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Developmental regulation of intracellular calcium transients during cardiomyocyte differentiation of mouse embryonic stem cells[1]

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Key words

Ca^{2+} transients; cardiac differentiation; embryonic stem cells; Ca^{2+} handing proteins

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Abstract

Aim: To investigate the developmental regulation of intracellular Ca^{2+} transients, an essential event in excitation-contraction coupling, during cardiomyocyte differentiation. **Methods:** Using the embryonic stem (ES) cell *in vitro* differentiation system and pharmacological intervention, we investigated the molecular and functional regulation of Ca^{2+} handling proteins on the Ca^{2+} transients at early, intermediate and later differentiation stages of ES cell-derived cardiomyocytes (ESCM). **Results:** Nifedipine, a selective antagonist of L-type Ca^{2+} channels, totally blocked Ca^{2+} transients even in the condition of field-electric stimulation in ESCM at three differentiation stages. The Ca^{2+} transients of ESCM were also inhibited by both ryanodine [an inhibitor of ryanodine receptors (RyRs)] and 2-aminoethoxydipheylborate [2-APB, an inhibitor of inositol-1,4,5-trisphosphate receptors (IP_3Rs)]. The inhibitory effect of ryanodine increased with the time of differentiation, while the effect of 2-APB decreased with the differentiation. Thapsigargin, an inhibitor of SR Ca^{2+}-pump ATPase, inhibited Ca^{2+} transients equally at three differentiation stages that matched the expression profile. Na^+ free solution, which inhibits Na^+-Ca^{2+} exchanger (NCX) to extrude Ca^{2+} from cytosol, did not affect the amplitude of Ca^{2+} transients of ESCM until the latter differentiation stage, but it significantly enhanced the basal Ca^{2+} concentration. **Conclusion:** The Ca^{2+} transients in ESCM depend on both the sarcolemmal Ca^{2+} entry via L-type Ca^{2+} channels and the SR Ca^{2+} release from RyRs and IP_3Rs even at the early differentiation stage; but NCX seems not to regulate the peak of Ca^{2+} transients until the latter differentiation stage.

Introduction

Intracellular Ca^{2+} signaling regulates a wide variety of cellular functions and organ development[1–4]. Intracellular Ca^{2+} transients, the cyclic variations in the concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_i$), play a crucial role in the contraction and relaxation of cardiomyocytes. The Ca^{2+} transients are the result of a spatio-temporal balance between cytosolic Ca^{2+} elevation and Ca^{2+} re-uptake by sarcoplasmic reticulum (SR) or cell extrusion. It arises via Ca^{2+}-induced Ca^{2+} release (CICR) mechanism in adult cardiomyocytes, where a relatively small Ca^{2+} influx through sarcolemmal L-type Ca^{2+} channels triggers greater amounts of SR Ca^{2+} release from type-2 ryanodine receptor (RyR2). This is the base of cardiac excitation-contraction (E-C) coupling[5,6]. Upon the recycling of a majority of cytosolic Ca^{2+} back to the SR by Ca^{2+}-pump ATPase (SERCA2) and a small portion of cytosolic Ca^{2+} out of the sarcolemma by Na^+-Ca^{2+} exchanger (NCX), a decrease of $[Ca^{2+}]_i$ occurs, leading to myocardial relaxation. Thus, SR plays a central role in the regulation of the contractile force of adult cardiac myocytes by modulating the amplitude and the rise or decay velocity of the Ca^{2+} transients. However, because of the known difficulties in obtaining cardiomyocytes from the very early mammalian embryos (eg, before d 12 to 13 of gestation in mice), there is only limited knowledge on the developmental aspects and the regulation of Ca^{2+} transients.

The heart is the first organ that becomes functional in the vertebrate embryo. On approximately embryonic day (E) 7.25 in mice, the precardiac mesoderm forms a primitive tubular heart that starts beating at E8[7]. The heart is continuously remodeled until the four-chambered organ is formed, and maintains its physiologic pumping function in response to increasing circulatory demands[7]. The ensuing development of E-C coupling is fundamental to the embryonic cardiac function during embryogenesis. In the embryonic heart the mRNA and protein abundance of the main Ca^{2+} handing proteins, such as RyR2, SERCA2, phospholamban (PLB), and NCX1, however, is different from those in neonatal and adult hearts[8,9], suggesting that the regulation of Ca^{2+} transients in embryonic cardiomyocytes may be different from that in adult cardiac myocytes.

The embryonic stem (ES) cell-derived cardiomyocytes (ESCM) represent specialized cell types of the heart, such as atrial-like, ventricular-like, sinus nodal-like, and Purkinje-like cells[10]. Published ultrastructural[11], molecular biological[12] and electrophysiological[10,13] studies have demonstrated that within the ES cell-formed embryoid bodies (EB), the various stages of cardiomyocytes closely recapitulate the developmental pattern of murine early cardiogenesis. Therefore, the ES cell *in vitro* differentiation system can be used to investigate early cardiogenesis[10–12, 14–16]. ESCM are also one possible source of transplantable cells. It is a therapeutic prerequisite to investigate the regulation of Ca^{2+} transients, one of the critical functional properties of potential replacement cells. Recently, we observed that RyR2-mediated SR Ca^{2+} release directly contributed to the spontaneous and β-adrenergic receptor-stimulated Ca^{2+} transients and contraction of ESCM even at very immature stages of development[17]. However, the importance of sarcolemmal Ca^{2+} handing proteins, such as L-type Ca^{2+} channels and NCX, and SR Ca^{2+} release channels inositol triphosphate receptors (IP_3Rs) on Ca^{2+} transients of ESCM have not yet been fully clarified.

