RhoA GTPase regulates L-type Ca\textsuperscript{2+} currents in cardiac myocytes

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Yatani, Atsuko, Keiichi Irie, Takayuki Otani, Maha Abdellatif, and Lei Wei. RhoA GTPase regulates L-type Ca\textsuperscript{2+} currents in cardiac myocytes. Am J Physiol Heart Circ Physiol 288: H650–H659, 2005. First published October 7, 2004; doi:10.1152/ajpheart.00268.2004.—Regulation of ionic channels plays a pivotal role in controlling cardiac function. Here we show that the Rho family of small G proteins regulates L-type Ca\textsuperscript{2+} currents in ventricular cardiomyocytes. Ventricular myocytes isolated from transgenic (TG) mice that overexpress the specific GDP dissociation inhibitor Rho GDI-\alpha exhibited significantly decreased basal L-type Ca\textsuperscript{2+} current density (~40%) compared with myocytes from nontransgenic (NTG) mice. The Ca\textsuperscript{2+} channel agonist BAY K 8644 and the \beta-adrenergic agonist isoprotolennol increased Ca\textsuperscript{2+} currents in both NTG and TG myocytes to a similar maximal level, and no changes in mRNA or protein levels were observed in the Ca\textsuperscript{2+} channel alpha\textsubscript{1}-subunits. These results suggest that the channel activity but not the expression level was altered in TG myocytes. In addition, the densities of inward rectifier and transient outward K\textsuperscript{+} currents were unchanged in TG myocytes. The amplitudes and rates of basal twitches and Ca\textsuperscript{2+} transients were also similar between the two groups. When the protein was delivered directly into adult ventricular myocytes via TAT-mediated protein transduction, Rho GDI-\alpha significantly decreased Ca\textsuperscript{2+} current density, which supports the idea that the defective Ca\textsuperscript{2+} channel activity in TG myocytes was a primary effect of the transgene. In addition, expression of a dominant-negative Rac-1 or Cdc42 also significantly decreased Ca\textsuperscript{2+} current density, which indicates that inhibition of Ca\textsuperscript{2+} channel activity by overexpression of Rho GDI-\alpha is mediated by inhibition of RhoA. This study points to the L-type Ca\textsuperscript{2+} channel activity as a novel downstream target of the RhoA signaling pathway.

GDP dissociation inhibitor; TAT-mediated protein transduction; K\textsuperscript{+} channel; ventricular; cardiomyocyte

RHO GTPASE FAMILY PROTEINS, which include RhoA, Rac-1, and Cdc42, control a wide variety of cellular processes such as cell morphology, motility, proliferation, differentiation, and apoptosis (11, 28). A considerable amount of attention has centered lately on their role in cardiac myocyte hypertrophy, which is an important adaptive growth response to pressure or volume overload (9). In cultured cardiomyocytes, RhoA mediates hypertrophic signals induced by the \alpha\textsubscript{1}-adrenergic agonist phenylephrine (13), angiotensin II (2), and mechanical stress (1). Similarly, expression of an activated form of Rac-1 appears to stimulate the hypertrophic program, whereas expression of a dominant-negative Rac-1 is inhibitory in cultured cardiomyocytes (20).

RhoA, Rac-1, and Cdc42 were recently reported to mediate receptor-coupled G protein signaling for regulating ion channels in a variety of cell culture systems. RhoA was found to suppress the activity of the delayed rectifier K\textsuperscript{+} channel K\textsubscript{1.2} (6). RhoA and Rac-1 have been shown to regulate the ether-\textsubscript{a}-go-go-related K\textsuperscript{+} channel in a rat pituitary cell line (24). Rac-1 was found to mediate inhibition of voltage-dependent Ca\textsuperscript{2+} currents by bradkin in a neuronal cell line (32). Although there is considerable evidence that Rho GTPases regulate ion channel activity in other cell systems, their roles in regulating cardiac ion channel activities remain unknown.

We have previously (29) generated transgenic (TG) mice with cardiac-specific inhibition of Rho family proteins by expressing the specific GDP dissociation inhibitor Rho GDI-\alpha under the control of the cardiac-specific \alpha-myosin heavy chain promoter, which is activated during early cardiogenesis [from embryonic day 8 (E8.0); Ref. 26]. We observed that first-generation TG mice that expressed the highest levels of the transgene died around E10.5, and that heart tube looping and ventricular maturation were disrupted in these TG embryos (29). Heterozygotes of middle-copy lines had no early-lethal embryonic phenotype but did display progressive atrioventricular conduction defects (30), which suggests that Rho GTPases are involved in the regulation of cardiac electrical activity. Because L-type Ca\textsuperscript{2+} channels are crucial for cardiac excitation-contraction coupling and are regulated by intracellular signals such as heterotrimmeric G proteins, protein kinases, and calmodulin (4, 7, 12, 18, 25), we examined the effects of Rho GTPase in ventricular myocytes isolated from these TG mice. Here we present evidence indicating that L-type Ca\textsuperscript{2+} channel activity is a downstream target of the RhoA-signaling pathway in cardiac myocytes.

