Ca²⁺ removal mechanisms in mouse embryonic stem cell-derived cardiomyocytes

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Guo A, Yang HT. Ca²⁺ removal mechanisms in mouse embryonic stem cell-derived cardiomyocytes. Am J Physiol Cell Physiol 297: C732–C741, 2009. First published July 15, 2009; doi:10.1152/ajpcell.00025.2009.—In mammalian adult cardiomyocytes, sarcoplasmic reticulum (SR) Ca²⁺-ATPase (SERCA) plays a major role in controlling the decline of cytosolic free Ca²⁺ concentration ([Ca²⁺]i) in comparison with sarcolemmal Na⁺/Ca²⁺ exchanger (NCX). However, the functional importance of SERCA and NCX in cytosolic Ca²⁺ removal during early cardiomyogenesis is still debated. In this study, the functional contributions of Ca²⁺ transporters to [Ca²⁺]i decline in mouse embryonic stem cell-derived cardiomyocytes (mESCMs), a suitable model for investigation of early cardiogenesis, at various differentiation stages were investigated. We estimated that even at early differentiation stages of mESCMs, SERCA was responsible for ~76% of total Ca²⁺ removal, while NCX was responsible for ~21%. The contributions of SERCA and NCX to cytosolic Ca²⁺ clearance were increased to ~88% and decreased to ~10%, respectively, at late differentiation stage. Dynamical analysis of the transient decay phases in normal and Na⁺-free solutions suggests that the contribution of NCX to [Ca²⁺]i to NCX to [Ca²⁺]i is more apparent in the terminal slow decay phase than that in the initial fast phase. When SR function was suppressed in type 2 ryanodine receptor-null mESCMs or with ryanodine receptor and SERCA inhibitors (ryanodine and thapsigargin), NCX acted as the main pathway for [Ca²⁺]i decline. We conclude that the rapid [Ca²⁺]i decline is mainly achieved by the SR uptake even at the early differentiation stage of mESCMs, while NCX acts as the main Ca²⁺ remover when SR function is suppressed. These findings suggest a critical role of SR in the regulation of [Ca²⁺]i homeostasis even in differentiating cardiomyocytes.

Sarcoplasmic reticulum; calcium transporters; sarcoplasmic reticulum Ca²⁺-ATPase; sodium/calcium exchanger.

IT IS GENERALLY ACCEPTED THAT Ca²⁺ uptake via sarcoplasmic reticulum (SR) Ca²⁺-ATPase (SERCA) and Ca²⁺ extrusion via sarcolemmal Na⁺/Ca²⁺ exchanger (NCX) are main pathways for decline of cytosolic free Ca²⁺ concentration ([Ca²⁺]i) during twitch relaxation of adult mammalian cardiomyocytes. Plasma membrane Ca²⁺-ATPase (PMCA) and mitochondrial Ca²⁺ uniporter, referred to as “slow system”, contribute much less to the decline of [Ca²⁺]i (2, 4). Though the relative contributions of SERCA and NCX to [Ca²⁺]i decline differ among species, it has been demonstrated that, in adult mammalian cardiomyocytes, the relative contribution of SERCA to cytosolic Ca²⁺ removal is much greater than that of NCX (2–4, 11, 19, 27, 29). However, the relative contributions of these Ca²⁺ transporters during mammalian embryonic cardiogenesis remain controversial. Basically, to maintain intracellular Ca²⁺ homeostasis, the amount of Ca²⁺ removed by sarcolemmal or SR Ca²⁺ transporters should be equal to the sarcolemmal Ca²⁺ influx or SR Ca²⁺ release during steady-state of [Ca²⁺]i transients. Several studies with fetal cardiomyocytes have shown the SR undeveloped and suggested that the sarcolemmal Ca²⁺ influx may be a main resource of [Ca²⁺]i, transients in such cells (21, 26, 28), and high expression and current density of NCX were detected in embryonic cardiomyocytes (17, 21, 31, 32). It is, therefore, postulated that NCX would play a more prominent role in cytosolic Ca²⁺ removal than the contribution of SERCA Ca²⁺ uptake during early differentiation of cardiomyocytes. This is supported by the study with mouse embryonic stem (mES) cell-derived cardiomyocytes (mESCMs) showing that >50% of cytosolic Ca²⁺ removal is mediated by NCX at the early stage of differentiation (16). On the other hand, recent reports showed that functional SR and SR proteins are already present in embryonic cardiomyocytes and contribute to the regulation of [Ca²⁺]i transients (24, 25, 33). Our recent study using wild-type (RyR2+/+ ) and type 2 ryanodine receptor-deficient (RyR2−/−) mESCMs also demonstrated the crucial role of RyR2 in regulating [Ca²⁺]i transients and contraction in differentiating cardiomyocytes (12). In the same study, concomitant with functional traits, the expression of transcripts of SR proteins including SERCA2a was also detected before the initiation of contractions. A recent study provides more direct evidence showing that a majority (~74%) of cytosolic Ca²⁺ removal is mediated by SERCA in mouse embryonic cardiomyocytes 8.5–9.5 days old (32). These observations suggest that SERCA may be an important Ca²⁺ transporter even during early cardiomyogenesis. Thus, the relative functional importance of SERCA and NCX to Ca²⁺ removal in differentiating cardiomyocytes remains to be further clarified.

mESCMs have been established as a suitable model for the investigation of early cardiomyogenesis (1, 5, 15, 41). Ultrastructural, molecular biological, and electrophysiological studies have demonstrated that the in vitro differentiation of mESCMs within the embryoid bodies (EBs) closely recapitulates the developmental pattern of murine embryonic cardiomyocytes (1, 5, 9, 10, 15, 23, 38, 41). This in vitro model system is especially useful to identify gene function during cardiac development when “loss of function” studies result in early embryonic lethality (5, 15, 41). In addition, this in vitro system provides a unique tool for cardiac-related drug screening (6, 35). Therefore, identification of physiological properties...
of mESCMs is important not only for understanding the function of differentiating cardiomyocytes, but also for further genetic studies and drug screening. Here, to reveal the importance of Ca\(^{2+}\) transporters to cytosolic Ca\(^{2+}\) removal in differentiating cardiomyocytes, we analyzed 1) dynamics of [Ca\(^{2+}\)]; 2) relative participation of SERCA and NCX to [Ca\(^{2+}\)]; 3) decline in mESCMs; and 3) pathways for [Ca\(^{2+}\)] removal under lower SR function in mESCMs. Our results reveal a predominant contribution of SERCA to [Ca\(^{2+}\)], decline in mESCMs even at very early differentiation stages, while the contribution of NCX is limited and mainly to the terminal decay phase of [Ca\(^{2+}\)], transients. These data provide new insights into the regulatory mechanisms of [Ca\(^{2+}\)], homeostasis in early differentiating cardiomyocytes.

