Effects of Overexpression of the Na\(^+\)-Ca\(^{2+}\) Exchanger on [Ca\(^{2+}\)]\(_i\) Transients in Murine Ventricular Myocytes

Atsushi Yao, Zhi Su, Akihiko Nonaka, Iram Zubair, Liyan Lu, Kenneth D. Philipson, John H.B. Bridge, William H. Barry

Abstract—We measured [Ca\(^{2+}\)]\(_i\), and [Na\(^+\)]\(_i\) in isolated transgenic (TG) mouse myocytes overexpressing the Na\(^+\)-Ca\(^{2+}\) exchanger and in wild-type (WT) myocytes. In TG myocytes, the peak systolic level and amplitude of electrically stimulated (ES) [Ca\(^{2+}\)]\(_i\) transients (0.25 Hz) were not significantly different from those in WT myocytes, but the time to peak [Ca\(^{2+}\)]\(_i\) was significantly prolonged. The decline of ES [Ca\(^{2+}\)]\(_i\) transients was significantly accelerated in TG myocytes. The decline of a long-duration (4-s) caffeine-induced [Ca\(^{2+}\)]\(_i\), transient was markedly faster in TG myocytes, and [Na\(^+\)]\(_i\), was identical in TG and WT myocytes, indicating that the overexpressed Na\(^+\)-Ca\(^{2+}\) exchanger is functionally active. The decline of a short-duration (100-ms) caffeine-induced [Ca\(^{2+}\)]\(_i\), transient in 0 Na\(^+\)/0 Ca\(^{2+}\) solution did not differ between the two groups, suggesting that the sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase function is not altered by overexpression of the Na\(^+\)-Ca\(^{2+}\) exchanger. There was no difference in L-type Ca\(^{2+}\) current density in WT and TG myocytes. However, the sensitivity of ES [Ca\(^{2+}\)]\(_i\), transients to nifedipine was reduced in TG myocytes. This maintenance of [Ca\(^{2+}\)]\(_i\), transients in nifedipine was inhibited by Ni\(^{2+}\) and required SR Ca\(^{2+}\)-ATPase function. The decline of [Ca\(^{2+}\)]\(_i\), content, consistent with enhanced Ca\(^{2+}\) influx by reverse Na\(^+\)-Ca\(^{2+}\) exchange, and the resulting Ca\(^{2+}\)-induced Ca\(^{2+}\) release from SR. The rate of rise of [Ca\(^{2+}\)]\(_i\), transients in nifedipine in TG myocytes was much slower than when both the L-type Ca\(^{2+}\) current and the Na\(^+\)-Ca\(^{2+}\) exchange current function together. In TG myocytes, action potential amplitude and action potential duration at 50% repolarization were reduced, and action potential duration at 90% repolarization was increased, relative to WT myocytes. These data suggest that under these conditions, overexpression of the Na\(^+\)-Ca\(^{2+}\) exchanger in TG myocytes accelerates the decline of [Ca\(^{2+}\)]\(_i\), during relaxation, indicating enhanced forward Na\(^+\)-Ca\(^{2+}\) exchanger function. Increased Ca\(^{2+}\) influx also appears to occur, consistent with enhanced reverse function. These findings provide support for the physiological importance of both these modes of Na\(^+\)-Ca\(^{2+}\) exchange. (Circ Res. 1998;82:657-665.)

Key Words: Na\(^+\)-Ca\(^{2+}\) exchanger ■ transgenic mouse ■ [Ca\(^{2+}\)]\(_i\), transient ■ myocyte

In cardiac ventricular myocytes, an increase in [Ca\(^{2+}\)]\(_i\), is considered the mechanism of excitation-contraction coupling. It has been generally accepted that Ca\(^{2+}\) influx through the SL L-type Ca\(^{2+}\) channel induces Ca\(^{2+}\) release (CICR) from the SR, which leads to contraction.\(^1\,\text{2}\) Subsequently, Ca\(^{2+}\) is taken up by the SR Ca\(^{2+}\)-ATPase and extruded from the myocyte by the SL Na\(^+\)-Ca\(^{2+}\) exchanger operating in the “forward” mode. Therefore, the Na\(^+\)-Ca\(^{2+}\) exchanger competes with the SR Ca\(^{2+}\)-ATPase to lower [Ca\(^{2+}\)]\(_i\), and cause relaxation. The Na\(^+\)-Ca\(^{2+}\) exchanger can also operate in a “reverse” mode, causing the influx of 1 Ca\(^{2+}\) in exchange for 3 Na\(^+\) early during excitation.\(^2\) The reverse function of the Na\(^+\)-Ca\(^{2+}\) exchanger has been proposed to increase the content of Ca\(^{2+}\) in SR, \(^1\,\text{3}\,\text{4}\) directly activate the contractile elements,\(^5\) and/or trigger CICR, contributing to contraction.\(^6\,\text{9}\) However, it remains unclear the extent to which these various components of Na\(^+\)-Ca\(^{2+}\) exchanger function are important in physiological excitation-contraction coupling and relaxation.