Therefore, in the present study, we investigated the developmental regulation of the main Ca^{2+} handling proteins on the Ca^{2+} transients in ESCM during cardiogenesis. Our results demonstrate that both sarcolemmal Ca^{2+} entry and SR Ca^{2+} release contribute to the Ca^{2+} transients even at the early differentiation stage, while NCX plays more crucial roles in maintaining normal basic Ca^{2+} concentration during whole ESCM differentiation and only regulates peak Ca^{2+} transients at the latter differentiation stage.

Materials and methods

Cell culture, differentiation and isolation of beating cardiomyocytes R1 ES cell lines were cultivated and differentiated into spontaneously beating cardiomyocytes as described in a previous study[16]. Undifferentiated ES cells were cultivated on mitomycin C-inactivated mouse feeder layers in the presence of leukemia inhibitory factor. The differentiation of ES cells into cardiac cells was initiated by a hanging drop technique to form embryoid bodies (EB). After 7 d in suspension, EB were plated onto gelatin-coated tissue culture dishes. Cardiomyocytes appeared in the form of spontaneously contracting cell clusters, and single cardiomyocytes were isolated at three distinct differentiation stages [early (EDS, 7+2–4 d); intermediate (IDS, 7+6–8 d), and late differentiation stages (LDS, 7+11–14 d)] by enzymatic dissociation with collagenase followed by plating on laminin/gelatin-coated glass coverslips[16]. All cultivation medium and other substances for cell cultures were purchased from Gibco BRL (Grand Island, NY, USA).

Detection of gene transcripts ES cells, EB and adult mouse hearts were used to isolate total RNA[16]. In brief, 0.5 μg total RNA from each tissue was converted to cDNA by using Superscript II reverse transcriptase (Life Tech, MD) and oligodT (T16, 500 ng) in a final volume of 20 μL, according to the manufacturer's instructions, and 0.4 μL of this was used for each PCR reaction. Semi-quantitative reverse transcriptase polymerase chain reactions (RT-PCR) were carried out with Tth DNA polymerase (Promega, Madison, WI, USA)) and DNA amplifications were carried out according to the manufacturer's instructions. Reactions were carried out in a Mastercycler gradient (Eppendorf, Hamburg, Germany) under the following conditions. PCR amplification involved 5 min at 95 ºC followed by 30–35 cycles of 45 s at 95 ºC, 45 s at the appropriate annealing temperature and 45 s at 72 ºC for elongation ending with 5 min at 72 ºC for final PCR product extension. DNA was visualized on a 1% agarose gel containing ethidium bromide. The primers of L-type Ca^{2+} channel (L-type channel, forward: 5'-GTTCCTGAAGGAGGTGTGCTGGACG-3', reverse: 5'-AAAGGCAGTTCCCATGCCGG-3'), cardiac Na^+/Ca^{2+} exchanger (NCX1, forward: 5'-CAGCTTCCAAAACTGAAATCGA-3', reverse: 5'-GTCCCTCTCATCGACTTCCAAAA-3'), RyR2 (forward: 5'-GACGGCAGAAGCCACTCACCTGCG-3', reverse: 5'-CCTGCAGAGAAACTGACAACTGG-3'), type 2 IP_3R (IP_3R2, forward: 5'-GGCTCGGTCAATGGCTTC-3', reverse: 5'-CCCCTGTTTCGCCTGCTT-3'), SERCA2a (forward: 5'-TGTGTGATGTGGAGGAAATGTGTA-3', reverse: 5'-TACAACTGAAGGCATGCATTACAA-3'), and house-keeping gene β-tubulin (forward: 5'-GGAACATAGCCGTAAA-CTGC-3', reverse: 5'-TCACTGTGCCT GAACTTACC-3') were used in RNA samples.

Measurement of Ca^{2+} transients Isolated ESCM were loaded with 5 μmol/L Indo-1AM and 0.45% pluronic F-127

(Molecular Probes, Eugene, Oregon, USA) for 10 min at room temperature[17,18]. Loaded cells were washed with a solution containing 140 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 1.0 mmol/L $MgCl_2$, 5.0 mmol/L $NaHCO_3$, 10.0 mmol/L glucose and 10 mmol/L HEPES (pH 7.4 at 35 ºC). Fluorescence signals of Indo-1 were detected by a Fluorescence/ Contractility System (IonOptix, Milton, MA, USA). Fluorescence signals were excited at 360±5 nm with an ultraviolet light source, and the emitted fluorescence was measured at 405 and 480 nm using two photomultipliers attached to an inverted microscope (Olympus, Tokyo, Japan). After subtraction of background fluorescence, the ratio of fluorescence (R) emitted at 405 and 480 nm was recorded[19] and analyzed by IonWizard 4.4 software (IonOptix).

Sodium free solutions were produced by equimolar replacement of Na^+ by Li^+. 2-Aminoethoxydipheylborate (2-APB), thapsigargin (Calbiochem, Darmstadt, Germany), nifedipine and, ryanodine (Sigma, St Louis, MO, USA) were used in our experiments.

Statistics Data are expressed as mean±SEM. Statistical significance of differences in means was estimated by one-way ANOVA, by Student's *t*-test or a paired *t*-test, where appropriate (StatSoft, Version 5.1, StatSoft, Tulsa, OK, USA). $P<0.05$ was considered significant.

Results

Expressions of genes coding main Ca^{2+} handling proteins during cardiac differentiation of ES cells ES cells formed spontaneously contracting cardiomyocytes that were visible 1 d after EB plating during *in vitro* differentiation. The number of EB with spontaneous contracting cardiomyocytes increased significantly and reached maximum on d 5 after plating as was observed in a previous study[17]. Concomitant with the differentiation of ESCM, transcripts of sarcolemmal L-type Ca^{2+} channels and NCX1 increased with the cardiac development; transcripts of SR Ca^{2+} release-related proteins IP_3R2 was expressed very early and increased in early differentiation stages but not in the latter one. RyR2 was present prior to the occurrence of spontaneous beating activity and increased in abundance from early to late differentiation stages. SERCA2a was also present prior to initial contractions but had no obvious changes during ESCM differentiation (Figure 1). Therefore, the main Ca^{2+} handling proteins were already expressed, even at the early differentiation stage.