**MATERIALS AND METHODS**

Culture and differentiation of mouse embryonic stem cells. Murine RyR2\(^{+/+}\) and RyR2\(^{-/-}\) R1 mES cell lines (12, 42) were cultivated and differentiated into spontaneously beating cardiomyocytes, as described previously (41). Briefly, undifferentiated mES cells were cultivated on mitomycin C-inactivated mouse feeder layers in the presence of leukemia inhibitory factor. Differentiation of mES cells into cardiac cells was initiated by a hanging drop technique to form EBs, and after 7 days in suspension, EBs were plated onto gelatin-coated tissue culture dishes. Beating cardiomyocytes were isolated at three distinct differentiation stages [early (EDS, 9–11 days), intermediate (IDS, 13–15 days), and late differentiation stages (LDS, 18–21 days)] as described previously (12, 41). All cultivation media and other substances for cell cultures were purchased from Invitrogen.

**Solutions.** The control solution was composed of the following (in mM): 140 NaCl, 5.4 KCl, 1.8 CaCl\(_2\), 1.0 MgCl\(_2\), 5.0 NaHCO\(_3\), 10.0 glucose, and 10 HEPES (pH 7.4 at 35°C). In Na\(^{+}\)-free (0 Na\(^{+}\)) solution, LiCl substituted for NaCl. In Ca\(^{2+}\)-free (0 Ca\(^{2+}\)) solution, CaCl\(_2\) was omitted and 1 mM EGTA was added. In Na\(^{+}\)-free and Ca\(^{2+}\)-free (0 Na\(^{+}\)-0 Ca\(^{2+}\)) solution, used in caffeine experiments, CaCl\(_2\) was omitted, 1 mM EGTA was added, and NaCl was replaced with LiCl as described previously (2).

[Ca\(^{2+}\)]\(_i\) injections. mESCMs were loaded with 5 μM Fura-2 AM and 0.45% pluronic F-127 (Molecular Probes, Carlsbad, CA) for 10 min at room temperature and washed in control solution for 15 min. Fluorescence signals of Fura-2 were detected by a Fluorescence System (IonOptix, Milton, MA). After subtraction of background fluorescence, the 340- to 380-nm fluorescence ratio (R) was recorded. (Ca\(^{2+}\))\(_i\) was estimated according to the following equation (13): (Ca\(^{2+}\))\(_i\) = K\(_d\)β(R - R\(_{\text{min}}\))/(R\(_{\text{max}}\) - R), where K\(_d\) is the apparent dissociation constant and is assumed to be 224 nM in the cytosolic environment, and β is the ratio of fluorescence at 380 nm at zero and saturation of [Ca\(^{2+}\)]. R\(_{\text{min}}\) and R\(_{\text{max}}\) are the ratios at minimal and saturating [Ca\(^{2+}\)], respectively, which were determined in vivo by permeabilizing the cells with 5 μM ionomycin. Carbonyl cyanide p-(trifluoromethoxy)phenylhydrazine (FCCP) and glucose-free solution were used to prevent the hypercontraction in Ca\(^{2+}\) saturating solution (20).

Caffeine-induced [Ca\(^{2+}\)]\(_i\) transients. First, mESCMs were exposed in control solution to reach steady-state spontaneous twitches. Caffeine-induced [Ca\(^{2+}\)]\(_i\) transients were evoked by rapid and continuous application of 10 mM caffeine in 0 Ca\(^{2+}\) solution. The decay rate of caffeine-evoked transients was used as a measure of non-SERCA Ca\(^{2+}\) removal (2). After washout, the perfusion was switched to 0 Na\(^{+}\)-0 Ca\(^{2+}\) solution, and 0 Na\(^{+}\)-0 Ca\(^{2+}\) solution containing 10 mM of caffeine was rapidly and continuously applied. This inhibited both SR Ca\(^{2+}\) accumulation via SERCA and Ca\(^{2+}\) extrusion via NCX (2). Time constants (τ) of [Ca\(^{2+}\)]\(_i\), decay were estimated by fitting the decay phase of each [Ca\(^{2+}\)], transient with a monoexponential function.

**Quantitative analysis of [Ca\(^{2+}\)]\(_i\) decay.** We took the assumption by Bassani et al. (2) that 1) the rate of total intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)\(_{\text{total}}\)] removal during a normal transient decay phase is the sum of Ca\(^{2+}\) removal fluxes mediated by the SR, NCX, and slow system with a subtraction of negligible constant leak; and 2) the Ca\(^{2+}\) removal fluxes are [Ca\(^{2+}\)]\(_i\) dependent. Because the intrinsic passive Ca\(^{2+}\)-buffering characteristics in mESCMs are unknown, we did not convert the [Ca\(^{2+}\)], into the [Ca\(^{2+}\)\(_{\text{total}}\)]\(_i\). Instead, since the Ca\(^{2+}\) concentration of spontaneous transients is low and the amplitudes are small in our preparations, we assumed the rate of change of [Ca\(^{2+}\)\(_i\)], (d[Ca\(^{2+}\)]/dt) to be proportionally linearly proportional to d[Ca\(^{2+}\)\(_{\text{total}}\)]/dt in the [Ca\(^{2+}\)\(_i\)] range of mESCMs. Therefore, the decline rate of [Ca\(^{2+}\)\(_i\)] can be calculated by