TG mice overexpressing the Na\(^+\)-Ca\(^{2+}\) exchanger in ventricular myocardium have been produced by K.D. Philipson and associates, and initial studies of voltage-clamped isolated myocytes from these animals have demonstrated that forward function of the exchanger is enhanced 2.5-fold.\(^10\) These myocytes provide a model to examine further the effects of increased function of the Na\(^+\)-Ca\(^{2+}\) exchanger in intact myocytes. We therefore compared values of diastolic and peak systolic [Ca\(^{2+}\)]\(_i\), and the time course of rise and decline of [Ca\(^{2+}\)]\(_i\), transients in TG and WT myocytes. We also examined the effects of overexpression of the Na\(^+\)-Ca\(^{2+}\) exchanger on the action potential, \(I_{\text{Ca}}\), [Na\(^+\)]\(_i\), and SR Ca\(^{2+}\) content and the extent to which the Na\(^+\)-Ca\(^{2+}\) exchanger contributes to Ca\(^{2+}\) influx.

Materials and Methods 

Production of TG Mice

TG mice were produced as described by Adachi-Akahane et al.\(^10\) Briefly, the transgene construct consisted of the open reading frame

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of the canine cardiac Na\(^+-\)Ca\(^{2+}\) exchanger connected downstream to the \(\alpha\)-myosin heavy chain promoter. This promoter contains 4.5 kb of 5\('\) upstream sequence and 1 kb of the \(\alpha\)-myosin heavy chain gene encompassing exons 1 through 3 of the untranslated region. An SV40 transcriptional terminator was used to provide a polyadenyl-
tation device. TG mice lines were heterozygous so that non-TG littermates could be used as control WT mice in the present study.

**Dissociation of Adult Mouse Ventricular Myocytes**

Adult mouse myocyte isolations were performed as previously described. Briefly, hearts were removed from anesthetized mice and immediately attached to an aortic cannula. After perfusion with Ca\(^{2+}\)-free modified Tyrode’s solution for 5 minutes, hearts were digested with 0.90 mg/mL collagenase D (Boehringer-Mannheim Biochemicals) in 25 \(\mu\)mol/L CaCl\(_2\)-containing modified Tyrode’s solution for 7 to 12 minutes. These two solutions consisted of (mmol/L) NaCl 126, KCl 4.4, MgCl\(_2\), NaHCO\(_3\), 18, glucose 11, HEPES 4, and BDM 20, along with 0.13 U/mL insulin, and were gassed with 5% CO\(_2\)/95% O\(_2\), which maintained the pH at 7.4. After a train of steady-state conditioning pulses (eight 200-ms pulses of alternating polarity, and [Ca\(^{2+}\)]\(_i\) to decline from 90% to 50% of peak, [Ca\(^{2+}\)]\(_i\) when the SR was disabled by the continued presence of caffeine. In this form, F is the measured fluorescence intensity of the myocyte, and \(K_t\) is the dissociation constant. All experiments were performed at 25°C. The \(K_t\) of fluo 3 is known to be temperature dependent and is reported to be 400 and 864 nmol/L at 22°C and 37°C, respectively. Therefore, we used 493 nmol/L as the \(K_t\) at 25°C, assuming a linear relationship between \(K_t\) and temperature. In some experiments, we reported fluorescence intensity in arbitrary units, normalized to the peak amplitude of the electrically stimulated transient.

**Caffeine-Induced Ca\(^{2+}\) Transients**

In some experiments, we abruptly exposed a myocyte to 10 mmol/L caffeine with an SW. With this device, the bulk solution bathing a myocyte can be changed within 4 ms. Myocytes were exposed to caffeine for 4 s (long caffeine pulse) or 100 ms (short caffeine pulse). The long caffeine pulse protocol permitted assessment of the rate of decline of [Ca\(^{2+}\)]\(_i\), when the SR was disabled by the continued presence of caffeine. In the short caffeine pulse protocol, the Ca\(^{2+}\) released from the SR by the initial exposure to caffeine could be resequenced by the SR as the caffeine effects were rapidly washed out and the function of Na\(^+-\)Ca\(^{2+}\) exchanger was blocked in 0 Na\(^+\)/70 Ca\(^{2+}\) solution.
**Measurement of [Na+]i**

[Na+]i was measured with a modified method of Harootunian et al and Levi et al using two Na+-sensitive fluorescent dyes, Sodium Green and SBFI (Molecular Probes). Myocytes on laminin-coated coverslips were incubated at 25°C for 30 minutes in 5 μmol/L Sodium Green tetraacetate–containing HEPES solution or for 120 minutes in 10 μmol/L SBFI acetoxymethyl ester–containing HEPES solution. The loading solution for each dye was prepared with the same method as described for fluo-3 AM. The loaded myocytes were then washed and incubated in dye-free HEPES solution at 25°C. For Sodium Green–loaded myocytes, the fluorescence was collected with exactly the same method as that for fluo 3, because the excitation and the emission wavelengths are both the same as those of fluo 3. As described, the fluorescence intensity at 530 nm was used as F, an indicator for [Na+]i. SBFI has two different excitation (340- and 380-nm) and one emission (510-nm) wavelength. Myocytes were illuminated sequentially at 60 Hz by 340- and 380-nm excitation light passing through band-pass filters (P10–340, and P10–380, Corion) with an optical switcher (DX-1000, Solamere Technology Group), and the fluorescence at 510 nm (P10–510, Corion) was continuously recorded. The ratio of the fluorescence intensities during excitation with 340-nm light to that with 380-nm light used as R, an indicator for [Na+]i. After recording the emission intensities, an in vivo calibration was performed for each dye. For calibration, the myocyte was sequentially exposed to three calibration solutions of 5, 10, and 15 mmol/L NaCl containing (μmol/L) gramicidin D 2, monensin 40, and strophanthidin 100. In each solution, [Na+]i was equilibrated to [Na+]i, and the stable fluorescence at each [Na+]i was then obtained. Calibration solutions were made from appropriate mixtures of high-Na+ solution and high-K+ solution. The former consisted of (μmol/L) NaCl 30, sodium gluconic acid 110, EGTA 2, and HEPES 10, and the latter was identical except for complete replacement of Na+ by K+. The pH of both solutions was adjusted to 7.2 with NaOH and KOH, respectively. Data were all digitized and directly acquired by a computer. The relationships between F or R and [Na+]i were fitted with the following curve:

\[
[\text{Na}^+]_i = \frac{K_i \times (T - T_{\text{min}})}{(T_{\text{max}} - T)}
\]

where T indicates F for Sodium Green and R for SBFI. Using this curve, the fluorescence intensity from the myocyte was then converted to [Na+]i.

**I\text{Ca}** Measurements

I\text{Ca} was measured as described by Chin et al. A myocyte was superfused with HEPES solution, which contained the same components as described above except for the elimination of KCl, insulin, and probenecid, and voltage-clamped (Axopatch 200A, Axon Instruments) with a suction pipette (2 to 3 MΩ) filled with solution containing (μmol/L) NaCl 20, MgCl, 0.3, EGTA 14, MgATP 3.0, glucose 5.5, and HEPES 10. The pH was adjusted to 7.1 with CsOH, and the concentration of Cs+ in the solution was brought to 140 mmol/L by adding CsCl. The myocyte was held at a potential of −40 mV, and I\text{Ca} was then activated by depolarizing the myocyte membrane to more positive values for 500 ms. Between measurements, the myocyte was maintained at a holding potential of −80 to −90 mV in order to prevent the rundown phenomenon. Data were all digitized, acquired, and analyzed by a computer with PCLAMP6 software (Axon Instruments Inc). The cell capacitance was also measured for the determination of the current density.

**Measurement of Action Potentials**

Action potentials were recorded in whole-cell current-clamp mode using an AxoClamp 2B (Axon Instruments Inc). The "physiological" pipette filling solution used for recording action potentials contained (mmol/L) KCl 113, NaCl 15, MgCl, 0.5, ATP (potassium salt) 5, HEPES 10, and dextrose 5.5, with pH adjusted to 7.1 with KOH. Myocytes were superfused in HEPES solution (same composition as used for the measurement of [Ca2+]i, transients) and stimulated at 0.25 Hz with 3-ms current pulses (1 nA). In a few experiments, simultaneous measurements of action potentials and [Ca2+]i were made from appropriate mixtures of high-Na+ solution and low-K+ solution. The former consisted of (mmol/L) NaCl 30, sodium gluconic acid 110, EGTA 2, and HEPES 10, and the latter was identical except for complete replacement of Na+ by K+. The pH of both solutions was adjusted to 7.2 with NaOH and KOH, respectively. Data were all digitized and directly acquired by a computer. The relationships between F or R and [Na+]i were fitted with the following curve:

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where T indicates F for Sodium Green and R for SBFI. Using this curve, the fluorescence intensity from the myocyte was then converted to [Na+]i.

**Results**

**[Ca2+]i Transients of Electrically Stimulated Beats in TG and WT Myocytes**

Figure 1 shows representative recordings of [Ca2+]i transients in a TG and a WT mouse ventricular myocyte. After pacing for 2 minutes at 0.25 Hz, very stable [Ca2+]i transients were observed in both types of the myocytes.

**Statistical Analysis**

For analysis, all records of [Ca2+]i transients and pacing signals were simultaneously digitized and acquired at the sampling rate of 1 kHz (Axo Scope, Axon Instruments Inc). Digitized [Ca2+]i transients were analyzed with Origin. Results were expressed as mean±SEM. An unpaired t test was performed for the comparison between WT and TG myocytes. Significance was also tested by ANOVA if multiple comparisons were made. Values of P<.05 were considered significant.

**TABLE 1. Characteristics of [Ca2+]i Transients**

<table>
<thead>
<tr>
<th></th>
<th>Systolic [Ca2+]i, nmol/L</th>
<th>Diastolic [Ca2+]i, nmol/L</th>
<th>Amplitude of [Ca2+]i, Transient, nmol/L</th>
<th>Time to Peak, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (n=20)</td>
<td>458±73</td>
<td>100±12</td>
<td>358±64</td>
<td>100±6</td>
</tr>
<tr>
<td>TG (n=27)</td>
<td>654±94</td>
<td>90±8</td>
<td>564±89</td>
<td>129±5</td>
</tr>
<tr>
<td>P*</td>
<td>.128</td>
<td>.475</td>
<td>.086</td>
<td>.001</td>
</tr>
</tbody>
</table>

*WT vs TG.
the end-diastolic [Ca^{2+}] level and C_o as the initial [Ca^{2+}] at the beginning of the decline. Values of t_{0.5-0.05} and t_{0.9-0.95} were also calculated. The t value was significantly shorter in TG myocytes (128±6 ms) compared with WT myocytes (166±12 ms) (P=.004, n=27 and 20). The most prominent difference was in the terminal phase of the decline of the [Ca^{2+}] transients (t_{0.9-0.95} 205±12 ms [TG] and 284±22 ms [WT]; P<.001). These data clearly show that in myocytes overexpressing the Na^+-Ca^{2+} exchanger, the decline of [Ca^{2+}] transients is accelerated, suggesting an enhanced effectiveness of Ca^{2+} extrusion by Na^+-Ca^{2+} exchange and thus a more effective competition with Ca^{2+} uptake by the SR Ca^{2+}-ATPase.