Contribution of L-type Ca^{2+} channels to Ca^{2+} transients during cardiomyocyte differentiation L-type Ca^{2+} channels are thought to be the main transporter for trans-sarcolemmal Ca^{2+} influx in adult cardiomyocytes[20], and play an important

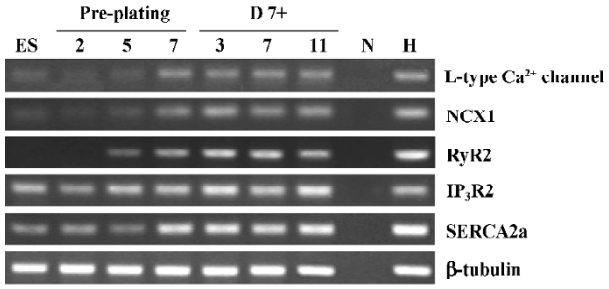

Figure 1. RT-PCR analyses of transcripts for L-type Ca^{2+} channel, cardiac Na^+/Ca^{2+} exchanger (NCX1), type 2 ryanodine receptor (RyR2), type 2 inositol triphosphate receptor (IP_3R2), SERCA2a, and housekeeping gene β-tubulin in ES cells at various differentiated stages. ES, undifferentiated ES cells; 2, 5, and 7, days prior to plating; D 7+3, 7, and 11, days after plating; N, no template negative control; H, adult mouse heart.

role in E-C coupling in early embryonic cardiomyocytes[21,22]. We therefore used an L-type Ca^{2+} channel selective antagonist, nifedipine, to identify the role of L-type Ca^{2+} channels in the regulation of Ca^{2+} transients during cardiomyocyte differentiation. When L-type Ca^{2+} channels were blocked by 3 μmol/L of nifedipine, spontaneous and field-electric stimulated Ca^{2+} transients were totally inhibited in ESCM at three differentiation stages. In order to evaluate the importance of L-type Ca^{2+} channels in the regulation of ESCM Ca^{2+} transients at different differentiation stages, we selected a lower concentration of nifedipine. Spontaneous Ca^{2+} transients in the ESD (n=17) and IDS (n=12) ESCM were completely inhibited by nifedipine at 1 μmol/L (Figure 2A), but were only partially inhibited in some of the LDS ESCM (8 out of 30 cells). Under field-electric stimulation, the Ca^{2+} transients in 24% EDS (n=17) and 8% IDS ESCM (n=12) were totally inhibited by nifedipine at 1 μmol/L and others were partially inhibited. But Ca^{2+} transients in all LDS ESCM examined were only partially inhibited (n=11, Figure 2A and 2B). We then analyzed the inhibitory degree of nifedipine on the cells showing partially inhibited Ca^{2+} transients. The inhibitory effect of nifedipine in EDS and IDS ESCM was obviously stronger than that in LDS cells ($P<0.05$, Figure 2C). These results indicate that the Ca^{2+} influx through the L-type Ca^{2+} channel is essential for Ca^{2+} transients of ESCM at the three differentiation stages, but it is more dominant in the EDS and IDS.

Contribution of SR to Ca^{2+} transients during cardiomyocyte differentiation The contraction in adult cardiomyocytes is highly dependent on the SR Ca^{2+} release from RyR2. To identify whether RyR2 contributes to the upstroke of Ca^{2+} transients during cardiac differentiation, ESCM were treated with ryanodine (10 μmol/L, 30 min) to inhibit RyRs. Ryanodine

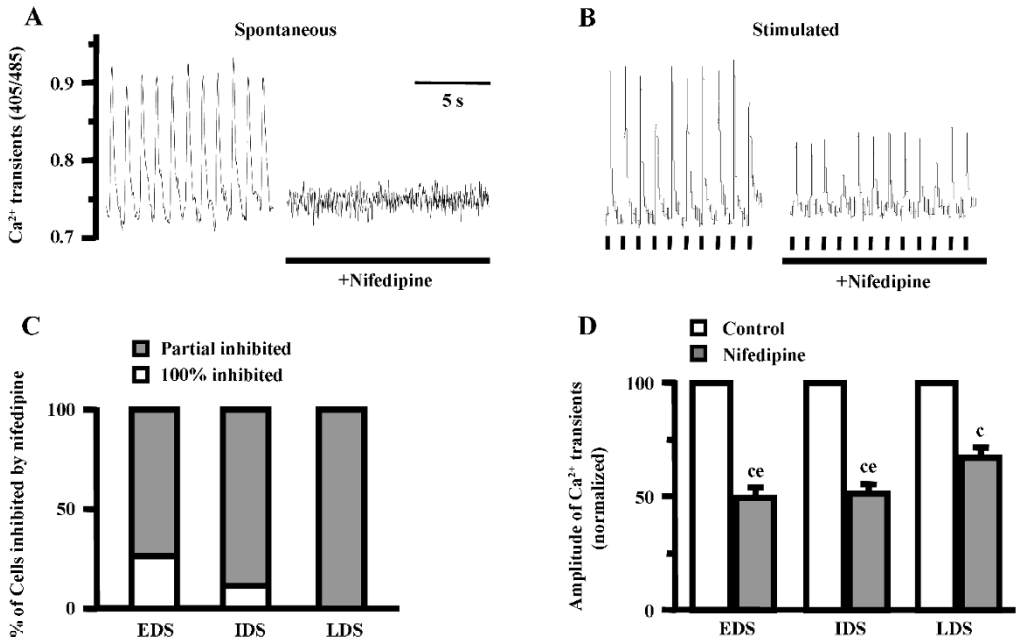

Figure 2. The inhibitory effects of nifedipine on Ca^{2+} transients during ESCM differentiation. A, Representative tracings of spontaneous and stimulated Ca^{2+} transients in IDS cardiomyoctyes before and after nifedipine (1 μmol/L, 5 min) treatment. B, Percentage of cell numbers with (partial inhibited) or without (100% inhibited) Ca^{2+} transients in the field-electric stimulated ESCM after nifedipine treatment. n=17, 12, and 11 of ESCM at early (EDS), intermediate (IDS) and late differentiation stage (LDS), respectively. C, The analysis of the normalized amplitude of Ca^{2+} transients in EDS, IDS, LDS ESCM. ^{c}P<0.01 *vs* corresponding control. ^{e}P<0.05 *vs* LDS.