MATERIALS AND METHODS

All experiments were conducted in accordance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals” and were approved by the Institutional Animal Care and Use Committee.

Generation of TG mice. FVB/N mice that expressed bovine Rho GDI-\alpha under the control of the murine \alpha-myosin heavy chain promoter have been described (29). The TG mice used in this study were heterozygotes of an M2 line with approximately sevenfold overexp-
pression of Rho GDI-α. TG and nontransgenic (NTG) littermates at 4 wk and 4 mo of age were used in the present study.

**Immunoblot analysis.** Protein samples were from a single heart of a TG or NTG mouse as previously described (29). Separation of cytosolic and membrane fractions and immunoblot analysis of the L-type Ca\(^{2+}\) channel Ca1.2 subunit using a rabbit polyclonal antibody against the Ca1.2 subunit (Alomone Labs; Jerusalem, Israel) were performed as previously described (29).

**RT-PCR analysis.** Transcription levels of the Ca1.2 subunit of L-type Ca\(^{2+}\) channels in TG hearts were assessed by semiquantitative RT-PCR as previously described (29). GAPDH (16–19 cycles) was amplified as a control marker with primers as described (8). The α1-subunit of the cardiac L-type Ca\(^{2+}\) channel was amplified (19–22 cycles) with the following primers: forward, 5′-CCAGC-GAGAAACCTCAACAGCAG-3′; reverse, 5′-GAGGACTACAGGT-TGCTGACG-3′.

**Cellular electrophysiological, mechanical, and Ca\(^{2+}\) transient measurements.** Left ventricular myocytes were isolated from the apical two-thirds of the left ventricle of NTG and TG mice, and whole cell currents were recorded using patch-clamp techniques as previously described (17, 34). Myocyte contraction and Ca\(^{2+}\) transients were measured as previously described (33, 35). Briefly, isolated left ventricular myocytes were perfused with Tyrode solution composed of (in mM) 135 NaCl, 1.0 CaCl\(_2\), 1.0 MgCl\(_2\), 5.4 KCl, 10 glucose, and 5 HEPES (pH 7.3) at 32°C and were field stimulated at 1.0 Hz. Myocyte contractile and relaxation functions were measured using a video motion-edge detector. For the Ca\(^{2+}\) transient measurements, cells were loaded with 2 μM fura 2-AM at room temperature for 1 h. Intracellular free Ca\(^{2+}\) was monitored as the ratio of 340-to-380 nm fluorescence of fura 2 using a Photocam dual-beam spectrofluorophotometer (Photon Technology). The changes in Ca\(^{2+}\) transience were evaluated by direct reading of the fluorescence intensity.

Sarcoplasmic reticulum (SR) Ca\(^{2+}\) content was evaluated by a caffeine-pulse protocol similar to that used by Puglisi et al. (21). In brief, cells were given a series of 10 stimulations (0.5 Hz) to load SR Ca\(^{2+}\). Once cells were loaded, electrical stimulation was stopped and we switched to Tyrode solution that contained caffeine (10 mM). SR Ca\(^{2+}\) content was assessed from caffeine-induced Ca\(^{2+}\) transient amplitudes.

Cell capacitance was measured using voltage ramps of 0.8 V/s from a holding potential of −50 mV. L-type Ca\(^{2+}\) currents (\(I_{\text{Ca}}\)) were recorded using an external solution that contained (in mM) 2 CaCl\(_2\) or BaCl\(_2\), 1 MgCl\(_2\), 135 tetraethylammonium chloride, 5 K-4-aminopyridine, 10 glucose, and 10 HEPES (pH 7.3). The pipette solution contained (in mM) 100 cesium aspartate, 20 CsCl, 1 MgCl\(_2\), 2 Mg-ATP, 0.5 GTP, 5 EGTA, and 5 HEPES (pH 7.3). These solutions provided isolation of \(I_{\text{Ca}}\) from other membrane currents and the Na\(^{+}\)-Ca\(^{2+}\) exchanger. For experiments with isoproterenol and forskolin, BAPTA (10 mM) was replaced with EGTA in the pipette solution to minimize Ca\(^{2+}\)-dependent inactivation.