Characteristics of Caffeine-Induced [Ca^{2+}] Transients
To confirm that the overexpression of the Na^+-Ca^{2+} exchanger induces enhanced Ca^{2+} extrusion, we exposed the myocytes to a long pulse of 10 mmol/L caffeine with the SW. In this protocol, a myocyte was paced at 0.25 Hz in HEPES solution in one stream of the SW for 2 minutes and then abruptly exposed for 4 s to a 10 mmol/L caffeine–containing HEPES solution stream with SW 4 s after the last pacing stimulus (see Fig 2). The myocyte was subsequently paced again for 2 minutes in control solution. After the solutions perfusing SW were changed to a 10 mmol/L caffeine–containing HEPES solution stream of the SW for 2 minutes and then abruptly exposed for 4 s a myocyte was paced at 0.25 Hz in HEPES solution in one

Figure 2. Effects of overexpression of the Na^+-Ca^{2+} exchanger on extrusion of intracellular Ca^{2+} during a long caffeine pulse–induced Ca^{2+} transient. a. After the last electrically stimulated beat, a caffeine-induced Ca^{2+} transient was induced by a 4-s exposure to caffeine in a WT and a TG myocyte (left tracings). Note that the rate of decline of [Ca^{2+}] in the WT myocyte when the SR is disabled by caffeine is ~1/10 that of a normal electrically stimulated transient. The decline of the caffeine-induced Ca^{2+} transient was markedly accelerated in the TG myocyte. In the right tracings, a caffeine-induced Ca^{2+} transient in the presence of 5 mmol/L Ni^{2+} is also shown in each myocyte. There is no apparent difference in the decline of these transients between the WT and the TG myocytes. Arbitrary units of fluo 3 fluorescence are shown. b. Average time courses of the decline of long caffeine pulse–induced [Ca^{2+}] transients in WT and TG myocytes are shown. Experiments were all performed with the same protocol as in panel a. Data were all normalized to the peak amplitude of each transient and expressed as mean±SEM. Without Ni^{2+} treatment, the time course of decline rate was significantly more rapid in TG myocytes, and at each time point (every 0.5 s) after the peak signal, the [Ca^{2+}] was significantly lower in TG myocytes (n=8) than in WT myocytes (n=7) (P<.01 and *P<.05, by ANOVA). On the other hand, there was no significant difference in the time course of decline in the long caffeine pulse–induced [Ca^{2+}] transients between two types of myocytes after treatment with 5 mmol/L Ni^{2+}. All transients with and without Ni^{2+} were serially obtained from the same myocytes.
changer and the SR Ca²⁺-ATPase. As shown in examples in Fig 2a, the speed of the decline of the [Ca²⁺] signal in caffeine was markedly faster in the TG myocyte, whereas the amplitudes of both fluorescence signals relative to the amplitude of the last electrically stimulated beats were identical in the two myocytes. In the presence of 5 mmol/L Ni²⁺, the [Ca²⁺] decline was prolonged in both types of myocytes, but there was no apparent difference in the rate of decline between the myocytes. Fig 2b shows average results. In the absence of Ni²⁺, the average decline of the long-pulse caffeine-induced [Ca²⁺], transient was significantly more rapid in TG myocytes. Time from the peak signal to 50% of the peak, which was measured with an interpolation method, was 464 ms in TG myocytes and 1750 ms in WT myocytes. This enhanced Ca²⁺ extrusion by Na⁺-Ca²⁺ exchange probably accounts for the more rapid decline in [Ca²⁺] in electrically stimulated transients in TG myocytes. The rate of decline did not differ in the presence of Ni²⁺, suggesting that there is no significant difference in the combined [Ca²⁺], reducing function of the SL Ca²⁺-ATPase, mitochondria, and Ca²⁺ buffering between WT and TG myocytes.

The amplitude of the caffeine-induced [Ca²⁺], transient in the presence of Ni²⁺ is caused only by the Ca²⁺ release from SR and is an indicator of SR Ca²⁺ content. In the presence of Ni²⁺, the amplitude of the caffeine-induced [Ca²⁺], transient relative to that of the last electrically stimulated transient was 196±33% for WT myocytes (n=6) and 213±37% for TG myocytes (n=7, P=NS). We also measured the inward exchange current (I_SNaC), induced by a rapid exposure to caffeine, under voltage-clamp conditions. The peak current was higher in TG than WT myocytes (5.2±1.3 versus 1.8±0.2 pA/pF). However, the amounts of Ca²⁺ released estimated from the integrals of the I_SNaC were similar (7.96±0.69 pmol/µF for WT and 8.61±0.56 pmol/µF for TG, P=NS, n=9 and 5). Thus, these results suggest that SR Ca²⁺ content is similar in WT and TG myocytes under these experimental conditions.