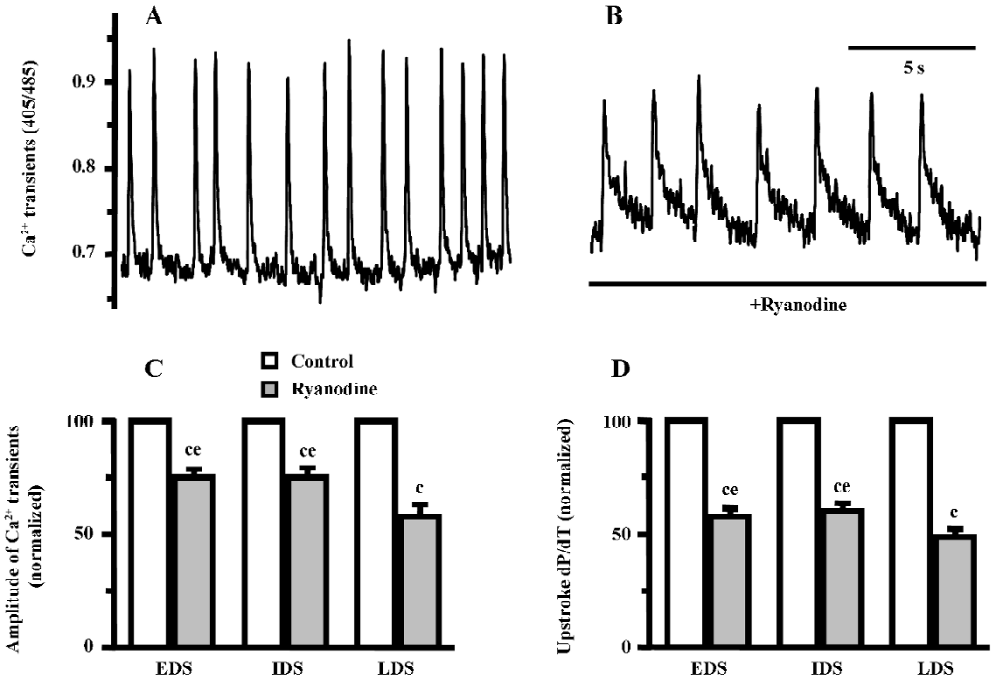

Figure 3. The inhibitory effects of ryanodine (10 μmol/L, 30 min) on Ca^{2+} transients during ESCM differentiation. A, Representative tracings of spontaneous Ca^{2+} transients in IDS cardiomyocytes before and after ryanodine treatment. B, The analysis of the normalized amplitude of Ca^{2+} transients in early (EDS), intermediate (IDS) and late differentiation stage (LDS) ESCM. C, The analysis of the normalized upstroke velocity (dP/dT) of Ca^{2+} transients in EDS, IDS, and LDS ESCM. n=10, 12, and 8 of ESCM at EDS, IDS, LDS, respectively; ^{c}P<0.01 *vs* corresponding control. ^{e}P<0.05 *vs* LDS.

significantly decreased the amplitude and reduced the upstroke velocity (dP/dT) of ESCM Ca^{2+} transients at the three differentiation stages (Figure 3). The inhibitory effects of ryanodine were larger in the LDS ESCM than in the EDS and IDS cells (Figure 3B, 3C). These results indicate that the SR Ca^{2+} release from RyR2 is one of the Ca^{2+} sources of Ca^{2+} transients even in early differentiating ESCM, and the role of RyR2 tends to increase with the development.

Besides RyRs, there is an IP_3 sensitive Ca^{2+} release channel IP_3R2 on the SR membrane of adult cardiomyocytes. We then used 2-APB, an IP_3R inhibitor[23], to investigate the role of IP_3R in the regulation of Ca^{2+} transients in the ESCM. 2-APB (20 μmol/L, 15 min) inhibited Ca^{2+} transients significantly by decreasing the amplitude and reducing the upstroke dP/dT of Ca^{2+} transients of ESCM from the EDS to LDS (Figure 4). The inhibitory effect of 2-APB was larger in EDS than in IDS and LDS ESCM (Figure 4B, 4C). These data demonstrate that IP_3Rs also contribute to the upstroke of Ca^{2+} transients, but this effect decreases with the ESCM differentiation.

To maintain steady-state contraction of cardiomyocytes, the SR released Ca^{2+} should be sequestered by the SR Ca^{2+}-pump ATPase. In our experiment thapsigargin, a Ca^{2+}-pump ATPase inhibitor, was used to detect the role of SR Ca^{2+}-pump ATPase in the regulation of Ca^{2+} transients during cardiac differentiation. Thapsigargin (0.5 μmol/L, 15 min) also significantly inhibited Ca^{2+} transients by decreasing the amplitude and the decay dP/dT of Ca^{2+} transients in differentiating ESCM, but unlike ryanodine and 2-APB, there was no obvious difference in the thapsigargin-induced changes between the EDS and LDS (Figure 5B, 5C). These results indicate that SR Ca^{2+}-pump ATPase functions in the Ca^{2+} reuptake into the SR and contributes to the Ca^{2+} decay of Ca^{2+} transients at three differentiation stages examined.