For K\(^+\) current recordings, myocytes were perfused with Tyrode solution. Nifedipine (10 μM) was added to block \(I_{\text{Ca}}\) and the patch-pipette solution contained (in mM) 110 potassium aspartate, 20 KCl, 2 MgCl\(_2\), 2 ATP, 0.5 GTP, 5 EGTA, and 5 HEPES (pH 7.3). The K\(^+\) voltage dependence of peak current activation was determined using an interactive nonlinear regression-fitting procedure to the Boltzmann equation as follows: \(I_{\text{norm}} = I/1 + e^{(V_{\text{m}} - V_{\text{hm}})/k}\), where \(I_{\text{norm}}\) is the normalized current to the maximal peak current during the test pulse to +60 mV, \(V_{\text{m}}\) is the membrane potential, \(V_{\text{hm}}\) is the midpotential, and \(k\) is the slope factor.

**TAT-mediated protein transduction into cultured cardiomyocytes.** Constitutively active RhoA-V14 and dominant-negative RhoA-N19, Rac-1-N17, Cdc42-N17, and Rho GDI-α were cloned in-frame into the bacterial expression vector pTAT-HA (kindly provided by Dr. Steven F. Dowdy). TAT fusion proteins were purified from BL21-CodonPlus(DE3) cells (Stratagene; La Jolla, CA) under native conditions using a nickel-nitrilo-triacetic acid (Ni-NTA) column. The purification protocol was adapted from the published procedure (3). Briefly, bacterial pellets were sonicated in 20 mM HEPES (pH 7.2) and 100 mM NaCl in the presence of protease inhibitors (buffer A). The clarified sonicate was equilibrated in 10 mM imidazole and then applied to a preequilibrated Ni-NTA column. After the column was washed with 10 bed volumes of buffer A and 10 mM imidazole, TAT fusion proteins were eluted with increasing imidazole concentrations and then desalted via a PD-10 column into the medium of choice. For the effects on \(I_{\text{Ca}}\) in ventricular myocytes isolated from NTG mice were preincubated for 2–4 h with TAT fusion proteins dialyzed with Tyrode solution for 2 h at concentrations ranging from 50 to 400 μg/ml. Effects of TAT proteins on actin cytoskeleton organization were examined using neonatal rat cardiac fibroblasts and cardiomyocytes by immunofluorescence analysis with a rhodamine-phalloidin conjugate (Molecular Probes; Junction City, OR) as previously described (31).

**Statistical analysis.** Data are reported as means ± SE. Comparisons between groups were analyzed by Student’s t-test or ANOVA as appropriate with \(P < 0.05\) considered as significant.

**RESULTS**

*L-type Ca\(^{2+}\) channel currents.* We previously described that Rho GDI-α TG mice (M2 line), in which the transgene level is approximately sixfold higher than endogenous Rho GDI-α (Fig. 1A), had no early-lethal embryonic phenotype, and that the activity of Rho family proteins was inhibited in the TG hearts (29, 30). Whole cell patch-clamp studies were performed on ventricular myocytes of M2 line mice. Myocyte capacitance values, which are a measure of cell size, were not different between TG and NTG myocytes at 4 wk of age (TG: 124 ± 6 pF, \(n = 29\); NTG: 122 ± 3 pF, \(n = 76\)) but became significantly increased at 4 mo of age in TG myocytes (TG: 153 ± 4 pF, \(n = 119\); NTG: 122 ± 6 pF, \(n = 75\); \(P < 0.01\)), which is consistent with mild hypertrophy observed at this age (~15% increase in ventricular weight in TG compared with NTG mice; Ref. 30).

