Characteristics of calcium sparks in cardiomyocytes derived from embryonic stem cells

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Characteristics of calcium sparks in cardiomyocytes derived from embryonic stem cells. Am J Physiol Heart Circ Physiol 281: H411–H421, 2001.—In embryonic stem (ES) cell-derived cardiomyocytes, spontaneous calcium sparks representing calcium release through ryanodine receptor (RyR) channels were characterized and correlated to the expression of RyRs as well as the calcium load of the sarcoplasmic reticulum (SR). In very early developmental stage (VEDS) cardiomyocytes, global intracellular calcium concentration ([Ca2+],) fluctuations occurred, whereas calcium sparks and contractions were absent. In early developmental stages (EDS), contractions as well as calcium sparks were obvious. During the further differentiation to late developmental stage (LDS) cardiomyocytes, a marked increase in the frequency of global [Ca2+], transient, the amplitude and the frequency of calcium sparks, as well as the expression of RyRs and the volume of RyR-positive SR, was observed. Furthermore, the caffeine-releasable SR calcium load was elevated in LDS compared with EDS cardiomyocytes. A high calcium (Ca2+) solution raised spark frequency as well as amplitude in EDS cardiomyocytes differentiated from embryonic stem (ES) cells were used. These cardiomyocytes have been applied to characterize the time course of expression of voltage-dependent calcium channels as well as the underlying signaling pathways and have been proven as a versatile tool to study the developmental aspects of cardiomyogenesis (17, 19, 22, 23).

We report for the first time on the occurrence of calcium sparks during the process of differentiation of cardiomyocytes from nonbeating cardiac precursor cells to terminally differentiated cardiomyocytes. We also demonstrate that the characteristics of calcium sparks in developing cardiomyocytes is related to the filling state of the SR with calcium as well as to the volume of the SR and the amount of RyRs expressed during different stages of cardiomyocyte differentiation.

MATERIALS AND METHODS

Spinner flask culture technique for cultivation of embryoid bodies. The ES cell line CCE (27) was grown on mitotically inactivated feeder layers of primary murine embryonic fibroblasts for a maximum of eight passages in Iscove’s medium (GIBCO Life Technologies) supplemented with 20% heat-inactivated (56°C, 30 min) fetal calf serum (GIBCO), 2 mM Glutamax (GIBCO), 100 μM 2-mercaptoethanol (Sigma; Deisenhofen, Germany), 1% minimal essential medium nonessential amino acid stock solution (GIBCO), 100 IU/ml penicillin, and 100 μg/ml streptomycin (GIBCO) in a humidified environment containing 5% CO2 at 37°C and passaged every 2 to 3 days. At day 0 of differentiation, adherent cells were enzymatically dissociated with the use of 0.2% trypsin and 0.05% EDTA in phosphate-buffered saline (PBS) (GIBCO).
and seeded at a density of 1·10⁷ cells/ml in 250 ml of siliconized spinner flasks (Integra Biosciences; Gernwald, Germany) containing 125 ml of Iscove’s medium supplemented with the same additives as described above. After 24 h, 125 ml of medium were added for a final volume of 250 ml. The spinner flask medium was stirred at 20 rpm with the use of a stirrer system (Cell Spin, Integra Biosciences) and was partly (125 ml) changed every day.