[Na⁺], in TG and WT Myocytes
[Na⁺], is recognized as one of the factors affecting the function of the Na⁺-Ca²⁺ exchanger. For comparison of the function of the Na⁺-Ca²⁺ exchanger in TG and WT myocytes, it is therefore necessary to measure [Na⁺], in both types of the myocytes. The resting [Na⁺] in WT myocytes was 16.4±0.7 and 15.6±0.7 mmol/L when measured with Sodium Green and SBFI, respectively, and was similar in TG myocytes (16.9±0.6 and 14.1±0.7 mmol/L, n=21 and 20 for Sodium Green and n=11 and 7 for SBFI). Using identical calibration protocols, we measured an [Na⁺] of 5 to 6 mmol/L in rabbit ventricular myocytes, a result similar to that reported by Levi et al. Thus, the [Na⁺], in murine ventricular myocytes is quite high compared with that in rabbit (4 to 7 mmol/L) and guinea pig (=8.0 mmol/L) ventricular myocytes and comparable to that in rat myocytes (=16 mmol/L). [Na⁺], although high in murine myocytes, is not affected by the overexpression of the Na⁺-Ca²⁺ exchanger; therefore, a difference in [Na⁺], seems unlikely to account for the difference in the function of the Na⁺-Ca²⁺ exchanger in TG and WT myocytes noted above.

SR Ca²⁺-ATPase Function in TG and WT Myocytes
To rule out the possibility that the acceleration of the decline of the electrically stimulated [Ca²⁺], transients in TG myocytes was in part mediated by modified function of SR Ca²⁺-ATPase, we directly examined the function of the SR in TG and WT myocytes. For this purpose, we superfused a myocyte with 0 Ca²⁺ solution for 1 minute, and then the solution was exchanged to 0 Na⁺/0 Ca²⁺ solution, with the complete replacement of Na⁺ by Li⁺. (If the bathing solution was simultaneously changed to 0 Na/0 Ca²⁺ solution, myocytes developed [Ca²⁺], oscillations.) The myocyte was then captured in one stream of 0 Na⁺/0 Ca²⁺ solution from the SW and subsequently abruptly exposed to 10 mmol/L caffeine–containing 0 Na⁺/0 Ca²⁺ solution for 100 ms. In this experiment, the time from the switching point to the peak of the caffeine-induced [Ca²⁺], was always >200 ms, so that during the decline of the [Ca²⁺], transient, the extracellular caffeine concentration was decreased to less than an effective concentration for releasing Ca²⁺ from the SR. Hence, this decline reflects sequestering of Ca²⁺ by a normally functioning SR Ca²⁺-ATPase, because the Na⁺-Ca²⁺ exchanger is disabled in 0 Na⁺/0 Ca²⁺ solution. Fig 3 shows a representative recording of the short caffeine pulse–induced [Ca²⁺], transients obtained in both types of myocytes. Both myocytes had a similar resting and peak [Ca²⁺], and showed an identical rate of decline of the [Ca²⁺], transient. The mean values of τ, tₜ₀₋₉₀₅, and tₜ₀₋₁₀₅ were similar in TG and WT myocytes. In addition, the mean diastolic and peak systolic [Ca²⁺], values did not differ significantly (Table 2). These data indicate that the SR Ca²⁺-ATPase function is not altered in TG myocytes and further support the conclusion that the overexpressed Na⁺-Ca²⁺ exchanger is most likely responsible for the more rapid decline of electrically stimulated [Ca²⁺], transients noted in Table 1.

Function of the L-type Ca²⁺ Channel
There is normally a balance between Ca²⁺ influx via the L-type Ca²⁺ channel and Ca²⁺ extrusion by Na⁺-Ca²⁺ exchange.
Overexpression of Na\textsuperscript+-Ca\textsuperscript{2+} Exchanger in Myocytes

Therefore, increased forward function of the Na\textsuperscript+-Ca\textsuperscript{2+} exchanger might be expected to result in increased Ca\textsuperscript{2+} influx via the L-type Ca\textsuperscript{2+} channel to maintain Ca\textsuperscript{2+} homeostasis. To examine whether the L-type Ca\textsuperscript{2+} channel is upregulated in TG myocytes, we measured $I_{\text{Ca}}$ in both types of myocytes. $I_{\text{Ca}}$ normalized to membrane capacitance, as a function of membrane potential, was very similar in the two groups of myocytes (Fig 4). This finding is consistent with that reported by Adachi-Akahane et al

Excitation-Contraction Coupling in the Presence of Ca\textsuperscript{2+} Channel Blockade

There has been disagreement as to whether the Na\textsuperscript+-Ca\textsuperscript{2+} exchanger operating in the reverse mode can trigger or contribute to CICR. To examine whether overexpression of the Na\textsuperscript+-Ca\textsuperscript{2+} exchanger results in a difference in the Ca\textsuperscript{2+} content is necessary for this response of TG myocytes. Hence, it seems possible that Ca\textsuperscript{2+} influx via the Na\textsuperscript+-Ca\textsuperscript{2+} exchanger is contributing to CICR rather than directly activating the contractile elements, and this maintains the electrically stimulated [Ca\textsuperscript{2+}], transient magnitude in TG myocytes under these experimental conditions.