The traces in Fig. 1C, a and b, indicate that TG myocytes exhibited significantly smaller \(I_{\text{Ca}}\) density (peak inward current amplitude normalized to cell capacitance, measured in pA/pF) compared with NTG myocytes. The peak \(I_{\text{Ca}}\) density was significantly decreased in TG compared with NTG myocytes at both 4 wk (TG: 5.0 ± 0.3 pA/pF, \(n = 88\); NTG: 8.5 ± 0.4 pA/pF, \(n = 25\); \(P < 0.01\); Fig. 1B) and 4 mo (TG: 4.4 ± 0.3 pA/pF, \(n = 32\); NTG: 8.6 ± 0.5 pA/pF, \(n = 35\); \(P < 0.01\)) of age, which indicates that the decreased \(I_{\text{Ca}}\) density was not secondary to myocyte hypertrophy. There was no change in the current-voltage (\(I-V\)) relationship (Fig. 1C, c). At the potential where \(I_{\text{Ca}}\) reached a maximum value (+10 mV), \(I_{\text{Ca}}\) inactivated rapidly during maintained depolarization in both groups (Fig. 1D). The time to half-decay (\(T_{1/2}\) of \(I_{\text{Ca}}\) in TG myocytes was significantly slower (31.0 ± 0.3 ms, \(n = 32\)) than in NTG myocytes (18.3 ± 1.1 ms, \(n = 35\); \(P < 0.01\)). Decay kinetics of Ca\(^{2+}\) channels are also an important parameter for Ca\(^{2+}\) entry. To examine whether the slower inactivation time was secondary to the smaller \(I_{\text{Ca}}\), we compared inactivation time courses using 2 mM Ba\(^{2+}\) as the charge carrier (Fig. 1E). Ba\(^{2+}\) currents showed significantly slower inactivation compared with \(I_{\text{Ca}}\) in both NTG and TG myocytes such that these were no longer different, which indicates that the slower \(I_{\text{Ca}}\) inactivation observed in TG myocytes may be due to Ca\(^{2+}\)-dependent fast inactivation.
Fig. 1. L-type Ca\(^{2+}\) channel currents (I\(_{\text{Ca}}\)) were reduced in GDP dissociation inhibitor Rho GDI-α transgenic (TG) ventricular myocytes. A: Western blot analysis was performed with proteins extracted from ventricles of 4-wk- or 4-mo-old nontransgenic (NTG) and TG mice using an anti-Rho GDI polyclonal antibody that recognizes both endogenous Rho GDI-α and the transgene. B: TG myocytes exhibited decreased I\(_{\text{Ca}}\) at both 4 wk and 4 mo of age. I\(_{\text{Ca}}\) was normalized to the cell capacitance to yield current densities (in pA/pF). C: representative I\(_{\text{Ca}}\) recorded in NTG (a) and TG (b) ventricular myocytes isolated from 4-wk-old mice. Traces show currents elicited from a holding potential of −50 mV to the indicated test potentials. Pooled current-voltage (I-V) relationships obtained from 4-wk- and 4-mo-old NTG and TG myocytes are shown (c). Voltage-dependent I\(_{\text{Ca}}\) activation was not changed in TG myocytes. D and E: time courses of I\(_{\text{Ca}}\) inactivation recorded from NTG (a) and TG (b) myocytes in the presence of 2 mM Ca\(^{2+}\) (D) or Ba\(^{2+}\) (E). Currents were scaled to the same peak current amplitude to compare waveforms (c). Currents were elicited by depolarizing steps to +10 (D) or 0 (E) mV from a holding potential of −50 mV. Mean times to half-decay of currents (T\(_{1/2}\)) obtained from 4-wk-old NTG and TG myocytes were compared (d). Data are means ± SE; n, no. of myocytes examined; *P < 0.01 vs. NTG myocytes.
Decreased $I_{Ca}$ density reflects either a decrease in channel activity or a decrease in the total number of channels. To examine these possibilities, we tested the effects of the dihydropyridine agonist BAY K 8644 on $I_{Ca}$. The $I-V$ relationships before and after the addition of BAY K 8644 were measured, and the peak $I_{Ca}$ (usually at a potential of 0 mV) was used to evaluate maximal increase. In the presence of BAY K 8644 (0.1 μM), the maximal $I_{Ca}$ density in TG myocytes was not significantly different from NTG myocytes (TG: 16.4 ± 1.1 pA/pF, n = 13; NTG: 18.2 ± 0.9 pA/pF, n = 25; Fig. 2A). Cardiac $I_{Ca}$ are regulated by a cAMP-dependent PKA pathway that results in the phosphorylation of the channels (15). We examined effects of forskolin, which directly activates adenylyl cyclase, on $I_{Ca}$. Forskolin (5 μM) increased $I_{Ca}$ in all myocytes (TG: 26.9 ± 2.3 pA/pF, n = 10; NTG: 27.2 ± 2.0 pA/pF, n = 17; Fig. 2A). We also compared the effects of isoproterenol, which is a β-adrenergic receptor agonist. The responsiveness to isoproterenol (as determined by maximal $I_{Ca}$ and EC50 values) was not significantly different between TG and NTG myocytes, and the difference in current density was abolished in the presence of isoproterenol (Fig. 2B).

In addition, we have examined expression levels of the α1C-subunit, which contains the ion-conducting pore, in TG hearts via immunoblot and RT-PCR analysis. As shown in Fig. 2C, expression of the α1C-subunit of the cardiac L-type Ca2+ channel was not significantly altered in TG hearts relative to NTG hearts. These results suggest that the decreased $I_{Ca}$ density in TG myocytes is not caused by decreased channel abundance but is probably due to a decrease in channel open probability.

$K^+$ channel currents. Figure 3A illustrates typical outward $K^+$ currents recorded in NTG and TG myocytes (a and b, respectively) at 4 wk of age. In both groups, depolarization positive to −30 mV activated outward currents, which then decayed slowly to a sustained outward current at the end of a 300-ms voltage step. Details of electrophysiological characteristics of the outward $K^+$ currents in mouse ventricular myocytes that exhibit a sum of fast and slow components have been described elsewhere (37). In the present study, we refer to the total $K^+$ current components simply as $I_{K}$. There were no significant changes in the $I_{K}$ amplitude and voltage dependence of activation between the two groups (Fig. 3B, a). The activation curves (Fig. 3B, b) generated from the original recordings (shown in Fig. 3B, a) revealed that $V_{0.5}$ and $k$ values in NTG and TG myocytes were similar. Similarly, there was no significant difference in the density of the inward rectifier $K^+$ currents ($I_{K1}$) between NTG and TG myocytes. The mean $I_{K1}$ densities at −100 mV in NTG and TG myocytes were similar (Fig. 3C). Thus it appears that changes in $I_{K1}$ are not associated with changes in repolarizing currents in Rho GDI-α TG myocytes.