Isolation procedure of cardiomyocytes. Whole 5- to 7-day-old embryoid bodies were enzymatically dissociated to obtain very early developmental stage (VEDS) cardiac precursor cells. To obtain early developmental stage (EDS) and late developmental stage (LDS) cardiomyocytes, embryoid bodies were removed from the spinner flasks on day 6 of cell culture and plated to 10-cm tissue culture petri dishes (Falcon, Becton-Dickinson; Franklin Lakes, NJ). During the subsequent days, an increasing number embryoid bodies displayed spontaneous contractions, indicating cardiomyocyte differentiation. Single cardiomyocytes were isolated from clusters of spontaneously contracting cells by a modified procedure of Isenberg and Klockner (14). Beating areas were excised with a sterile microscalpel and collected in low-Ca²⁺ solution containing (in mM) 120 NaCl, 5.4 KCl, 5 MgSO₄, 5 sodium pyruvate, 20 glucose, 20 taurine, and 20 HEPES (pH 6.9 with NaOH). The tissue was then incubated in enzyme medium (30 μM CaCl₂; 1 mg/ml collagenase B; Boehringer-Mannheim; Mannheim, Germany) for 20 min at 37°C. Tissue fragments were transferred into a medium containing (in mM) 85 KCl, 30 K₂HPO₄, 5 MgSO₄, 1 EGTA, 2 Na₂ATP, 5 Na pyruvate, 5 creatine, 20 taurine, and 20 glucose, pH 7.2, where they were kept at room temperature for 1 h and then resuspended in Iscove’s medium. Isolated cells were plated on sterile coverslips (Celllocate, Eppendorf; Hamburg, Germany) and kept in the incubator for 24–48 h. Spontaneously contracting myocytes were observed within 12 h after cell preparation. Because VEDS cardiac precursor cells could not be identified by spontaneous contractions, the cells were immunostained after the Ca²⁺ measurements with an antibody directed against sarcomeric α-actinin.

Ca²⁺ imaging and confocal laser scanning microscopy. Isolated cardiomyocytes on coverslips were loaded for 15 min at 37°C with 10 μM fluo 3-acetoxymethyl ester dissolved in dimethyl sulfoxide (final concentration 0.1%) and pluronic F-127 (Molecular Probes) (final concentration <0.025%). After the cells were loaded, they were washed in E1 buffer containing (in mM) 135 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4 at 37°C). Fluo 3 fluorescence imaging was performed on a laser scanning confocal microscope (model LSM 410, Zeiss; Jena, Germany) equipped with an argon ion laser and coupled to an inverted microscope (Axiovert 135, Zeiss). The objective lens was a Zeiss ×25 oil immersion Plan-Neofluar with a numerical aperture of 0.8. Fluo 3 fluorescence was excited with the use of the 488-nm line of the argon laser. The laser excitation beam was directed to the specimen through a 510-nm dichroic beam splitter, and the emitted fluorescence was collected through a 515-nm long-pass emission filter in front of a photomultiplier tube. For Ca²⁺ spark recording and quantitative analysis, the line-scan mode of the confocal laser-scanning microscope was used. Single myocytes were scanned repetitively (250 Hz) along a line positioned along the longitudinal axis of the cell avoiding the nuclei. The axial cell depth amounted to ~2 μm. A line-scan image was constructed by stacking all 512 lines vertically. The magnification was set by the objective and the hardware zoom factor of the laser scanning microscope to give a pixel size of ~0.1 μm². The volume of each pixel (voxel) in the line was ~0.1 μm³.

Spark detection was performed using an automated spark-detection algorithm based on a program recently developed by Cheng et al. (7) coded in the image-processing language IDL (Research Systems; Boulder, CO). To keep bias to a minimum, all steps in the analysis were performed automatically. Human intervention was requested only to confirm the initial parameters set up by the algorithm and the results presented at the end of the analysis. The cell edges were detected by the analysis routine. Potential spark regions that exceeded the overall standard deviation of the fluorescence baseline were excised to calculate a corrected baseline containing background noise only. The image was then normalized with the use of the corrected fluorescence baseline. As detailed by Cheng et al. (7), potential spark regions were detected as connected regions that exceeded a fluorescence threshold given by the sum of the cell fluorescence baseline and its standard deviation multiplied with a given constant. Only those spark sites that exceeded the normalized fluorescence intensity by 3.2 standard deviations were accepted. These locations were analyzed further to derive spatial and kinetic data from every given spark site, i.e., the spark amplitude, the half-time of rise and decay, and the full width at half maximum, i.e., the spatial spark diameter.