Some studies suggest that 2-APB is not only an antagonist to inhibit IP_3-induced Ca^{2+} release, but is also an inhibitor of SR Ca^{2+} pump ATPase in non-excitable cells[24]. To further confirm that the observed inhibitory effect of 2-APB is not related to its effect on SR Ca^{2+}-pump ATPase function. The ESCM were treated with a combination of ryanodine with thapsigargin or 2-APB, respectively. Ryanodine-inhibited peak Ca^{2+} transients were reversed by thapsigargin, although the duration of Ca^{2+} transients was still significantly prolonged (Figure 6A). In contrast, ryanodine-inhibited Ca^{2+}

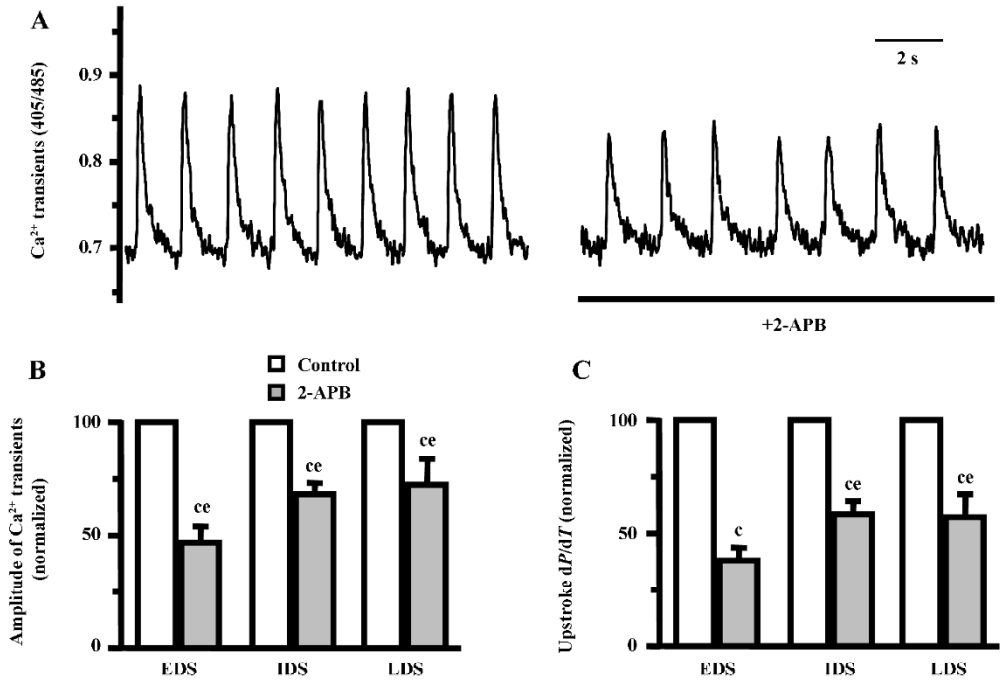

Figure 4. The inhibitory effects of 2-APB (20 μmol/L, 5 min) on Ca^{2+} transients during ESCM differentiation. A, Representative tracings of spontaneous Ca^{2+} transients in IDS cardiomyocytes before and after 2-APB treatment. B, The analysis of the normalized amplitude of Ca^{2+} transients in early (EDS), intermediate (IDS) and late differentiation stage (LDS) ESCM. C, The analysis of the normalized upstroke velocity (dP/dT) of Ca^{2+} transients in EDS, IDS, and LDS ESCM. n=6, 8, and 6 of ESCM at EDS, IDS, LDS, respectively. $^cP<0.01$ *vs* corresponding control. $^eP<0.05$ *vs* EDS.