\[
d[\text{Ca}^{2+}]_i/dt = J_{\text{SR}} + J_{\text{NCX}} + J_{\text{slow}}
\]

where the J terms refer to net [Ca\(^{2+}\)], decline flux mediated by SR, NCX, and slow system, and they are [Ca\(^{2+}\)]\(_i\)-dependent (2, 3, 19). For each cell, J\(_{\text{slow}}\) was defined by plotting d[Ca\(^{2+}\)]/dt as a function of the corresponding [Ca\(^{2+}\)], during a caf-00 decay phase when SR stores are unloaded by treatment with caffeine. SERCA activity is ineffective in removing Ca\(^{2+}\) from the cytosol, and NCX is inhibited in 0 Na\(^{+}\)-0 Ca\(^{2+}\) solution (2, 3, 19). J\(_{\text{SR}} + J_{\text{NCX}} + J_{\text{slow}}\) was defined by plotting d[Ca\(^{2+}\)]/dt against the corresponding [Ca\(^{2+}\)], during a caf decay phase when SR stores are unloaded by treatment with caffeine, but NCX remains active. Similarly, total flux (J\(_{\text{total}}\) = J\(_{\text{SR}}\) + J\(_{\text{NCX}}\) + J\(_{\text{slow}}\)) was defined by plotting d[Ca\(^{2+}\)]/dt vs. [Ca\(^{2+}\)], during spontaneous transient decline (average of at least 5 decay phases). Thus, J\(_{\text{SR}}\), J\(_{\text{NCX}}\), and J\(_{\text{slow}}\) had been defined. Finally, we plotted the [Ca\(^{2+}\)]\(_i\) decline rate by each system during the decline of spontaneous transients, and numerical integration of the curves gave the amount of net [Ca\(^{2+}\)]\(_i\) decayed by individual systems.

To estimate the fractional contribution of SERCA to the total Ca\(^{2+}\) removal, two methods were used and are described as follows.

In method 1, the fractional contribution of SERCA, NCX and slow system to the total Ca\(^{2+}\) removal was calculated according to the formulas (1/τ\(_{\text{caf-00}}\) - 1/τ\(_{\text{caf-00}}\)) (1/τ\(_{\text{caf-00}}\) - 1/τ\(_{\text{caf-00}}\)), and (1/τ\(_{\text{caf-00}}\)), respectively, as described previously (11, 16, 27, 19) and estimated the relative contributions of SERCA, NCX, and slow system to total Ca\(^{2+}\) leak from SR. As mentioned previously (2), J\(_{\text{SR}}\) can be described as simple quasiempirical [Ca\(^{2+}\)],-dependent expression of the following formula: J\(_{\text{SR}}\) = \(V_{\text{max}}[1 + (K_{\text{ca}}/[Ca^{2+}])^n]\). Assuming that L\(_{\text{SR}}\) is constant, then J\(_{\text{SR}}\) can be described as

\[
J_{\text{SR}} = V_{\text{max}}[1 + (K_{\text{ca}}/[Ca^{2+}])]\times n - L_{\text{SR}}
\]

We fitted J\(_{\text{SR}}\) to Eq. 2 for each experiment and determined the constants \(V_{\text{max}}\), \(K_{\text{ca}}\), n, and L\(_{\text{SR}}\). Then we simulated the action of J\(_{\text{SR}}\) during the decay phase (gray dashed curve in Fig. 1D) as described previously (2, 19) and estimated the relative contributions of SERCA, NCX, and slow system to total Ca\(^{2+}\) removal (supplemental Fig. 1; supplemental data for this article can be found online at the American Journal of Physiology-Cell Physiology website).

When calculating d[Ca\(^{2+}\)]/dt for each experiment, the decay phases of at least five spontaneous transients were averaged. The earliest time that [Ca\(^{2+}\)], decayed to the baseline was considered to be the end of a decay phase, and this time scale was used for the numerical integration. The numerical analysis was performed with OriginLab7 (OriginLab, Northampton, MA).

**Na\(^{+}\) perfusion procedure.** To avoid Ca\(^{2+}\) entry via NCX during Na\(^{+}\) perfusion, 0 Na\(^{+}\)-0 Ca\(^{2+}\) preperfusion procedure was used as
described previously (2). Briefly, the cells were first perfused in 0 Na+-0 Ca2+ solution (0.5 mM EGTA) for 5 min to deplete intracellular Na+ so that the subsequent reintroduction of extracellular Ca2+ would not result in Ca2+ entry via NCX (2). The perfusion solution was then switched to either control solution or 0 Na+ solution, and field stimulus was applied to evoke transients after the solution replacement was accomplished. The first transient was used for analysis. After the reintroduction of extracellular Ca2+, slowly linear rise of the diastolic [Ca2+]i level was usually observed. This may be due to the nonselective Ca2+ entry. To rule out the influence of the linear diastolic rise on the transient kinetics when analyzing the first transients, the slopes of the linear rise before the transients were subtracted from the transients. The cells were then paced by field stimulation until the transients reached steady state. The steady-state transients were analyzed. This procedure was repeated to get both control and 0 Na+ data from the same cell.

Electrophysiology. The NCX current (I_{NCX}) was measured as the bidirectional Ni2+ (5 mM)-sensitive current using standard whole cell patch-clamp techniques with a EPC-10 amplifier (Heka Electronics). Ramp pulse (+60 mV to −120 mV; 0.3 V/s) was applied at 0.1 Hz. The external solution contained (in mM) 135 NaCl, 1.8 CaCl2, 1.0 MgCl2, 10 glucose, 10 HEPES, 10 TEA-Cl (to block K+ currents), 0.01 niflumic acid (to block Ca2+-activated Cl− currents), and 0.01 verapamil (to block L-type Ca2+ currents), pH 7.4 with NaOH. The internal solution contained (in mM): 90 Cs-aspartate, 20 CsCl, 10 TEA-Cl, 3 MgCl2, 5 Na2ATP, 2 CaCl2, 5 EGTA, 5 creatine, and 5 HEPES (pH 7.2 with CsOH).