Calibration of [Ca²⁺], was performed as described previously (18). In brief, cardiac cells were superfused with E1 solution supplemented with the Ca²⁺ ionophore ionomycin (Sigma), which results in maximum Ca²⁺ saturation of the

![Fig. 1. Time course of cardiomyogenesis in embryoid bodies cultivated with the spinner flask technique. A: percentage of spontaneously beating embryoid bodies after plating. Embryoid bodies were cultivated for 6 days in spinner flasks and subsequently plated to cover slips. Number of beating embryoid bodies was evaluated by transmission light microscopy. B: beating frequency in relation to the age of the embryoid bodies.](http://ajpheart.physiology.org/DownloadedFrom/original 걸러내기)
fluorescence dye. The cells were subsequently superfused with a Ca^{2+}-free E1 solution containing 2 mM of Mn^{2+}, which brings fluo 3 to fluorescences (F) < 20% of that of the Ca^{2+} saturated dye, i.e., F_{\text{Mn}} = 0.2 - F_{\text{max}}. At the end of the experiment, the perfusate contained 0.1% Triton X-100 (Sigma), which resulted in the release of the fluorescence dye into the superfusate and permitted recording of the background signal F_{\text{bkg}}. Because F_{\text{Mn}} and F_{\text{bkg}} represent the fluorescence signals with ionomycin-Mn^{2+} before and after lysis, the maximum fluorescence F_{\text{max}} from the Ca^{2+} saturated dye can be calculated to (F_{\text{Mn}} - F_{\text{bkg}})/0.2 + F_{\text{bkg}}. Metal-free fluo 3 has 1/40 the fluorescence of the Ca^{2+} complex. Hence, the fluorescence signal F_{\text{min}} from the cellular dye is (F_{\text{max}} - F_{\text{bkg}})/40 + F_{\text{bkg}}. Cytosolic Ca^{2+} can then be estimated by the equation \[ [\text{Ca}^{2+}] = K_d (r - F_{\text{min}})/ (F_{\text{max}} - F) \], where \( K_d \) is 1,100 \( \mu \text{M} \) at intracellular ionic strength (12). Because both F_{\text{max}} and F_{\text{min}} can be expressed in terms of F_{\text{Mn}}, the only parameters that must be determined experimentally are F_{\text{Mn}} and F_{\text{bkg}}.

**Fluorescence staining of RyRs.** RyRs in cardiac cells were stained by use of the fluorescent ryanodine derivative BODIPY FL-X (Molecular Probes; Eugene, OR). Briefly, cardiac cells isolated from embryoid bodies were fixed for 30 min in methanol-acetone (7:3), permeabilized in PBS supplemented with 0.1% Triton X-100 (Sigma), and incubated for 20 min with 0.2 \( \mu \text{M} \) BODIPY FL-X ryanodine dissolved in dimethyl sulfoxide. The cells were subsequently washed three times in PBS, and the BODIPY fluorescence was analyzed by confocal laser scanning microscopy and the 488-nm line of the argon-ion laser. Emission was recorded using a long-pass 515-nm filter set.

**Immunohistochemistry.** Cardiac cells were identified by immunolabeling sarcomeric \( \alpha \)-actinin. Cardiac cells on coverslips were fixed in ice-cold methanol-acetone (7:3) for 30 min and were subsequently permeabilized with PBS supplemented with 0.1% Triton X-100. Blocking against unspecific staining was performed by incubation for 1 h in PBS containing 1% milk powder. For \( \alpha \)-actinin staining, a mouse monoclonal antibody (Sigma) was used in a concentration of 20 \( \mu \text{g}/\text{ml} \). The secondary antibody was a Cy3-labeled goat anti-mouse antibody (Dianova; Hamburg, Germany) in a concentration of 6.5 \( \mu \text{g}/\text{ml} \). Excitation was performed by use of a 543-nm He-Ne laser of the confocal setup. Emission was recorded with the use of a 575- to 640-nm band-pass filter set. The RyR was labeled by using a goat polyclonal antibody directed against the amino terminus of RyR (Santa Cruz Biotechnology; Santa Cruz, CA) and applied in a concentration of 4 \( \mu \text{g}/\text{ml} \). As a secondary antibody, a Cy5-labeled donkey-anti-goat IgG (Dianova) was used in a concentration of 5 \( \mu \text{g}/\text{ml} \) and was excited by a 633-nm He-Ne laser of the confocal setup. Emission was recorded with the use of a long-pass 650-nm filter set.

