stimulated cAMP-PKA signaling (Xiao et al., 1994; Altschuld et al., 1995; Kuschel et al., 1999b). Thus, β_2-R* differs qualitatively from β_2-**LR***; this difference might not be simply explained by different coupling efficiency to various targets. Taken together, not only the receptor type or subtype (e.g., Zhou et al., 1997), but also the conformational state of the same receptor is an important determinant of intracellular sorting of cAMP signaling. Selective shielding of cAMP signaling from a subset of target proteins implies that an additional counteracting mechanism(s) must be simultaneously engaged. In this respect, we have shown, in rat and dog, that the β_2-**LR***-G_i signaling pathway can fully antagonize the β_2-**LR***-G_s-cAMP-mediated effects in the bulk cytosolic compartment (Xiao et al., 1994; Altschuld et al., 1995; Kuschel et al., 1999a); but not in the vicinity of L-type Ca^{2+} channel (Xiao and Lakatta, 1993; Altschuld et al., 1995; Xiao et al., 1995; Zhou et al., 1997; Kuschel et al., 1999b). In the mouse, β_2-**LR***-G_i signaling dominates, negating β_2-**LR***-G_s effects in both sarcolemmal and cytosolic compartments (Xiao et al., 1999; also see Fig. 5, A and B). Hence, activation of G_i is involved in the intracellular sorting of β_2-**LR***-G_s-cAMP signal. However, the same mechanism cannot explain the inability of β_2-R* to modulate the L-type Ca^{2+} channel because there is little β_2-R*-G_i coupling (Gurdal et al., 1997), and in the present study, PTX treatment cannot potentiate the basal I_{Ca} in TG4 cells (Fig. 6B). Thus, some unidentified mechanisms must be involved in the differential cAMP signaling induced by β_2-R* versus β_2-**LR***. For example, β_2-R*

and β_2-**L**R* could couple to different isoforms of G_s (Seifert et al., 1998) or adenylyl cyclase (for review see Tang and Hurley, 1998), or to distinctively localized components of the cAMP signaling cascade, such as cAMP (Hohl and Li, 1991) or PKA (Buxton and Brunton, 1983). In addition, localized activation of phosphodiesterase (Jurevicius and Fischmeister, 1996), protein phosphatase (Kuschel et al., 1999a), or specific anchoring proteins of PKA (Gray et al., 1998) may also contribute to subcellular compartmentalization of cAMP or PKA during β_2-R* or β_2-**L**R* stimulation. The exact mechanism underlying the inability of β_2-R*-cAMP signaling to regulate I_{Ca} remains to be elucidated in future studies.

Possible Mechanism for β_2-R* to Augment Cardiac Contractility. Cardiac contractility is an integrated parameter determined by several effectors involved in the E-C coupling cascade. Although I_{Ca} is unaffected by β_2-R*, the increase in the adenylyl cyclase activity and cAMP production may modulate the E-C coupling cascade by PKA-dependent phosphorylation of target proteins downstream of L-type Ca^{2+} channels, e.g., the SR Ca^{2+} release channels, SR membrane protein PLB, and some contractile proteins. Indeed, our preliminary observations have shown that in TG4 ventricular myocytes, the frequency of "Ca^{2+} sparks" (i.e., the elementary SR Ca^{2+} release events) and the amplitude of whole cell Ca^{2+} transients are markedly increased in TG4 cells, and that both are sensitive to ICI. In addition, there is an adaptive down-regulation of PLB expression in TG4 hearts (Rockman et al., 1996) and thereby less basal inhibition of the SR Ca^{2+} pump in cardiac cells from these transgenic animals. Thus, the enhanced SR Ca^{2+} recycling may be sufficient to account for the augmentation of baseline contractility in TG4 heart. Regardless of the specific mechanism, the suppression of the enhanced basal contractility by Rp-CPT-cAMPS (Fig. 3) indicates that the β_2-R*-elicited contractile effect is largely cAMP/PKA dependent.