Statistical analysis. Data are expressed as means ± SE. Statistical significance of differences in means was estimated by one-way ANOVA or by Student’s t-test or a paired t-test, when appropriate (StatSoft, version 5.1, StatSoft, Tulsa, OK). P < 0.05 was considered significant.

RESULTS

Time course of [Ca2+]i, decline during cardiomyocyte differentiation. To characterize the dynamics of [Ca2+]i, transient decay and evaluate the relative contribution of NCX, SERCA, and slow system to the decline of [Ca2+]i, transients during cardiomyocyte differentiating, the time courses of [Ca2+]i, decline in spontaneous and caffeine-induced [Ca2+]i transients were analyzed at early, intermediate, and late differentiation stages of mESCMs. Figure 1A shows the procedure used here. The amplitudes of spontaneous [Ca2+]i transients (spon) were significantly higher at LDS as compared with those from mESCMs at EDS and IDS (Fig. 1A). The amplitudes of 10 mM of caffeine-induced [Ca2+]i transients in the presence of extracellular Na+ (caf) were also significantly upregulated in LDS mESCMs compared with those from EDS and IDS. The amplitudes of caffeine-induced transients in the absence of extracellular Na+ and Ca2+ were significantly upregulated in LDS mESCMs as compared with those from EDS, indicating that the SR Ca2+ content is increased during differentiation (Fig. 1B). Caffeine in 0 Na+-0 Ca2+ solution (caf-00) evoked significantly larger amplitude of [Ca2+]i transients than that evoked by caffeine in the presence of extracellular Na+, indicating that NCX contributes to the limitation of amplitudes of [Ca2+]i transient in differentiating mESCMs (Fig. 1B). In spontaneous beating myocytes, the time constant of [Ca2+]i decay, τ_{spon}, an inverse indicator of the rate constants of Ca2+ removal, was significantly smaller at LDS compared with EDS (Fig. 1C). To examine the Ca2+ removal ability of Ca2+ transporters other than SERCA, the time constant of the decay of [Ca2+]i transients induced by 10 mM of caffeine (τ_{caf}) was then determined when net Ca2+ accumulation by SR was prevented by a high caffeine concentration (2). The τ_{caf} values of Ca2+ transients were significantly larger at LDS than at EDS and IDS, and were −4.2-fold higher at EDS, −5.2-fold higher at IDS, and −9.0-fold higher at LDS than the corresponding τ_{spon} values (Fig. 1C). Next, [Ca2+]i, decline attributable to transports via the slow system was evaluated when both Ca2+ uptake by SR and Ca2+ extrusion by NCX were inhibited by applying 0 Na+-0 Ca2+ solution containing 10 mM of caffeine (2). Under such conditions, the decay time constants τ_{caf,00} were −10.6-fold higher at EDS (n = 17), −9.8-fold higher at IDS (n = 20), and −8.9-fold higher at LDS (n = 24) than the corresponding τ_{caf} value, but they did not differ among differentiation stages (Fig. 1C).

Relative contribution of Ca2+ transporters to [Ca2+]i decline. To further estimate the dynamical contribution of SR and NCX to [Ca2+]i decline, we estimated the [Ca2+]i, removed via each Ca2+ transporter by using the principles mentioned previously (2, 3, 19) (for details, see Quantitative analysis of [Ca2+]i, decay in MATERIALS AND METHODS). Figure 1D shows a typical integration of [Ca2+]i, fluxes calculated from the transients shown in Fig. 1A. During the spontaneous transient decay of mESCMs, the contribution of SR to the net [Ca2+]i, decline was much greater than that of NCX, especially during the initial rapid decay phase. We summarized the proportion of integrated [Ca2+]i, fluxes via each system to the total net [Ca2+]i, decay during the first 50% and 100% of the [Ca2+]i, decline, respectively (Fig. 1, E and F). Figure 1E shows that the SR was responsible for 73 ± 2% (EDS) and 80 ± 2% (LDS) of the first 50% of the net [Ca2+]i, decline. These fractions decreased to 56 ± 3% (EDS) and 69 ± 2.5% (LDS) at the end of the [Ca2+]i, decline (Fig. 1F), suggesting that the net Ca2+ removal by SR was less efficient for the terminal decay phase when compared with the initial phase. This was also supported by the observation that at the terminal slow decay phase, the rates of total net [Ca2+]i, flux and NCX-mediated [Ca2+]i, flux were similar (indicated by the dashed tangent in Fig. 1D), and the rate of SR-mediated net [Ca2+]i, flux was close to zero. But at the initial rapid decay phase, the rate of NCX-mediated [Ca2+]i, flux was much smaller than that of total net [Ca2+]i, flux. These observations indicate that the SR dominates the initial rapid decay phase and the NCX may take over the terminal slow decay in mESCMs.