To further assess this possibility, we abruptly blocked the L-type Ca\textsuperscript{2+} channel without changing SR Ca\textsuperscript{2+} content. Briefly, after pacing at 0.1 Hz, a myocyte was exposed to 20 μmol/L nifedipine with the SW for 9 s before the next electrical stimulation. A prominent [Ca\textsuperscript{2+}], transient was observed in the TG myocyte, although only a small transient could be seen in the WT myocyte (Fig 6). The myocyte was subsequently paced in control solution until the Ca\textsuperscript{2+} signal again stabilized and then was exposed to 5 mmol/L Ni\textsuperscript{2+}-containing solution with the same protocol. In the Ni\textsuperscript{2+} solution, the [Ca\textsuperscript{2+}], transient was completely eliminated in both myocytes. These experiments clearly indicate that nifedipine-insensitive and Ni\textsuperscript{2+}-sensitive Ca\textsuperscript{2+} influx, which is most likely caused by the reverse function of the Na\textsuperscript+-Ca\textsuperscript{2+} exchanger, is able to induce a [Ca\textsuperscript{2+}], transient in TG myocytes. In Fig 6b are shown superimposed high-speed recordings of the control [Ca\textsuperscript{2+}], transient and the [Ca\textsuperscript{2+}], transient after abrupt exposure to nifedipine. The rise in [Ca\textsuperscript{2+}], is clearly prolonged in the

| Table 2. Characteristics of [Ca\textsuperscript{2+}], Transients Induced by a 100-ms Caffeine Pulse |
|---------------------------------|-------------|-------------|-------------|-------------|-------------|
|                                | Systolic [Ca\textsuperscript{2+}], nmol/L | Diastolic [Ca\textsuperscript{2+}], nmol/L | $t_{0–10\%}$, ms | $t_{50–10\%}$, ms | $\tau$, ms |
| WT (n=6)                        | 389±89      | 85±14       | 132±14      | 230±41      | 184±22      |
| TG (n=5)                        | 315±50      | 106±13      | 105±12      | 238±30      | 166±19      |
| $P^*$                           | .514        | .375        | .201        | .883        | .556        |

*WT vs TG.

Figure 4. The $I_{\text{Ca}}$ density-voltage relationship in WT and TG myocytes. $I_{\text{Ca}}$ density was measured as described in “Materials and Methods” and averaged for WT (n=29, □) and TG (n=30, ●) myocytes. $I_{\text{Ca}}$ density was similar at each voltage from −40 to +60 mV in WT and TG myocytes. The insert shows examples of current traces elicited by a clamp from −40 to 0 mV. There was consistently a more prominent outward current in TG myocytes, but the transient due to $I_{\text{Ca}}$ was similar.
nifedipine-treated transient compared with the control transient. On the other hand, the decline of the two transients was similar. This result suggests that the rate at which Ca$^{2+}$ influx via reverse Na$^{+}$-Ca$^{2+}$ exchange contributes to CICR is slower than CICR caused by L-type Ca$^{2+}$ channel Ca$^{2+}$ influx. This is consistent with a previous report. 27

Effects of Overexpression of the Na$^{+}$-Ca$^{2+}$ Exchanger on Action Potentials

Examples of action potentials recorded from TG and WT myocytes are shown in Fig 7, and average parameter values are given in Table 3. The resting membrane potentials were similar. However, the TG myocyte action potentials had a lower peak amplitude (APA), a more rapid initial repolarization (APD$_{50}$), and a delayed terminal repolarization (APD$_{90}$). An increase in APD$_{90}$ in TG myocytes was also noted in a preliminary report by Silverman et al. 29 These action potential characteristics are consistent with but do not prove the hypothesis that the TG myocytes have a more marked reverse function of the Na$^{+}$-Ca$^{2+}$ exchanger early during depolarization, with an increase in outward current causing a decrease in APA and APD$_{50}$, and a more marked forward function during the [Ca$^{2+}$]$_i$ transient, causing an increase in inward current and an increase in APD$_{90}$. 

Figure 5. [Ca$^{2+}$]$_i$ transients in nifedipine-containing solution in WT and TG myocytes. a, After steady-state pacing in HEPES solution, myocytes were superfused with 10 μmol/L nifedipine-containing HEPES solution for 5 minutes while [Ca$^{2+}$]$_i$ transients were measured. The flow rate of the bathing solution was 5 mL/min, and the volume of the chamber was ~1.5 mL. In the WT myocyte (upper trace), the [Ca$^{2+}$]$_i$ transient was almost completely eliminated within 1 minute in the nifedipine solution. In contrast, the [Ca$^{2+}$]$_i$ transient was still present after 5 minutes in the nifedipine solution in the TG myocyte (middle trace). The [Ca$^{2+}$]$_i$ transient in a TG myocyte was almost eliminated within 3 minutes in the presence of 1 μmol/L ryanodine and 0.5 μmol/L thapsigargin in addition to 10 μmol/L nifedipine (lower trace). b, The time course of the decay in [Ca$^{2+}$]$_i$ transients in nifedipine solution in WT and TG myocytes is shown. The mean values of the [Ca$^{2+}$]$_i$ transients in nifedipine solution in the absence or presence of ryanodine and thapsigargin are plotted. Data were all normalized to the mean amplitude of the last three control transients and expressed as mean±SEM. The amplitude of the [Ca$^{2+}$]$_i$ transients in the nifedipine solution was significantly higher in TG myocytes (n=6, *) compared with WT myocytes (n=8, □) (*P<.01 and **P<.05). Also, the [Ca$^{2+}$]$_i$ transient decline in TG myocytes was enhanced after 3 minutes of treatment with nifedipine, ryanodine, and thapsigargin (○) (+P<.01 and ++P<.05, by ANOVA).