Loss of β_1AR Function Associated with β_2AR Overexpression. Although both β_1AR and β_2AR coexist in mouse ventricular myocyte, the function of β_1AR is undetectable in β_2AR overexpression transgenic (TG4) murine heart, as shown by the absence of I_{Ca} (Fig. 7, A and B) or contractile response (Fig. 7C; also see Bond et al., 1995; Du et al., 1996) to β_1AR stimulation by either NE plus prazosin or isoproterenol plus the β_2AR blocker, ICI. In contrast, in WT mouse ventricular myocyte, β_1AR stimulation produced a dose-dependent increase in contraction amplitude (Korzick et al., 1997) and I_{Ca} (Fig. 7, A and B). In TG4 myocytes, PTX treatment only rescues the contractile and I_{Ca} responses to β_2AR agonists, but not to β_1AR agonists (Fig. 7; also see Xiao et al., 1999). Although the exact mechanism for the loss of β_1AR function in TG4 heart is unknown, this phenotype seems to be linked to the overexpression of β_2AR, because the β_1AR function also disappeared in rat C_6 glioma cells overexpressed β_2AR (Zhong et al., 1996). These results indicate a complex interaction between βAR subtypes (Zhong et al., 1996).

β_2-AR Stimulation in TG4 Hearts at Different Ages. Recent studies have shown that I_{Ca} density is increased in embryonic/neonatal TG4 myocytes (An et al., 1999), but decreased in 3- to 8-month old TG4 mouse heart cells (Heubach et al., 1999) as compared with age-matched controls. In the present study, we found no evidence for any difference in I_{Ca} characteristics between transgenic and WT cells from young adult animals (2–3 months old). This apparent discrepancy may reflect an age-related change in βAR signaling cascade. In nontransgenic rat, there are striking developmental changes with respect to β_2AR agonist sensitivity and functions (Kuznetsov et al., 1995, Xiao et al., 1998), perhaps due to a developmental changes in β_2AR-G_i coupling. In this scenario, it is not surprising that spontaneous β_2AR activation may exhibit differential functions at different stages of development. Alternatively, it is possible that some compensatory changes (e.g., expression of L-type Ca^{2+} channel) may occur progressively as a result of the receptor overexpression, rendering divergent and even conflicting phenotypes at different ages. Nevertheless, as discussed above, a compensatory change in Ca^{2+} channel sensitivity to cAMP-PKA signaling cannot account for the inability of β_2-R*s to regulate I_{Ca} in the young mouse heart.

Additionally, it is noteworthy that there is a common thread among these reports: the effect of β_2-R*s in TG4 cardiac myocytes is highly compartmentalized and target protein-specific. In embryonic/neonatal TG4 cells, β_2-R*s augment I_{Ca} but not cAMP-sensitive potassium currents (I_K) (An et al., 1999). In young adult TG4 cells (2–3 months), baseline contraction is increased but I_{Ca} is unchanged (this study); whereas in older (3–8 months) TG4 cells, I_{Ca} is down-regulated without changing baseline contractility (Heubach et al., 1999). The results in adult TG4 cells also suggest a general pattern for dissociation between alterations in baseline contractility and I_{Ca} in this transgenic model.

In summary, we have provided several lines of evidence that in TG4 cardiac myocytes, ligand-independent, spontaneously activated β_2ARs, in contrast to the ligand-activated β_2ARs, do not regulate the L-type Ca^{2+} channel, despite the fact that both β_2-R* and β_2-**L**R* can increase cAMP and contractility. However, salient properties of L-type channels in TG4 cells are unaltered and I_{Ca} response to β_2-**L**R* (in PTX-treated cells) or forskolin remains intact. These results suggest that β_2-R* may differ from β_2-**L**R*, and thereby the two-state receptor model apparently needs to be expanded to accommodate additional active receptor species. These novel findings of the present study also raise many important unsolved questions. 1) What is the mechanism controlling the sorting of intracellular signals en route from the same receptor at different active states? 2) What are the effectors via which β_2-R* produce a positive inotropic effect? 3) Why are L-type Ca^{2+} channels inaccessible to β_2-R*-stimulated cAMP yet receptive to β_2-**L**R*- and adenylyl cyclase-elicited cAMP signaling? 4) What is the mechanism underlying the development- and age-associated differences in β_2-AR signaling? Future studies are required to further understand these detailed aspects of β_2-R* and β_2-**L**R* signaling.

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