To explain the asymmetric contribution of the SR to the net [Ca2+]i, decline, the homeostasis of SR Ca2+ content needs to be considered. It was postulated that RyR2 may open occasionally even at resting [Ca2+]i, level in fetal cardiomyocytes (37). Our previous study showed that the mRNA of SR Ca2+ buffering protein calsequestrin is not detectable in mESCMs before differentiation day 14 and the expression level in mESCMs at day 18 is lower compared with adult cardiomyocytes (12). Thus, the Ca2+ leak from SR may be vigorous in mESCMs though it is considered to be small in the adult cardiomyocytes (2). As a result, the net [Ca2+]i, decline flux mediated by SR may be hindered significantly by SR Ca2+ leak during the terminal decay phase in mESCMs, when the SERCA-mediated Ca2+ flux is relatively slow. With this consideration, it can be deduced that the contribution of SERCA to the total Ca2+ removal is greater than the contribution of SR to the net [Ca2+]i, decline. We calculated this fractional contribution of SERCA to the total Ca2+ removal by using two
calculation methods (method 1 and method 2; for details see Quantitative analysis of [Ca\(^{2+}\)]\(_i\) decay in MATERIALS AND METHODS) to make a more careful estimation. The calculation by method 1 showed that in EDS mESCMs, the contribution of NCX extrusion and SERCA uptake to total Ca\(^{2+}\) removal was 21.2% and 76.2%, respectively (Fig. 1G). Upon differentiating, the functional proportion of NCX decreased significantly by 48% at LDS from EDS, whereas that of SERCA increased significantly by 16% at LDS from EDS, but no significant differences of the proportions between EDS and IDS were detected (Fig. 1G). Correspondingly, the relative contribution of slow system was much less compared with those of NCX and SERCA, and it decreased from 2.8 ± 0.6% at EDS to 1.3 ± 0.1% at LDS (Fig. 1G). Similar results were obtained by using method 2 and are shown in supplemental Fig. 1.

NCX is important for terminal phase of [Ca\(^{2+}\)]\(_i\) decline but not for the initial phase. To further explore the role of NCX as a Ca\(^{2+}\) remover in mESCMs, we then investigated field-stimulated [Ca\(^{2+}\)]\(_i\) transients of mESCMs in 0 Na\(^+\) solution, which inhibits NCX-mediated Ca\(^{2+}\) extrusion from the cytosol (2). To prevent Ca\(^{2+}\) entry via NCX during 0 Na\(^+\) perfusion
while keeping SR Ca\(^{2+}\) store, the 0 Na\(^{+}\)-0 Ca\(^{2+}\) preperfusion procedure was used (2). Figure 2A shows the transients in control or 0 Na\(^{+}\) solution after preperfusion with 0 Na\(^{+}\)-0 Ca\(^{2+}\) solution. The short vertical lines below the tracings represent field stimulation. B: representative tracings of the first [Ca\(^{2+}\)]\(_i\) transient in control or 0 Na\(^{+}\) solution after preperfusion with 0 Na\(^{+}\)-0 Ca\(^{2+}\) solution. The slope of the linear rise of baseline before the first transients had been subtracted from the transients. C and D: time to 50\% (T50\%) and 90\% decline (T90\%) of maximum amplitude of the first [Ca\(^{2+}\)]\(_i\) transient after 0 Na\(^{+}\)-0 Ca\(^{2+}\) preperfusion, normalized to those in control solution after 0 Na\(^{+}\)-0 Ca\(^{2+}\) preperfusion, respectively. E: steady-state [Ca\(^{2+}\)]\(_i\) transients in 0 Na\(^{+}\) solution or control solution. F and G: T50\% and T90\% of the steady-state (ss) transients, normalized to those in control solution, respectively. n = 14, 10, and 8 of mESCMs at EDS, IDS, and LDS, respectively. ###P < 0.01 as indicated.
achieved mainly by SERCA uptake, while NCX is mainly responsible for the terminal phase of \([\text{Ca}^{2+}] \text{i}\) decline.

NCX is the main \([\text{Ca}^{2+}] \text{i}\) remover in mESCMs with functionally suppressed SR. The above results suggest a dominant role of SR in regulating \([\text{Ca}^{2+}] \text{i}\) homeostasis in mESCMs. However, our previous work showed that in RyR2\(^{-/-}\) mESCMs, the amplitude of \([\text{Ca}^{2+}] \text{i}\) transient is similar to that of RyR2\(^{+/+}\) myocytes though the kinetics of \([\text{Ca}^{2+}] \text{i}\) transient upstroke is significantly slower, suggesting that some other intracellular or transsarcolemmal mechanisms may compensate for the loss of SR \([\text{Ca}^{2+}] \text{i}\) release to a great degree (12). Alteration in \([\text{Ca}^{2+}] \text{i}\) transient activation mechanism could possibly accompany changes in \([\text{Ca}^{2+}] \text{i}\) removal mechanisms. To further reveal potential compensatory \([\text{Ca}^{2+}] \text{i}\) resources in differentiating cardiomyocytes with suppressed SR, we analyzed the \([\text{Ca}^{2+}] \text{i}\) decay in these mESCMs.

To block the SR function, mESCMs were treated with 0.5 \(\mu\)M of thapsigargin for 15 min to inhibit SERCA or 10 \(\mu\)M of ryanodine for 30 min to inhibit RyRs. Ryanodine or thapsigargin treatment significantly attenuated \([\text{Ca}^{2+}] \text{i}\) transient amplitude and prolonged \([\text{Ca}^{2+}] \text{i}\) upstroke and decline phases in RyR2\(^{+/+}\) mESCMs (Fig. 3A), similar to our previous observations (12). To reveal the participation of NCX in the decay of \([\text{Ca}^{2+}] \text{i}\) transients in these ryanodine- or thapsigargin-treated mESCMs, field-stimulated \([\text{Ca}^{2+}] \text{i}\) transients in control or 0 \(\text{Na}^{+}\) solution were compared. During the perfusion, 0.5 \(\mu\)M thapsigargin or 10 \(\mu\)M ryanodine was always present in the solutions. In the 0 \(\text{Na}^{+}\) solution, an extremely slower \([\text{Ca}^{2+}] \text{i}\) decay rate was observed in ryanodine- (Fig. 3B) or thapsigargin-treated (Fig. 3C) mESCMs compared with that in control solution, indicating that NCX-mediated \([\text{Ca}^{2+}] \text{i}\) efflux is the main mechanism for \([\text{Ca}^{2+}] \text{i}\) removal in mESCMs with functionally suppressed SR. This further suggests that the sarcolemmal \([\text{Ca}^{2+}] \text{i}\) influxes are the compensatory \([\text{Ca}^{2+}] \text{i}\) resource for the \([\text{Ca}^{2+}] \text{i}\) transients in mESCMs when SR is functionally suppressed.