Figure 6. Examples of the relative effects of rapid exposure to nifedipine and Ni$^{2+}$ on [Ca$^{2+}$]$_i$ transients in WT and TG myocytes. a, Myocytes were paced at 0.1 Hz. After the transient stabilized, the myocytes were abruptly exposed (SW) to 20 μmol/L nifedipine for 9 s before the next pacing stimulus. With exposure to nifedipine, the [Ca$^{2+}$]$_i$ transient was almost eliminated in the WT myocyte, but a prominent [Ca$^{2+}$]$_i$ transient was still induced in the TG myocyte. After washout of nifedipine, the myocytes were subsequently paced until the signal recovered and then abruptly exposed to 5 mmol/L Ni$^{2+}$-containing solution with the same protocol. In the presence of Ni$^{2+}$, [Ca$^{2+}$]$_i$ transients were completely eliminated in both the WT and the TG myocytes. Similar results were observed in another four WT myocytes and two TG myocytes. b, Example of the effects of nifedipine on the time course of the [Ca$^{2+}$]$_i$ transient in TG myocytes is shown. The [Ca$^{2+}$]$_i$ transients in the control and the nifedipine-treated beats in the TG myocyte shown in panel a are displayed at high speed to compare the time course of the [Ca$^{2+}$]$_i$ transient. The time to peak was markedly prolonged in the nifedipine-treated transient, whereas the decline was unchanged.
Discussion

Degree of Functional Overexpression of the Na\(^{+}\)-Ca\(^{2+}\) Exchanger in TG Myocytes

The Na\(^{+}\)-Ca\(^{2+}\) exchanger is functionally overexpressed in TG myocytes, as shown by the accelerated decline of [Ca\(^{2+}\)]\(_i\), in the long caffeine pulse–induced [Ca\(^{2+}\)]\(_i\) transients. This result was not due to an alteration in [Na\(^{+}\)], and is consistent with the findings of Adachi-Akahane et al\(^{10}\) in voltage-clamped myocytes. The difference in the rates of the decline of the long caffeine pulse–induced [Ca\(^{2+}\)]\(_i\) transients between TG (t\(_{1/2}\)=464 ms) and WT (t\(_{1/2}\)=1750 ms) myocytes and in the magnitudes of the peak I\(_{\text{sec},\text{Ca}}\) measured during abrupt exposure to caffeine is consistent with the 2.5- to 3-fold increase in intracellular Na\(^{+}\)–dependent 45Ca uptake in sarclemmal vesicles obtained from TG and WT myocytes.\(^{10}\)

Alteration in [Ca\(^{2+}\)]\(_i\), Transients and Action Potential Morphology Associated With Overexpression of the Na\(^{+}\)-Ca\(^{2+}\) Exchanger

In electrically stimulated [Ca\(^{2+}\)]\(_i\) transients, the decline of [Ca\(^{2+}\)]\(_i\), was significantly accelerated in TG myocytes, particularly during the terminal phase of decline. This was associated with a prolonged APD\(_{90}\). Our recent findings in rabbit ventricular myocytes\(^{12}\) indicate that Na\(^{+}\)-Ca\(^{2+}\) exchanger function is most apparent during the terminal phase of Ca\(^{2+}\) decline. On the basis of the short-pulse caffeine results, SR Ca\(^{2+}\)-ATPase function appears similar in both types of myocytes. Taken together, these results indicate that the acceleration of decline of the Ca\(^{2+}\) transient is due to the functional overexpression of the Na\(^{+}\)-Ca\(^{2+}\) exchanger and the enhanced forward exchange in TG myocytes.

<table>
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<tr>
<th>TABLE 3. Action Potential Characteristics</th>
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<tr>
<td>RMP, mV</td>
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<tr>
<td>WT (n=6)</td>
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<tr>
<td>TG (n=8)</td>
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RMP indicates resting membrane potential; APA, peak APA.

*WT vs TG.*

Since I\(_{\text{Ca}}\), a major source of Ca\(^{2+}\) influx, was similar in the two groups of myocytes (Fig 4), one might predict that enhanced forward function of the Na\(^{+}\)-Ca\(^{2+}\) exchanger would result in Ca\(^{2+}\) depletion and a reduced [Ca\(^{2+}\)]\(_i\), transient magnitude. However, in electrically stimulated cells, the magnitude of the Ca\(^{2+}\) transient was somewhat greater in TG than in WT myocytes, although this difference did not reach statistical significance. In addition, as shown in Table 1, diastolic levels did not differ significantly in TG and WT myocytes. The amplitudes of both the long- and short-duration caffeine pulse–induced Ca\(^{2+}\) transients were similar, as was the integrated inward current activated by abrupt caffeine exposure, suggesting that SR Ca\(^{2+}\) loading was similar in TG and WT myocytes. The presence of a normal resting [Ca\(^{2+}\)], SR Ca\(^{2+}\) content, and a normal [Ca\(^{2+}\)]\(_i\), transient amplitude in TG myocytes therefore indicates that Ca\(^{2+}\) influx in these myocytes is enhanced, probably by augmented reverse Na\(^{+}\)-Ca\(^{2+}\) exchange. The changes in the early action potential morphology (decreased APA and decreased APD\(_{90}\)) are consistent with this hypothesis.