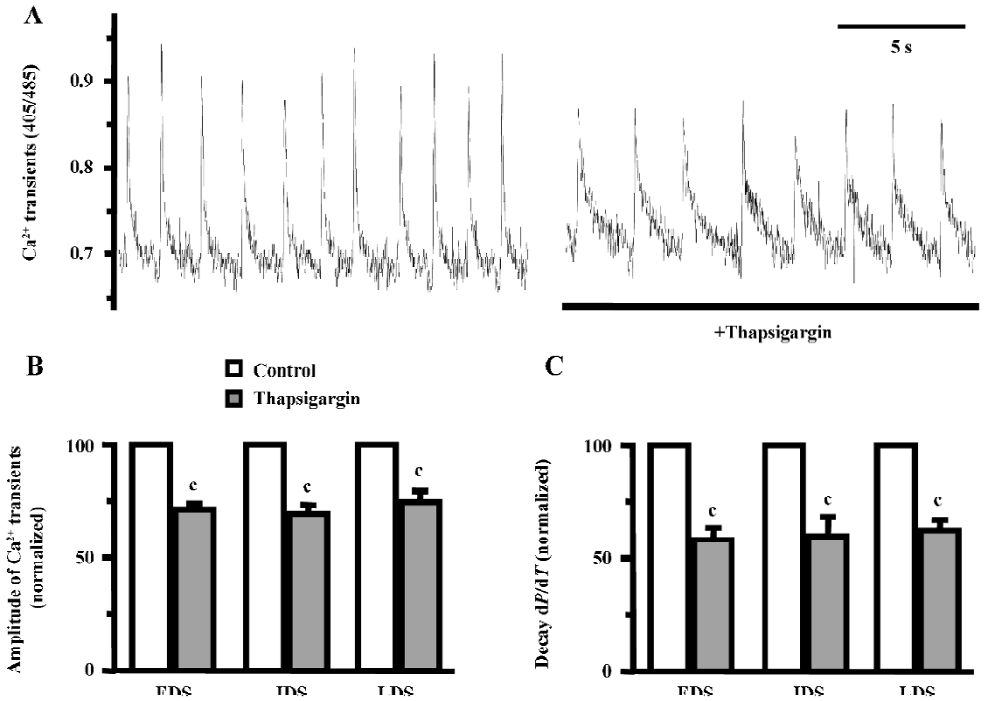

Figure 5. The inhibitory effects of thapsigargin (0.5 μmol/L, 15 min) on Ca^{2+} transients during ESCM differentiation. A, Representative tracings of spontaneous Ca^{2+} transients in IDS cardiomyocytes before and after thapsigargin treatment. B, The analysis of the normalized amplitude of Ca^{2+} transients in early (EDS), intermediate (IDS) and late differentiation stage (LDS) ESCM. C, The analysis of the normalized decay velocity (d*P*/d*T*) of Ca^{2+} transients in EDS, IDS, and LDS ESCM. n=6, 8, and 6 in ESCM at EDS, IDS, LDS, respectively. $^{c}P<0.01$ *vs* corresponding control.

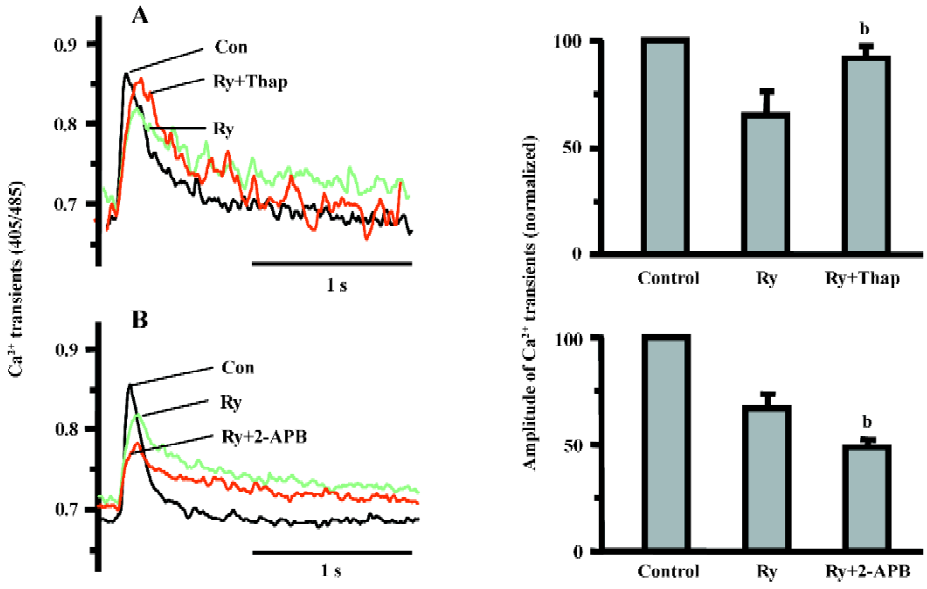

Figure 6. A, Effects of thapsigargin on spontaneous Ca^{2+} transients in ryanodine-treated EDS ESCM (n=4). B, Effects of 2-APB on spontaneous Ca^{2+} transients in ryanodine-treated EDS ESCM (n=5). $^{b}P<0.05$ *vs* Ry.

transients were decreased further by 2-APB (Figure 6B). These observations confirm that the target of 2-APB is not the same as that of thapsigargin.

Contribution of NCX to Ca^{2+} transients during cardiomyocyte differentiation In the normal resting myocytes, the NCX functions in a "forward" (Na^+ in/Ca^{2+} out) mode and serves as the main extrusion mechanism. It is clear that in adult cardiomyocytes NCX is critical in maintaining a low cytosolic $[Ca^{2+}]_i$ and that it extrudes the intracellular Ca^{2+} before the subsequent contraction[5]. To evaluate the role of NCX in the regulation of the cardiac Ca^{2+} transients with differentiation, we used the Na^+ free solution, which inhibits NCX to extrude Ca^{2+} from cytosol[5]. From EDS to LDS, the basal $[Ca^{2+}]_i$ of ESCM increased dramatically when cells were treated with the Na^+ free solution, indicating that NCX is already functional in maintaining the low cytosolic $[Ca^{2+}]_i$ even in early differentiating ESCM (Figure 7A). But Na^+ free solution had no effect on the Ca^{2+} transients in EDS ESCM, while it completely blocked the spontaneous Ca^{2+} transients in the LDS cells (Figure 7A). To evaluate the relative role of NCX in the decay of Ca^{2+} transients, we analyzed the stimulated Ca^{2+} transients of ESCM using field-electric stimulation. The Na^+ free solution did not inhibit the amplitude of Ca^{2+} transients of ESCM until the LDS (Figure 7A, 7B). Moreover, the decay dP/dT of Ca^{2+} transients were significantly increased in EDS cells but reduced in LDS ESCM. These results demonstrated that NCX already regulates basal $[Ca^{2+}]_i$ and the exclusion of cytosolic Ca^{2+} during differentiation of ESCM, but it does not regulate the amplitude of Ca^{2+} transients until the LDS.