**Statistical analysis.** Data are given as means ± SE, with \( n \) denoting the number of experiments with either cardiac cells or embryoid bodies derived from at least three independent preparations. Student's t-test for unpaired data was applied as appropriate. A value of \( P < 0.05 \) was considered significant.

**RESULTS**

**Time course of cardiac differentiation of ES cells.** ES cells of the cell line CCE were cultivated in spinner flask culture to the three-dimensional multicellular tissue of embryoid bodies. Within the embryoid bodies, cardiac precursor cells (5- to 7-day-old), EDS (8- to 11-day-old), and LDS cardiomyocytes (15- to 25-day-old) were differentiated. Spontaneous contractions were not visible in VEDS embryoid bodies but started 1 day after plating (day 7). The number of contracting embryoid bodies increased during subsequent days and
reached a maximum of 83 ± 4% on day 5 after plating \((n = 7)\) (Fig. 1A). In parallel to the increase in spontaneously contracting embryoid bodies, a marked increase in the frequency of contractions from 60 contractions per minute on day 3 after plating to 80 ± 2 contractions per minute on days 9–10 after plating was observed during the time course of cardiomyocyte differentiation \((n = 7)\) (Fig. 1B).

During the time course of cardiac muscle development, far-reaching changes in the morphology of the cardiac cells were observed as evaluated by immunostaining of sarcomeric \(\alpha\)-actinin (Fig. 2, A–C). In clusters of VEDS cardiac precursor cells, sarcomeric organization was totally absent, although positive immunostaining for \(\alpha\)-actinin was evident predominantly in the near membrane region of the cells \((n = 3)\) (see Fig. 2A). In clusters of EDS cardiomyocytes, the beating areas of cardiomyocytes showed distinct Z bands; however, the orientation of the myofibrils was random \((n = 3)\) (see Fig. 2B). In clusters of LDS cardiomyocytes, a strandlike parallel organization of the myofibrils was observed, which resembled the myofibril organization in the adult cardiac muscle \((n = 3)\) (see Fig. 2C).

\(\lbrack Ca^{2+}\rbrack_i\) transients in cardiomyocytes differentiated from ES cells. It has been shown (32) that EDS cardiomyocytes derived from the D3 ES cell line \((\text{days 8–9})\) were characterized by small fluctuations of \(\lbrack Ca^{2+}\rbrack_i\) from intracellular stores, which were independent from the membrane potential as well as the activity of voltage-dependent Ca\(^{2+}\) channels. In the present study, \(\lbrack Ca^{2+}\rbrack_i\) oscillations of cardiomyocytes were recorded at different stages of cardiomyocyte differentiation, i.e., in 5- to 7-day-old VEDS cardiac precursor cells, in which spontaneous contractions were absent, as well as in spontaneously contracting EDS \((\text{days 8–11})\) and LDS \((\text{days 15–25})\) cardiomyocytes. As shown in Fig. 3, A–D, slow \(\lbrack Ca^{2+}\rbrack_i\) fluctuations with largely variable frequency and sometimes irregular shape occurred in cardiac precursor cells \((n = 10)\). Incubation with 10 mM of caffeine resulted in a transient rise in \(\lbrack Ca^{2+}\rbrack_i\), indicating the presence of functional RyRs. Furthermore, superfusion of VEDS cardiac precursor cells with 140 mM K\(^+\) resulted in an...