Cardiomyocyte contractions and Ca2+ transients. Figure 4 shows twitch contractions and Ca2+ transients in myocytes.
isolated from NTG and TG mice (at 4 wk of age) under steady-state conditions (1.0 Hz). The amplitudes of myocyte contraction (percent cell shortening) and Ca\(^{2+}\)/H\(_{11001}\) transient were similar between the two groups. The pooled data (Table 1) indicate that the maximum rates of myocyte contraction (\(dL/dt\)) and relaxation (\(dL/dt\)) were also similar between the two groups. Ca\(^{2+}\)/H\(_{11001}\) transient results are consistent with the myocyte contraction data. There was no significant difference in peak amplitude between the two groups (Table 1). Because the rate of Ca\(^{2+}\) transient decline during twitch primarily reflects Ca\(^{2+}\) removal via the SR Ca\(^{2+}\) uptake, we compared the time course of Ca\(^{2+}\) transients. The \(T_{1/2}\) value for Ca\(^{2+}\) transients during twitch was unchanged in TG compared with NTG myocytes (Table 1).

To understand myocyte contraction and Ca\(^{2+}\)/H\(_{11001}\) transient results, we compared the amount of Ca\(^{2+}\) influx through L-type Ca\(^{2+}\)/H\(_{11001}\) channels and release of Ca\(^{2+}\) from the SR. However, the difference was not significant, probably because decay of \(I_{Ca}\) in TG myocytes was slower than in NTG myocytes. SR Ca\(^{2+}\) content was also assessed by caffeine-induced Ca\(^{2+}\)/H\(_{11001}\) transients upon termination of a steady train of stimulation. Peak Ca\(^{2+}\) transients were not significantly different between NTG (0.47 ± 0.1, \(n = 20\)) and TG (0.46 ± 1.2, \(n = 3\); \(P = NS\)) myocytes, which is consistent with the previous observation (30) that the mRNA abundances of sarco(endo)plasmic reticulum Ca\(^{2+}\)/H\(_{11001}\)-ATPase and phospholamban in TG ventricles were unchanged at 4 wk of age. The data suggest that a submaximal \(I_{Ca}\) can trigger a maximal SR Ca\(^{2+}\)/H\(_{11001}\) release, and that the reduction in peak \(I_{Ca}\) may not result in serious contractile alterations in TG hearts assuming that the SR Ca\(^{2+}\)/H\(_{11001}\)-loading function is normal (14). However, it is also possible that the preservation of contractility observed with TG myocytes could involve enhanced coupling of the Ca\(^{2+}\) entry through L-type Ca\(^{2+}\)/H\(_{11001}\) channels and release of Ca\(^{2+}\) from the SR.
Direct delivery of Rho GDI-α protein into ventricular myocytes decreased \( I_{Ca} \). The decreased \( I_{Ca} \) at 4 wk of age may be a direct effect of Rho GDI-α expression or an indirect effect of transgene expression on the developmental modulation of cellular architecture during cardiomyocyte differentiation. To distinguish between these possibilities, we examined the consequences of acute expression of Rho GDI-α using human immunodeficiency virus TAT-mediated delivery (protein transduction) of Rho GDI-α into adult mouse cardiomyocytes. It has been shown (23) that proteins fused to the 11-amino acid protein-transduction domain of the human immunodeficiency virus TAT protein can be transferred directly to a variety of tissues, organs, or cells. The small, positively charged protein-transduction domain makes contacts with the negatively charged outer membrane of the cell and can freely cross cell membranes independently of receptors and transporters. Neo-

![Cell shortening](image1)

**A** Cell shortening

- **a** NTG
- **b** TG

- **5%**
- **0.5 sec**

**B** \( \text{Ca}^{2+} \) transients

- **a** NTG
- **b** TG

- **0.2**
- **0.5 sec**

**C**

- **a** NTG
- **b** TG

- **2 pA/pF**
- **100 ms**

- **d** Area 300 ms (pC/pF)

**Fig. 4.** A: typical examples of myocyte contractions recorded in 4-wk-old NTG (a) and TG (b) myocytes. B: fura 2 \( \text{Ca}^{2+} \) transients. Constructions and \( \text{Ca}^{2+} \) transients were recorded during field stimulation at frequencies of 1.0 and 0.5 Hz, respectively. C: \( I_{Ca} \) integrals. Typical examples of \( I_{Ca} \) elicited at +10 mV in 4-wk-old NTG (a) and TG (b) are shown with corresponding \( I_{Ca} \) integrals (c). Average \( I_{Ca} \) integrals are indicated (d).