**Fig. 3.** Analysis of \([\text{Ca}^{2+}] \text{i}\) decline in mESCMs with functionally disabled SR. A: ryanodine (RY) or thapsigargin (TG) treatment attenuated \([\text{Ca}^{2+}] \text{i}\) transients in wild-type (RyR2\(^{+/+}\)) mESCMs but not in RyR2-deficient (RyR2\(^{-/-}\)) mESCMs. B–D: superimposed representative field-stimulated \([\text{Ca}^{2+}] \text{i}\) transients (normalized amplitudes) of ryanodine-treated (B) or thapsigargin-treated (C) RyR2\(^{+/+}\) (B and C) or RyR2\(^{-/-}\) (D) mESCMs in the presence (control) or absence (0 \(\text{Na}^{+}\)) of extracellular \(\text{Na}^{+}\) (left) and the corresponding time constants (\(\tau\)) of \([\text{Ca}^{2+}] \text{i}\) decline normalized to that of control (right). \(n = 7, 5, \text{and } 6\) for EDS, IDS, and LDS in B. \(n = 6, 7, \text{and } 6\) for EDS, IDS, and LDS in C. \(n = 5, 9, \text{and } 5\) for EDS, IDS, and LDS in D. \(^{**}P < 0.01\) as indicated.
To further determine whether the long-term inactivation of SR Ca\(^{2+}\) release may evoke other compensatory Ca\(^{2+}\) resources, the characteristics of [Ca\(^{2+}\)]\(_i\) transients in mESCMs were examined. In these cells, inhibition of SERCA by thapsigargin affected little the dynamics of [Ca\(^{2+}\)]\(_i\) transients (Fig. 3A), while inhibition of NCX by 0 Na\(^+\) perfusion caused a significantly prolonged [Ca\(^{2+}\)]\(_i\) decay phase (Fig. 3D). These data demonstrated that in RyR2\(^{-/-}\) mESCMs, [Ca\(^{2+}\)]\(_i\) transients are mainly decayed by Ca\(^{2+}\) extrusion via NCX, suggesting that these [Ca\(^{2+}\)]\(_i\) transients are mainly generated by sarcolemmal Ca\(^{2+}\) influxes.

Considering that NCX is the main Ca\(^{2+}\) remover in RyR2\(^{-/-}\) mESCMs, the long-term suppression of SR function may lead to a functional compensation of NCX. We thus compared the density of I\(_{\text{NCX}}\) in RyR2\(^{+/+}\) and RyR2\(^{-/-}\) but did not detect the difference between these cells (Fig. 4). The results demonstrated that though NCX acts as the main Ca\(^{2+}\) remover when SR function is suppressed in RyR2\(^{-/-}\) mESCMs, the function of NCX is not increased.

**DISCUSSION**

In the present study, we examined contributions of Ca\(^{2+}\) extrusion via NCX and intracellular sequestration via SERCA in differentiating mESCMs. The major findings here are 1) the time for [Ca\(^{2+}\)]\(_i\), decay is shortened with differentiation of mESCMs; 2) SERCA is a major Ca\(^{2+}\) transporter responsible for [Ca\(^{2+}\)]\(_i\) decline even at the early differentiation stage, and the relative contribution of SERCA to [Ca\(^{2+}\)]\(_i\) decline increases with time of in vitro cardiomyogenesis; and 3) the relative contribution of NCX to [Ca\(^{2+}\)]\(_i\) decline is much smaller than that of SERCA and its role in [Ca\(^{2+}\)]\(_i\) decline decreases during mESCMs differentiation; moreover, it mainly contributes to the terminal phase of [Ca\(^{2+}\)]\(_i\) decline; and 4) when SR is functionally suppressed, NCX acts as the main Ca\(^{2+}\) remover responsible for the [Ca\(^{2+}\)]\(_i\) decline. The findings offer new insights into the functional regulation of [Ca\(^{2+}\)]\(_i\) decline in differentiating cardiomyocytes and extend previous findings demonstrating the crucial role of SR as a major intracellular Ca\(^{2+}\) sink during cardiomyogenesis.

**SERCA but not NCX is the major Ca\(^{2+}\) remover in mESCMs.** It is believed that in embryonic cardiomyocytes that the myocardial contraction is predominantly dependent on transsarcolemmal Ca\(^{2+}\) influx (26, 36). Therefore, it is expected that in embryonic cardiomyocytes, NCX might be a prominent Ca\(^{2+}\) remover and even more important than SERCA. However, it has been recently shown that in murine embryonic cardiomyocytes 8.5–9.5 days old, the majority of Ca\(^{2+}\) removal (~74%) is accomplished by SERCA (32). The studies on mESCMs showed that the SERCA is the major Ca\(^{2+}\) remover in 17-day-old mESCMs (16). Here, we provided evidence showing that the contribution of SERCA to Ca\(^{2+}\) removal is upregulated with the time of mESCMs differentiation, accompanied with a downregulation of NCX to Ca\(^{2+}\) removal, a similar pattern as reported by Kapur and Banach (16). The functional changes with differentiation also coincide with the gene expression pattern of SERCA and NCX during embryonic development (21, 32) or mESCMs differentiation (12). Moreover, our data demonstrated that ~76% of Ca\(^{2+}\) is removed by SERCA even in mESCMs at EDS, indicating the importance of SR in regulating Ca\(^{2+}\) homeostasis in early differentiating mouse cardiomyocytes. This supports our recent observation of the crucial contribution of RyR2 to Ca\(^{2+}\) transients in mESCMs (12). Consistently, NCX is only responsible for ~21% of Ca\(^{2+}\) removal in EDS mESCMs and ~10% in LDS mESCMs. In addition, the contribution of NCX to [Ca\(^{2+}\)]\(_i\) decline is limited to the terminal decay phase of the transients. These findings indicate that even in early differentiating cardiomyocytes, SERCA is a predominant Ca\(^{2+}\) remover and NCX plays a much smaller role. The latter is supported by the observation that cardiac-specific NCX-knockout mice live into adulthood (14) and the finding that NCX-deficient embryonic cardiomyocytes have normal field-stimulated [Ca\(^{2+}\)]\(_i\) transients under basal conditions (7).