![Fig. 3](http://ajpheart.physiology.org/)

Fig. 3. Spontaneous intracellular calcium concentration \((\lbrack Ca^{2+}\rbrack_i)\) transients in cardiomyocytes differentiated from embryonic stem (ES) cells. A–D: \(\lbrack Ca^{2+}\rbrack_i\) transients of VEDS cardiac cells. Tracings were recorded from different cells within the same cell cluster. \(Ca^{2+}\) responses displayed large differences in amplitude and frequency, indicating the absence of intercellular coupling; E: presence of caffeine-sensitive \(Ca^{2+}\) stores and voltage-dependent \(Ca^{2+}\) influx pathways in VEDS cardiomyocytes. VEDS cardiomyocytes were superfused with solution containing 10 mM of caffeine, which led to transient increase in \(\lbrack Ca^{2+}\rbrack_i\). Cells were then superfused with high \((140\, \text{mM})\) K\(^+\) solution, which resulted in an elevation of \(\lbrack Ca^{2+}\rbrack_i\), indicating the presence of voltage-dependent \(Ca^{2+}\) influx pathways. Tracing was recorded from a cell cluster different from the cluster displayed in A–D. F: \(\lbrack Ca^{2+}\rbrack_i\) transients in a 10-day-old early developmental stage (EDS) cardiac cell, which displayed spontaneous contractions at a frequency of 66 beats/min.
increase of \([\text{Ca}^{2+}]_i\), indicating the presence of voltage-activated \(\text{Ca}^{2+}\) channels (see Fig. 3E). Interestingly, no propagation of the \(\text{Ca}^{2+}\) responses between different cardiac precursor cells organized in cardiac cell clusters was observed, indicating an absence of intercellular coupling. In EDS and LDS cardiomyocytes, the frequency of \([\text{Ca}^{2+}]_i\) oscillations increased to 40–70 per minute (see Fig. 3F), which correlated to the frequency of contractions. In cell clusters of these cardiac cells propagating \([\text{Ca}^{2+}]_i\) waves were observed, indicating intercellular coupling presumably via gap junctions (data not shown).

**Ca\(^{2+}\) \text{sparks during the time course of cardiomyocyte differentiation.}** Investigations on the occurrence of \(\text{Ca}^{2+}\) sparks in embryonic cardiomyocytes have not yet been undertaken. In adult cardiomyocytes, E-C coupling is mediated by the release of \(\text{Ca}^{2+}\) from the SR via RyRs. It is generally accepted that local \([\text{Ca}^{2+}]_i\) transients, i.e., sparks are triggered by depolarization-induced \(\text{Ca}^{2+}\) entry through L-type \(\text{Ca}^{2+}\) channels. The RyRs are thought to be situated very close to the L-type \(\text{Ca}^{2+}\) channel, where they sense an increase in local \([\text{Ca}^{2+}]_i\) when a nearby \(\text{Ca}^{2+}\) channel opens (33).