### Table 1. Properties of baseline myocyte shortening and \( \text{Ca}^{2+} \) transients

<table>
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<tr>
<th></th>
<th>Nontransgenic*</th>
<th>Transgenic†</th>
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<td><strong>Contractile parameters</strong></td>
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<tr>
<td>Shortening, %</td>
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<td>7.6±0.6</td>
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<tr>
<td>( +dL/dt ), ( \mu m/s )</td>
<td>225.3±9.3</td>
<td>224.9±16.4</td>
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<tr>
<td>( -dL/dt ), ( \mu m/s )</td>
<td>180.0±11.4</td>
<td>165.3±12.5</td>
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### Ca\(^{2+}\) kinetics

<table>
<thead>
<tr>
<th></th>
<th>Nontransgenic*</th>
<th>Transgenic†</th>
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<tr>
<td>Amplitude, 340/380 nm</td>
<td>0.28±0.02</td>
<td>0.28±0.02</td>
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<tr>
<td>( T_{1/2} ), ms</td>
<td>287.2±13.9</td>
<td>273.2±19.1</td>
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Data are means ± SE; *\( n = 35 \) and †\( n = 21 \) myocytes examined. Cell twitch and \( \text{Ca}^{2+} \) transients were determined under steady-state conditions at 1.0 and 0.5 Hz, respectively. \( +dL/dt \), myocyte relaxation; \( -dL/dt \), myocyte contraction; \( T_{1/2} \), time to 50% decline of \( \text{Ca}^{2+} \) transient.
natal rat cardiac myocytes and fibroblasts were used to test protein delivery into cultured cells as well as the function of the TAT fusion proteins before the whole cell patch-clamp study was performed with adult mouse ventricular myocytes.

TAT-Rho GDI-α fusion protein was produced in bacteria and purified under native conditions. A TAT-β-galactosidase (β-gal) fusion protein purified under native conditions was used as a control protein. Via β-gal staining, we observed that 100% of cultured neonatal rat cardiomyocytes were uniformly transduced with TAT-β-gal in a concentration-dependent manner after 1 h of incubation (data not shown). Western blot analysis indicated that TAT-Rho GDI-α was transduced into neonatal rat cardiomyocytes within 15 min of incubation and was stable for several hours (Fig. 5A). The level of TAT-Rho GDI-α transduced into neonatal rat cardiomyocytes increased in a dose-dependent manner upon incubation with TAT-Rho GDI-α (ranging from 50 to 500 μg/ml). The level is similar to that of endogenous Rho GDI-α when incubated with 100 μg/ml of TAT-Rho GDI-α for 1 h (Fig. 5A). We then tested whether TAT-Rho GDI-α was functional. After 1 h of incubation with 200 μg/ml TAT-Rho GDI-α but not with TAT-β-gal (≤500 μg/ml), the majority of neonatal rat cardiac fibroblasts exhibited cell shape changes due to altered stress-fiber formation (Fig. 5B), which are indicative of inhibition of the Rho GTPases by TAT-Rho GDI-α. On the other hand, no significant cell shape changes were detected in TAT-Rho GDI-α-treated neonatal rat cardiomyocytes (Fig. 5B), in which actin fibers organized into sarcomeres and could not be disrupted by inhibition of Rho GTPases.

We then tested the effects of TAT-Rho GDI-α on I_Ca in adult mouse ventricular myocytes. Reduced I_Ca density and slower T1/2 values were observed in myocytes incubated with TAT-Rho GDI-α (100 μg/ml) but not in those incubated with TAT-β-gal (Fig. 5C). These results suggest that decreased I_Ca density is a direct consequence of increased expression of Rho GDI-α in the TG myocytes.