Ca\(^{2+}\) Influx by Reverse Na\(^{+}\)-Ca\(^{2+}\) Exchange in TG Myocytes

To assess further the possible enhanced contribution of reverse Na\(^{+}\)-Ca\(^{2+}\) exchange to Ca\(^{2+}\) influx, we examined the effects of nifedipine. We found less sensitivity of the [Ca\(^{2+}\)]\(_i\), transient to nifedipine in TG myocytes. Furthermore, the maintenance of [Ca\(^{2+}\)]\(_i\), transients in nifedipine required SR Ca\(^{2+}\) content, suggesting that the Na\(^{+}\)-Ca\(^{2+}\) exchanger–mediated Ca\(^{2+}\) influx can contribute to CICR in TG myocytes. On the basis of our results, it is not clear whether the Ca\(^{2+}\) influx due to reverse Na\(^{+}\)-Ca\(^{2+}\) exchange actually “triggers” SR Ca\(^{2+}\) release itself or potentiates the effects of the very small residual Ca\(^{2+}\) influx possibly occurring via unblocked L-type Ca\(^{2+}\) channels. It should be noted that Adachi-Akahane et al\(^{10}\) reported only inconsistent triggering of CICR in TG ventricular myocytes by Ca\(^{2+}\) influx through the Na\(^{+}\)-Ca\(^{2+}\) exchanger during large (+80-mV) voltage clamps and failed to observe sufficient influx of Ca\(^{2+}\) via the exchanger to cause CICR in the physiological range of membrane potentials (–10 to +20 mV). This different result may have been obtained because Adachi-Akahane et al used 10 mmol/L Na\(^{+}\) in the voltage-clamp pipette solution, although the measured [Na\(^{+}\)], in our present study was =16 mmol/L. Using two values of [Na\(^{+}\)], (7 and 10 mmol/L), Bers\(^{8}\) simulated a [Ca\(^{2+}\)], transient and an action potential in a rabbit cardiac myocyte. He predicted that with [Na\(^{+}\)], of 10 mmol/L, Ca\(^{2+}\) influx by Na\(^{+}\)-Ca\(^{2+}\) exchange occurred during most of the action potential but that at [Na\(^{+}\)], of 7 mmol/L, this occurred only in the initial phase of AP. This simulation implies that just a 3-mol/L difference in [Na\(^{+}\)], could lead to a significant difference in Na\(^{+}\)-Ca\(^{2+}\) exchanger–mediated Ca\(^{2+}\) influx. Kohmoto et al\(^{10}\) also found that the magnitude of shortening in ventricular myocytes sensitive to the Na\(^{+}\)-Ca\(^{2+}\) exchange inhibitor XIP (indicating exchanger inhibitory peptide) was increased with increasing [Na\(^{+}\)]. A difference in [Na\(^{+}\)], may thus account for our detection of apparent Na\(^{+}\)-Ca\(^{2+}\) exchanger contribution to Ca\(^{2+}\) release in intact non–voltage-clamped TG myocytes under relatively physiological conditions. This possibility is also supported by work of Bers et al\(^{8}\), who showed a decreased sensitivity to nifedipine
in rabbit myocardium treated with acetylstrophanthinidin to increase [Na⁺].

We found that the rate of Ca²⁺ release induced by the Na⁺-Ca²⁺ exchanger is somewhat slow (Fig 6). It remains to be determined how much reverse Na⁺-Ca²⁺ exchange contributes to the [Ca²⁺], transient in the presence of a functioning L-type Ca²⁺ channel, since Ca²⁺ influx via the L-channel could enhance Na⁺-Ca²⁺ exchange via a catalytic effect of Ca²⁺ on the exchanger. In addition, the triggering by IsCC and IC may not add up in a simple linear fashion, because the relationship between open probability and [Ca²⁺] for the ryanodine receptor is sigmoid. The activity of Na⁺-Ca²⁺ exchange alone may, for example, bring Ca²⁺ in the vicinity of the ryanodine receptors close to the foot of this relationship, whereas the presence of both IC and IsCC could bring pCa levels onto the steep region of the relationship. Under this circumstance, the effect of both triggers would not be a simple linear combination of each trigger acting independently. However, the prolongation of time to peak systolic [Ca²⁺] in TG myocytes (Table 1) may reflect an increased and delayed contribution to Ca²⁺ release by the Na⁺-Ca²⁺ exchanger operating in the reverse mode, although further analysis will be necessary to examine this possibility.

In conclusion, the overexpressed Na⁺-Ca²⁺ exchanger is shown to function in vivo in TG myocytes. A significant increase in the rate of decline of electrically stimulated and long caffeine pulse–induced [Ca²⁺], transients indicates enhanced forward Na⁺-Ca²⁺ exchange function. In both TG and WT mouse ventricular myocytes, the [Na⁺] was high compared with that in other species. This seems to favor an enhanced reverse Na⁺-Ca²⁺ exchange, with increased influx of Ca²⁺, which maintains SR Ca²⁺ loading and contributes to triggering of CICR in TG myocytes. The L-type Ca²⁺ channel and SR Ca²⁺-ATPase function appear to be unaltered by overexpression of the Na⁺-Ca²⁺ exchanger in murine ventricular myocytes.

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