To investigate the occurrence of \(\text{Ca}^{2+}\) sparks during the time course of cardiomyocyte differentiation, confocal line-scan recordings were performed in VEDS, EDS, and LDS cardiomyocytes (Figs. 4–6). These spontaneous sparks occurred after the decline of the global \([\text{Ca}^{2+}]_i\) transients to baseline \([\text{Ca}^{2+}]_i\). Representative line-scan images are presented in Fig. 4. The tracings of the fluo 3 fluorescence changes along the scanned line in a series of consecutive line-scan images (see Fig. 5) revealed that no \(\text{Ca}^{2+}\) sparks occurred in VEDS cardiac precursor cells (days 5–7) \((n = 30)\) (see Fig. 5A) despite the presence of functional caffeine-sensitive \(\text{Ca}^{2+}\) stores (see Fig. 3E). During further differentiation, more cardiac cells displayed typical \(\text{Ca}^{2+}\) sparks, i.e., 9% in 8-day-old EDS cardiomyocytes \((n = 49)\), 51% in 9- to 10-day-old EDS cardiomyocytes \((n = 154)\), and 74% in 15- to 25-day-old cardiomyocytes \((n = 177)\). In EDS cardiomyocytes, spontaneous \(\text{Ca}^{2+}\) sparks occurred with a frequency of 4 ± 0.4 s and were characterized by an amplitude \((F/F_0)\) of 1.5 ± 0.04 (Fig. 6, A and B) corresponding to an \([\text{Ca}^{2+}]_i\) of ~145 nM. In LDS cardiomyocytes (days 15–25) a significant increase of the frequency as well as the amplitude \((F/F_0)\) of the sparks to 6.8 ± 0.7 ls\(^{-1}\) and 1.8 ± 0.1, respectively, corresponding to ~194 nM \([\text{Ca}^{2+}]_i\), was observed \((n = 20)\) (see Fig. 6, A and B). The spatial diameter of sparks tended to higher values in LDS compared with EDS.

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**Fig. 4.** Representative false color line-scan images of nonstimulated VEDS cardiac precursor cells (A), EDS (B), and late developmental state (LDS; C) cardiomyocytes differentiated from ES cells. Line-scan images were recorded after spontaneous global \([\text{Ca}^{2+}]_i\) transients had declined to baseline values. Each image includes 512 lines scanned at 250 Hz. Time is running from top to bottom. Localized \(\text{Ca}^{2+}\) sparks were absent in noncontracting VEDS cardiac precursor cells, whereas typical, spatially restricted sparks were observed in contracting EDS and LDS cardiomyocytes. Arrowheads indicate from where in the images the tracings of Fig. 5 were recorded. Vertical bar, 0.2 s. Horizontal bar, 5 μm.
cardiomyocytes with 3.54 ± 0.4 and 2.7 ± 0.3 μm (n = 20), respectively, which, however, did not reach statistical significance (see Fig. 6C). The same held true for the half-time to rise, which amounted to 15.4 ± 2.8 and 20 ± 3.3 ms in EDS and LDS cardiomyocytes, respectively, as well as for the half-time to decay, which amounted to 27.9 ± 4.8 and 37.6 ± 6.3 ms, respectively.

Expression of RyRs during cardiomyocyte differentiation. The observed changes in the characteristics of Ca\textsuperscript{2+} sparks in EDS and LDS cardiomyocytes may be owing to differences in the expression of L-type Ca\textsuperscript{2+} channels and RyRs, as well as the Ca\textsuperscript{2+} load of the SR.

A threefold increase in the voltage-dependent Ca\textsuperscript{2+} current density during the time course of cardiomyocyte differentiation of ES cells has been reported (19). To evaluate the expression of RyRs, beating areas of cardiomyocytes in whole mount embryoid bodies were labeled with BODIPY FL-X ryanodine, which has been recently used to localize RyRs in the rat parotid gland (35) and in pancreatic β-cells (11). Furthermore, RyR expression was evaluated in single cell preparations by a polyclonal anti-RyR antibody and immunohistochemical methods (Fig. 7). BODIPY FL-X ryanodine staining revealed that RyRs were expressed in EDS as well as in LDS cardiomyocytes. However, the level of expression of RyRs as evaluated by quantification of BODIPY FL-X ryanodine fluorescence was significantly increased by 53 ± 3% in LDS cardiomyocytes compared with EDS cardiomyocytes (n = 3). Immunohistochemistry of the RyR in single VEDS, EDS, and LDS cardiomyocytes revealed a continuous increase in RyR immunofluorescence intensity in LDS cardiomyocytes compared with VEDS and EDS cardiomyocytes, indicating an increasing density of RyRs during cardiomyocyte differentiation (n = 3). Furthermore, a significant increase in the volume of the SR positive for RyRs was observed during cardiac cell maturation (see
In VEDS and EDS cardiomyocytes, the most pronounced staining was observed in the near-nuclear region. In LDS cardiomyocytes, the cell compartment displaying SR positive for RyR-immunofluorescence was significantly increased compared with VEDS and EDS cardiomyocytes and occupied nearly the whole cytoplasmic compartment.