Fig. 5. Acute expression of Rho GDI-α decreased I_Ca density. A: immunoblot analysis of TAT-Rho GDI-α transduced into neonatal rat cardiomyocytes. Cardiomyocytes were incubated with 100 μg/ml TAT-Rho GDI-α for 15 min to 2 h. Immunoblotting was then performed with protein extracted from cardiomyocytes using specific antibodies as indicated. Equal amounts of protein (20 μg) were loaded on each lane. B: functional analysis of TAT-fusion proteins on actin cytoskeleton organization in neonatal rat cardiac fibroblasts as shown by phalloidin staining. TAT-Rho GDI-α induced cell shape changes in fibroblasts but not in myocytes. C: effects of TAT-Rho GDI-α on I_Ca in adult mouse ventricular myocytes. Holding potential was −50 mV, and test pulse was +10 mV. TAT-Rho GDI-α decreased I_Ca and increased T1/2 values for I_Ca. TAT-Rho GDI-α had no significant effect on membrane capacitance. *P < 0.01 vs. untreated myocytes.
RhoA but not Rac-1 and Cdc42 regulated ICa in ventricular myocytes. To further identify which Rho GTPase family member is involved in the regulation of ICa, we examined the consequences of acute expression of dominant-negative mutants of RhoA, Rac-1, and Cdc42 (RhoA-N19, Rac-1-N17, and Cdc42-N17, respectively) and a constitutively active mutant of RhoA (RhoA-V14) in cultured cardiomyocytes through TAT fusion delivery. As shown for TAT-Rho GDI-α, TAT-RhoA-N19, TAT-RhoA-V14, TAT-Rac-1-N17, and TAT-Cdc42-N17 were transduced into neonatal rat cardiomyocytes within 15 min of incubation and remained stable for several hours. When incubated with 100 μg/ml, the levels of TAT-RhoA-N19, TAT-RhoA-V14, TAT-Rac-1-N17, or TAT-Cdc42-N17 were 5–10-fold greater than those of endogenous RhoA, Rac-1, or Cdc42 in cardiomyocytes, respectively (Fig. 6A). All of these TAT fusion proteins were functional as they induced cell shape changes when incubated with neonatal rat cardiac fibroblasts at a concentration of 100 μg/ml for 1 h. No significant cell shape changes were detected in these TAT fusion protein-treated neonatal rat cardiomyocytes.

Importantly, when incubated with adult mouse ventricular myocytes at a concentration of 100 μg/ml, only TAT-RhoA-N19 but not TAT-Rac-1-N17 or TAT-Cdc42-N17 significantly reduced ICa density (Fig. 6C). In addition, TAT-RhoA-V14 induced a small but significant increase in ICa density (Fig. 6C). These results demonstrate that inhibition of ICa by overexpression of Rho GDI-α is mediated by inhibition of RhoA but not by inhibition of Rac-1 and Cdc42.

Fig. 6. Acute expression of a dominant-negative mutant of RhoA but not of Rac-1 and Cdc42 decreased ICa density in ventricular myocytes. A: immunoblot analysis of TAT fusion proteins transduced into neonatal rat cardiomyocytes. Cardiomyocytes were incubated with 100 μg/ml of the positive mutant TAT-RhoA-V14 and the negative mutants TAT-RhoA-N19, TAT-Rac-1-N17, and TAT-Cdc42-N17 for 1 h. Immunoblotting was then performed with protein extracted from cardiomyocytes using specific antibodies as indicated. Equal amounts of protein (20 μg) were loaded on each lane. B: functional analysis of TAT-RhoA-N19 on actin cytoskeleton organization in neonatal rat cardiac fibroblasts as shown by phalloidin staining. TAT-RhoA-N19 induced cell shape changes in fibroblasts but not in myocytes. C: effects of TAT fusion proteins on ICa in adult mouse ventricular myocytes. Holding potential was −50 mV and test pulse was +10 mV. TAT-RhoA-N19 decreased whereas TAT-RhoA-V14 slightly increased ICa, and TAT-Rac-1-N17 and TAT-Cdc42-N17 had no significant effect on ICa. All of these TAT fusion proteins had no significant effect on membrane capacitance. *P < 0.01 vs. untreated myocytes.
DISCUSSION

In the present study, we identified cardiac $I_{Ca}$ as a target of the RhoA signaling pathway in ventricular myocytes. We observed that ventricular myocytes isolated from TG mice with cardiac-specific overexpression of Rho GDI-$\alpha$ exhibited significantly decreased $I_{Ca}$ density. In addition, altered $I_{Ca}$ density was also observed in myocytes isolated from young TG mice before the onset of cardiac hypertrophy; thus it is not a secondary event related to the development of myocyte hypertrophy. Furthermore, using TAT-mediated protein delivery, we demonstrated that acute expression of Rho GDI-$\alpha$ or a dominant negative of Rac-1 or Cdc42 reproduced the phenotype observed in the TG myocytes, which supports the idea that altered $I_{Ca}$ density is a primary effect of enhanced expression of Rho GDI-$\alpha$ and is mediated through inhibition of RhoA, a direct target of Rho GDI.$\alpha$.

To our knowledge, our results represent the first description for a role of RhoA in the regulation of ion channel activity in cardiomyocytes. Previous studies of the Rho GTPases in cardiomyocytes have focused on their role in mediating hypertrophic signals (9), because their primary cellular functions are thought to be regulation of actin cytoskeleton and transcription factor activities. In other cells, RhoA and Rac-1 were previously shown to regulate the delayed rectifier K$^+$ channel Kir-1.2 (6) and the ether-à-go-go-related K$^+$ channel (24). However, the contribution of these channels to mouse ventricular myocyte repolarization is very small. In our study, both $I_{K1}$ and outward K$^+$ current densities were not altered in TG myocytes at 4 wk of age.