Discussion

In this study, we used the ES cell *in vitro* differentiation system to focus on the developing regulation of Ca^{2+} tran-

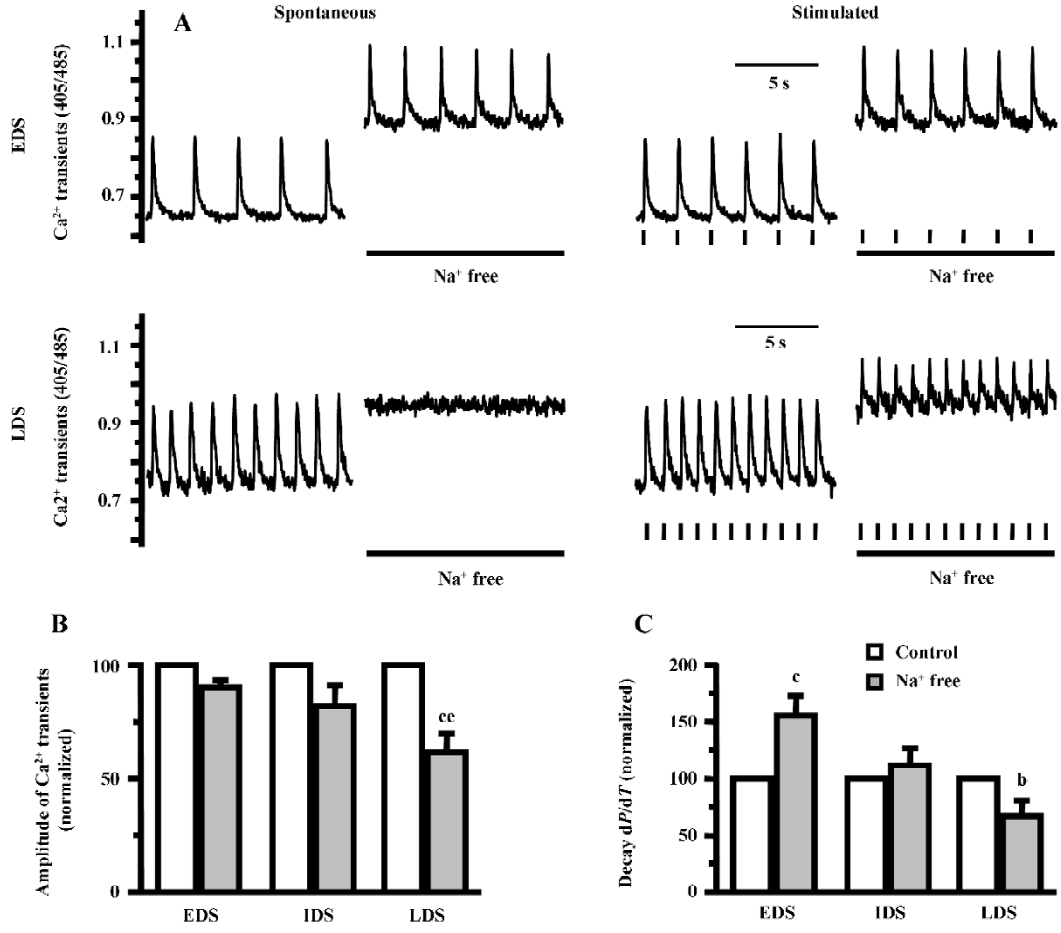

Figure 7. The effects of the Na^+ free solution on Ca^{2+} transients during ESCM differentiation. A, Representative tracings of spontaneous and stimulated Ca^{2+} transients in EDS and LDS cardio-myocytes before and after the Na^+ free solution treatment. B, The analysis of the normalized amplitude of Ca^{2+} transients in EDS, IDS LDS ESCM. C, The analysis of the normalized decay velocity (d*P*/d*T*) of Ca^{2+} transients in EDS, IDS, and LDS ESCM. n=5, 6, and 6 of ESCM at EDS, IDS, LDS, respectively. $^bP<0.05$, $^cP<0.01$ *vs* corresponding control. $^eP<0.05$ *vs* EDS.

sients in ESCM, which until now has not yet been fully clarified. We have demonstrated that: (i) the Ca^{2+} influx through L-type Ca^{2+} channels is essential for Ca^{2+} transients in ESCM at three differentiation stages, but it is more dominating in the earlier stage; (ii) Ca^{2+} release from IP_3Rs also contributes to the amplitude and upstroke of Ca^{2+} transients, but its contribution decreases with the cardiomyocyte differentiation. This temporal change is complementary to the development of RyR2 in the regulation of Ca^{2+} transients during cardiac differentiation; (iii) NCX already functions in EDS in the regulation of basal $[Ca^{2+}]_i$, but it does not regulate the peak Ca^{2+} transients until the LDS.

Ca^{2+} influx in the E-C coupling of the developing cardiomyocytes In adult cardiac myocytes, a relatively small Ca^{2+} influx via voltage-activated L-type Ca^{2+} channels triggers greater amounts of SR Ca^{2+} release from the RyR2 by the process of CICR, which leads to a rapid and high enough increase of $[Ca^{2+}]_i$ to initiate the interaction of contractile filaments and subsequent contractions[5]. For cardiac contraction, an extracellular Ca^{2+}-influx is required because the removal of Ca^{2+} from extracellular solution abolishes cardiac contraction. This phenomenon is also observed in ESCM during differentiation (data not shown). L-type Ca^{2+} channels are thought to be the main transporter for trans-sarcolemmal Ca^{2+} influx in adult cardiomyocytes[20]. In the early stage (3 d) of development, the L-type Ca^{2+} channels occur in embryonic chick-heart cells, and the density of L-type Ca^{2+} current in 3-d cells was higher than in 17-d cells[25]. In 9.5 d postcoitum (dpc) mouse heart[21] and in early-stage ESCM[22], β-adrenergic receptor stimulation already modulates L-type Ca^{2+} channel currents. In this study, nifedipine (>2 μmol/L) blocked the Ca^{2+} transients of ESCM during differentiation. Those results demonstrate that the Ca^{2+} influx via L-type Ca^{2+} channels is required for E-C coupling in early embryonic cardiomyocytes. The decreased inhibitory effects of nifedipine with differentiation, observed in our experiment, can be partially explained by the increased density of Ca^{2+} current in mouse and rat hearts during fetal development[26,27], but it may also indicate that the contribution of the L-type Ca^{2+} channels in the regulation of Ca^{2+} transients decreases with the differentiation upon the development of RyR2.