We showed that in the 9- to 11-day-old mESCMs, SERCA and NCX are responsible for 76% and 21% of Ca\(^{2+}\) removal, respectively, which is very close to the values obtained from embryonic mouse cardiomyocytes at corresponding embryonic stage (embryonic day E8.5–9.5) by Reppel et al. (32). This supports the view that the in vitro differentiated mESCMs are temporally and functionally comparable to the in vivo differentiated embryonic cardiomyocytes (9, 10). Therefore, mESC cell in vitro differentiation model is an ideal system to study functional regulation of early differentiating cardiomyocytes.

The slow system is generally considered to be PMCA and mitochondrial uniporter. Among them, PMCA appears to be more prominent in neonatal rat cardiomyocytes (3). Our data showed that the slow system is responsible for <3% of total Ca\(^{2+}\) removal during early, intermediate, and later differentiation stages (Fig. 1G). The individual contributions of these two slow transporters to Ca\(^{2+}\) removal need to be investigated further.
By using the similar caffeine-induced \([\text{Ca}^{2+}]_i\), transient method as we did, Kapur and Banach (16) estimated that less than half of \(\text{Ca}^{2+}\) removal is mediated by SERCA in mESCs at EDS (9 days old). This fraction is much smaller than that estimated by us. The inconsistent observation may be due to the different mES cell lines used in these two experiments. In the Kapur and Banach study, \(\alpha\)-myosin heavy chain (\(\alpha\)-MHC) promoter-driven neomycin resistance gene-selected mESCs derived from cytomegalovirus mES cell line were used (16). It has been shown that \(\alpha\)-MHC-positive embryonic cardiomyocytes do not include all types of the cardiomyocytes, and they are mostly atrial- or pacemaker-like myocytes (18), while in the present study, the nonselected mESCMs derived from R1 mES cells were used. Further studies are needed to determine whether the cell lines or cardiomyocyte types are relevant to the present study, the nonselected mESCMs derived from R1 cytops, do not include all types of the cardiomyocytes, and they has been shown that derived from cytomegalovirus mES cell line were used (16). It promoter-driven neomycin resistance gene-selected mESCMs the Kapur and Banach study, the different mES cell lines used in these two experiments. In estimated by us. The inconsistent observation may be due to at EDS (9 days old). This fraction is much smaller than that half of \(\text{Ca}^{2+}\) removal is mediated by SERCA in mESCMs. When fitting the \([\text{Ca}^{2+}]_i\) dependence of SR-mediated net \([\text{Ca}^{2+}]_i\) flux to Eq. 2, we did not take the cellular \(\text{Ca}^{2+}\) buffering into account. This may cause some system error to the final estimates. However, the fitting gave an averaged Hill coefficient \(n = 3.4\) and \(K_m = 216 \text{mM}\) for SERCA, which is very approximate to that estimated with consideration of \(\text{Ca}^{2+}\) buffering in adult cardiomyocytes (2). This indicated that our analysis can faithfully reflect the behavior of SERCA. We estimated the contributions of each system to total \(\text{Ca}^{2+}\) removal by using two kinds of calculation methods. Though these two methods gave very similar estimates, their accuracy could be refined by better knowledge of the characteristics of cellular \(\text{Ca}^{2+}\) buffering in mouse embryonic cardiomyocytes. This needs to be further explored.

It is generally assumed that caffeine can abolish SERCA-mediated net \(\text{Ca}^{2+}\) removal by unloading the SR in cardiomyocytes (2–4, 11, 19, 27, 29, 32). Cardiomyocytes express both caffeine-sensitive RyRs and caffeine-insensitive inositol 1,4,5-trisphosphate (\(\text{IP}_3\)) receptors (\(\text{IP}_3\)Rs). If the \(\text{IP}_3\)Rs do not share the same \(\text{Ca}^{2+}\) stores with the RyRs, the net \(\text{Ca}^{2+}\) removal mediated by SERCA on \(\text{IP}_3\)R-regulated \(\text{Ca}^{2+}\) stores may not be abolished by caffeine. However, it has been shown that \(\text{IP}_3\)Rs colocalize with RyRs in atrial cardiomyocytes (22), and application of \(\text{IP}_3\) decreases SR \(\text{Ca}^{2+}\) loading in ventricular cardiomyocytes (8). These observations suggest that at least a part of SR \(\text{Ca}^{2+}\) stores is shared by both \(\text{IP}_3\)Rs and RyRs in cardiomyocytes. These \(\text{IP}_3\)R-regulated \(\text{Ca}^{2+}\) stores will not affect the \(\text{Ca}^{2+}\) removal in the presence of caffeine because they can also be depleted by caffeine via activation of RyRs. Furthermore, application of caffeine in the absence of extracellular \(\text{Na}^+\) slowed the \([\text{Ca}^{2+}]_i\), decay significantly (Fig. 1, A and C), and the \(\text{Ca}^{2+}\) removal fluxes insensitive to caffeine and \(\text{Na}^+\) were only responsible for \(<3\%\) of total \(\text{Ca}^{2+}\) removal at all three differentiation stages (Fig. 1G). Thus, even if \(\text{IP}_3\)R-regulated \(\text{Ca}^{2+}\) stores still uptake \(\text{Ca}^{2+}\) via SERCA in the presence of caffeine, their contributions to total \(\text{Ca}^{2+}\) removal is negligible and would not fundamentally affect the conclusion.