Our results also suggest that Ca$^{2+}$ channel activity but not channel expression level is altered in TG myocytes. The Ca$^{2+}$ channel agonist BAY K 8644, the $\beta$-adrenergic agonist isoproterenol, or direct activation of adenyl cyclase by forskolin increased $I_{Ca}$ density to a similar level in both TG and NTG myocytes. In addition, the expression level of Cav1.2 in the membrane fraction was not significantly altered in TG hearts. These results support the idea that the channel abundance and pharmacological properties of the channels are not altered in TG myocytes. Recently, small G protein kir/Gem (4) and Rem and Rad (10) were found to interact with the $\beta$-subunit of the L-type Ca$^{2+}$ channel and thereby regulate the trafficking of the $\alpha_1$-subunit to the plasma membrane. These GTPases are members of a Ras-related GTPase subfamily (RGK family). Because the expression level of Cav1.2 at the plasma membrane was not reduced in Rho GDI-$\alpha$ TG myocytes, the mechanism by which RhoA regulates Ca$^{2+}$ channels appears to be different than that employed by kir/Gem, Rem, and Rad.

One potential mechanism is that RhoA may regulate $I_{Ca}$ density through its effects on cytoskeleton organization. Recent studies (16) suggested a role of actin filament organization in the regulation of $I_{Ca}$, which is upregulated in cardiomyocytes devoid of the actin-severing protein gelsolin or in cardiomyocytes treated with phalloidin, an actin filament stabilizer, whereas downregulation was observed in cytochalasin D-treated cardiomyocytes. However, inhibition of RhoA but not Rac-1 and Cdc42 decreases $I_{Ca}$ density, whereas all of these GTPases regulate actin cytoskeleton organization. In addition, treatment of cardiomyocytes with Y-27632, a specific inhibitor of Rho kinase, did not significantly reduce $I_{Ca}$ density (data not shown). It is thus likely that RhoA regulates cardiac $I_{Ca}$ density through an actin-independent signal pathway. On the other hand, cardiac $I_{Ca}$ is regulated by a variety of second-messenger pathways including PKA, PKG, PKC, protein tyrosine kinases, calmodulin, and Ca$^{2+}$ (7, 12, 18, 25). In other cell systems, RhoA produces many biological responses through cross-talks with signaling pathways involving PKA, PKG, and PKC (28). Whether the activity and/or cellular localization of these protein kinases are altered in cardiomyocytes upon inhibition of RhoA merits further investigation.

In ventricular myocytes, L-type Ca$^{2+}$ channels provide the major pathway for entry of extracellular Ca$^{2+}$ into the cytoplasm and thereby initiate excitation-contraction coupling. Pharmacological agents that either enhance or reduce $I_{Ca}$ density also cause changes in myocardial and myocyte contractility. However, both contractile and relaxation functions were largely preserved in Rho GDI-$\alpha$ transgenic hearts (30), which suggests functional compensation in this animal model under basal physiological conditions. Consistent with in vivo observations, there were no alterations in myocyte contractility and Ca$^{2+}$ transients in TG ventricular myocytes, which suggests that a submaximal $I_{Ca}$ can trigger a maximal SR Ca$^{2+}$ release, and that this reduction in peak $I_{Ca}$ may not result in serious contractile alterations in TG ventricular myocytes assuming that SR Ca$^{2+}$ loading function is normal. In other animal models in which excitation-contraction coupling processes are defective due to other abnormalities such as impaired SR Ca$^{2+}$ release, altered myocyte geometry, and alterations in other ionic channel processes, altered $I_{Ca}$ could exacerbate the defects in excitation-contraction coupling processes in these disease backgrounds.

It is worth noting that both cardiac-specific inhibition (30) and activation (22) of RhoA signaling resulted in alteration of cardiac rhythm and conduction. Ventricular cardiac L-type Ca$^{2+}$ channels are predominantly formed by the Cav1.2 subunit, which is also expressed at high levels in atria (5, 27). Cav1.3 subunits, which are only expressed in atria at much lower levels than Cav1.2 subunits, control pacemaker activity (19, 36). Whether RhoA also regulates the activity of Cav1.2 and Cav1.3 channels in atrial myocytes is worth additional investigation.

In summary, the present study provides important new insights into a novel function of RhoA in regulating cardiac $I_{Ca}$ density in ventricular myocytes. Although the signaling pathways regulating cardiac $I_{Ca}$ appear to be conserved in mammalian hearts, species-specific differences may exist. Future studies that examine the generality of the RhoA-dependent regulation of cardiac $I_{Ca}$ in other species including humans are warranted.

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REFERENCES