Importance of SR Ca^{2+} release in the E-C coupling of the developing cardiomyocytes In fetal heart cells the SR is scarce when observed under electron microscopy[28]. Isolated SR vesicles from the fetal heart have a lower volume, lower density, and a lower capability to load Ca^{2+} compared to those isolated from the mature heart[29–31]. Ryanodine, a specific inhibitor of RyR, has little to no effect on Ca^{2+} transients in fetal cells[8,28]. Therefore, it was proposed that the contraction of fetal cardiomyocyte is regulated predominantly by sarcolemmal Ca^{2+} influx rather than Ca^{2+} release from SR. However, RyR2 knockout fetal cardiomyocytes[32] and ESCM[17,18] have slow, weak, and irregular Ca^{2+} transients, which demonstrates that RyR2-released Ca^{2+} is critical for Ca^{2+} homeostasis and normal Ca^{2+} transients in early developmental cardiomyocytes. The observation of the amplitude of Ca^{2+} transients in early differentiating ESCM being inhibited by either ryanodine or thapsigargin is consistent with our recent data[17] and with the findings from the fetal cardiomyocytes[33].

Not like the inhibitory effect of ryanodine or thapsigargin when used alone, ryanodine-inhibited Ca^{2+} transients in ESCM could be reversed by thapsigargin. The latter is in line with the observation that the Ca^{2+} transients of the fetal cardiomyocytes at 9.5 dpc was unaffected by the combined presence of both ryanodine and thapsigargin, while the Ca^{2+} transients in the adult stage are largely inhibited by this combination[8]. Therefore, care should be taken in interpreting the results from using a combination of thapsigargin and ryanodine in these early cardiomyocytes, although we do not know why this combination can reverse the inhibitory effect of ryanodine or thapsigargin when used alone.

IP_3Rs, another Ca^{2+} release channel on the SR membrane, played an important role in the regulation of cellular proliferation and apoptosis, whereas RyR-released Ca^{2+} is required for muscle contraction[34]. The IP_3R mRNA expression and IP_3-induced intracellular Ca^{2+} release are detected as early as 5.5 dpc in the mouse embryo, which is earlier than the time of expression of RyR2 mRNA[35], which is also supported by our in vitro study. In adult guinea pig ventricular myocytes, the low concentrations of IP_3 (1–10 μmol/L) transiently increases isotonic contractions, which is in accordance with the receptor-initiated SR Ca^{2+} release[36]. Recent studies showed that IP_3-dependent shuttle of free Ca^{2+} in and out of the SR is essential for a proper generation of pacemaker activity during early cardiomyogenesis and fetal life[37]. From EDS to LDS, the Ca^{2+} transients of ESCM were inhibited significantly by 2-APB, but its inhibitory effect decreased with development. Although 2-APB is also an inhibitor of SR Ca^{2+}-pump in non-excitable cells[24], the different effects between 2-APB and thapsigargin we observed indicate that IP_3Rs have a critical role in regulation of Ca^{2+} transients in early developing cardiomyocytes. The complementary temporal changes in the molecular and function of RyR2 and IP_3Rs on the regulation of Ca^{2+} transients demonstrates a developmental mechanism of the Ca^{2+} release from SR.

Decay of $[Ca^{2+}]_i$ in the E-C coupling of the developing cardiomyocytes The decay of the Ca^{2+} transients occurs in adult cardiomyocytes because of a reuptake of Ca^{2+} into the SR through the Ca^{2+}-pump ATPase and extrusion of Ca^{2+} from the myocytes by the NCX[20]. The two fundamental principles for maintaining a steady-state contraction of cardiac physiological function are that[5]: (i) a balance exits between the amount of Ca^{2+} entering the cells mainly via the L-type Ca^{2+} channels and the amount of Ca^{2+} extruded via the NCX; and (ii) the amount of Ca^{2+} released by the SR equals that sequestered by the SR Ca^{2+}-pump ATPase. There is dynamic competition among NCX and SERCA2 during relaxation, and the SERCA2 and NCX contribute a variable amount toward $[Ca^{2+}]_i$ decline depending on species, development stages, and physiologic conditions.

The expression of SERCA2 increases at the time when beating cardiomyocytes appear and maintains a stable level during ESCM differentiation. This is consistent with the observation that the inhibitory effect of thapsigargin does not appear significantly different during ESCM differentiation. The NCX expression level increases upon the appearance of cardiomyocytes as observed here and its level in early developed cells is twice that in adult myocytes[8,9,38,39]. This is supported by the experiment with the Na^+ free solution showing that NCX is functional to maintain the low cytosolic $[Ca^{2+}]_i$ even in the early differentiating ESCM. But the Na^+ free solution did not affect the peak Ca^{2+} transients until the LDS. This is consistent with the observation that KB-R 7943, an inhibitor of the reverse mode of NCX, has no effect on the Ca^{2+} transients in the 9.5 dpc fetal cardiomyocytes[8]. Thus, the sarcolemmal Ca^{2+}-ATPase may play a major role in maintaining the balance between the amount of Ca^{2+} entering the cells and the amount of Ca^{2+} extruded at the early developmental period.

As discussed above, the dynamic changes in the regulation of Ca^{2+} transients occur during the cardiac development, and the sources of Ca^{2+} for producing contraction is altered during development. The failing heart has an altered program of gene expression with embryonic characteristics[40,41]. Moreover, transplantation of exogenous cells into injured myocardium, such as fetal cardiomyocytes[42], bone marrow cells[43] and ESCM[44], has emerged for regeneration of damaged myocardium and for improvement of cardiac function in post-infarcted hearts in recent years. Therefore, it is significant to further investigate the establishment of the E-C coupling and the regulation of Ca^{2+} homeostasis during development, which is important for a better understanding of normal development aspects and abnormalities in cardiac diseases.

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