Contributions of NCX to the initial and terminal decay phase of field-stimulated \([\text{Ca}^{2+}]_i\), transients are asymmetric. Dynamical analysis of the contribution of SR and NCX to \([\text{Ca}^{2+}]_i\), decline indicated that SR dominates the initial rapid decay phase and that NCX takes over the terminal slow decay phase in mESCs. This idea was confirmed by our observation that abolishing NCX function by \(0 \text{Na}^+\) perfusion only prolongs the terminal decay phase of field-stimulated transients but not the initial rapid decay phase in mESCs, coinciding with the observation by Yao et al. (43) in adult ventricular myocytes. The \(\text{Ca}^{2+}\)-binding affinity of NCX is much lower than that of SERCA (30); however, it is interesting to see that the NCX contributes predominantly during the terminal decay phase when \([\text{Ca}^{2+}]_i\) is near its lowest. The possible explanations are that 1) the \(\text{Ca}^{2+}\) removal rate of SERCA is much faster than that of NCX and, therefore, the contribution of NCX to \(\text{Ca}^{2+}\) removal is hardly detected at the rapid decay phase of the transients until the \(\text{Ca}^{2+}\) removal rate of SR becomes slow when the SR is reloaded; 2) the plateau phase of membrane depolarization may limit the \(\text{Ca}^{2+}\) extrusion by NCX at a certain extent during the initial decay phase as the NCX is a membrane potential dependent transporter; and 3) the \([\text{Ca}^{2+}]_i\), transients may be spatially and temporally heterogeneous in mESCs. The third possibility is supported by the observation that T-tubule network is absent in embryonic cardiomyocytes (33) with a large part of SR located in the interior of neonatal cardiomyocytes (39), and the existence of \(\text{Ca}^{2+}\)-binding regions near the sarcolemma of neonatal cardiomyocytes (40). These subcellular structural traits of immature cardiomyocytes suggest that in such cells, SERCA and NCX may take \(\text{Ca}^{2+}\) from different compartments. If so, the asymmetric contribution of NCX and SERCA to \([\text{Ca}^{2+}]_i\), decay phase can be explained as follows. SERCA may rapidly uptake the SR-released \(\text{Ca}^{2+}\), and thus, shaping the rapid early decay phase, while NCX may extrude part of the transsarcolemmal \(\text{Ca}^{2+}\) influx accumulated in the sarcosomal \(\text{Ca}^{2+}\)-binding region at the time of membrane repolarization. Whether the observed phenomenon is due to the large difference between the \(\text{Ca}^{2+}\) removal rate of SERCA and NCX, and/or to the compartmentalization of cytosolic \(\text{Ca}^{2+}\) needs to be further examined.

The \(\text{Ca}^{2+}\) influx compensates for the functional suppression of SR in mESCs. The present study showed NCX as the major \(\text{Ca}^{2+}\) remover in RyR2\(-/\) mESCs and in RyR2\(+/\) cells treated with high concentrations of ryanodine or thapsigargin, suggesting that transsarcolemmal \(\text{Ca}^{2+}\) influxes are the major source of \([\text{Ca}^{2+}]_i\), transients in these cells. It has been demonstrated that the amplitudes of \([\text{Ca}^{2+}]_i\), transients in RyR2\(-/\) mESCs are similar to that of RyR2\(+/\) mESCs (12). These results together suggest that compensatory \(\text{Ca}^{2+}\) influxes can raise the \([\text{Ca}^{2+}]_i\), to a comparable level as that raised by SR \(\text{Ca}^{2+}\) release in differentiating cardiomyocytes. This was supported by our previous finding that L-type \(\text{Ca}^{2+}\) current \((I_{\text{CAL}})\) was enhanced in RyR2\(-/\) mESCs compared with RyR2\(+/\) mESCs (12). This compensation may be partially due to the reduced \(\text{Ca}^{2+}\) release-induced \(\text{Ca}^{2+}\) current inactivation (34). This compensatory mechanism may have functional importance during development. As the heart is one of the most important organs during embryonic development, such compensatory mechanisms are needed to minimize functional depression of the heart even when the development of the SR is interrupted or delayed such as in RyR2\(-/\) mESCs. The long-term suppression of SR function as in RyR2\(-/\) mESCs may lead to a functional compensation of NCX to match the compensatory increase of \(I_{\text{CAL}}\) as observed previously (12). However, we did not detect a significant difference in \(I_{\text{NCX}}\) densities between RyR2\(+/\) and RyR2\(-/\) mESCs, indicating that the long-term functional suppression of SR does not lead to an equally compensatory increase of \(I_{\text{NCX}}\) as that of

\[\text{Ca}^{2+}\] REMOVAL PATHWAYS IN DEVELOPING CARDIOMYOCYTES C739

AJP-Cell Physiol • VOL 297 • SEPTEMBER 2009 • www.ajpcell.org
This may cause an accumulation of the influxed Ca\(^{2+}\) in the organelles such as SR, and, at least partially, explains the observation of the large vacuolated SR containing high Ca\(^{2+}\) concentration in RyR2\(^{-/-}\) mouse embryonic cardiomyocytes (37). Another Ca\(^{2+}\) transporter responsible for Ca\(^{2+}\) efflux is PMCA. Whether the long-term suppression of SR function in mESCMs leads to a functional compensation of this slow Ca\(^{2+}\) transport by altering its expression and activation needs to be investigated further.

In conclusion, our results revealed the differential roles of Ca\(^{2+}\) transporters in [Ca\(^{2+}\)]\(_{i}\), decay of differentiating cardiomyocytes derived from mES cells. The SERCA is a major Ca\(^{2+}\) transporter responsible for [Ca\(^{2+}\)]\(_{i}\) decay even when the NCX contribution is maximal. Moreover, the effect of NCX on [Ca\(^{2+}\)]\(_{i}\) decline is apparent only in the late phase of [Ca\(^{2+}\)]\(_{i}\) decay. However, when SR is suppressed in mESCMs, NCX becomes a major Ca\(^{2+}\) transporter responsible for Ca\(^{2+}\) decay. These results suggest a critical role of SR in regulating [Ca\(^{2+}\)]\(_{i}\), homeostasis in mES cell-derived differentiating cardiomyocytes. Whether embryonic cardiomyocytes have the similar regulatory mechanism needs to be determined further.

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