**Ca**\textsuperscript{2+} load of the SR in EDS and LDS cardiomyocytes. The observed differences in the frequency and amplitude of Ca\textsuperscript{2+} sparks in EDS and LDS cardiomyocytes may be related to differences in the Ca\textsuperscript{2+} load of the SR and/or differences in cytoplasmic [Ca\textsuperscript{2+}]. To evaluate these issues, calibration measurements for [Ca\textsuperscript{2+}] were performed, and Ca\textsuperscript{2+} was released from intracellular stores by superfusion with 10 mM of caffeine (Fig. 8, A and B). It was observed that basal [Ca\textsuperscript{2+}], was not significantly different in EDS and LDS cardiomyocytes with 92.8 ± 9.5 nM \((n = 22)\) and 99.8 ± 4.3 nM \((n = 18)\), respectively. Superfusion with solutions containing 10 mM of caffeine resulted in a transient increase in [Ca\textsuperscript{2+}]. The amplitude of the [Ca\textsuperscript{2+}] transient was significantly increased in LDS cardiomyocytes and amounted to 2,120 ± 180 nM \((n = 49)\), whereas in EDS cardiomyocytes a peak [Ca\textsuperscript{2+}] of 780 ± 30 nM \((n = 63)\) was observed, which clearly indicates an elevated Ca\textsuperscript{2+} content of the SR in LDS compared with EDS cardiomyocytes. However, the reduced amplitude of the caffeine-induced Ca\textsuperscript{2+} response in EDS cardiomyocytes may result from the lower number and density of RyRs in EDS compared with LDS cardiomyocytes. To exclude this possibility, EDS cardiomyocytes were preincubated before caffeine application in solution containing 10 mM of Ca\textsuperscript{2+}. This experimental protocol resulted in a significant increase in the peak [Ca\textsuperscript{2+}], to 1,990 ± 315 nM, which was not significantly different from the values obtained in LDS cardiomyocytes \((n = 54)\) (Fig. 8B, inset). Interestingly, [Ca\textsuperscript{2+}], spiking was restored in ~80% of EDS cardiomyocytes after the decline of the caffeine-

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**Fig. 7.** Expression of ryanodine receptor channels (RyRs) in VEDS (A), EDS (B), and LDS (C) cardiac cells differentiated from ES cells as evaluated by RyR immunofluorescence using a polyclonal antibody directed against the RyR. Top: RyR immunofluorescence; the color code bar represents RyR immunofluorescence intensity. Middle: α-actinin staining by the use of a monoclonal anti-α-actinin antibody. Bottom: overlay images of RyR immunofluorescence (yellow-red) and α-actinin immunofluorescence (green). RyR immunofluorescence, as well as the extension of the SR positive for RyRs, is increasing during the course of cardiomyocyte differentiation. Bar represents 10 μm.
CA\(^{2+}\) SPARKS IN ES CELL-DERIVED CARDIOMYOCYTES

Fig. 8. Ca\(^{2+}\) load of the SR in EDS (A) and LDS (B) cardiomyocytes. Fluo 3-loaded cardiomyocytes were superfused with 10 mM caffeine (arrows), which released Ca\(^{2+}\) from the SR, resulting in a transient increase in [Ca\(^{2+}\)]\(_{i}\). B, inset: absolute peak [Ca\(^{2+}\)]\(_{i}\) values after caffeine treatment of EDS and LDS cardiomyocytes under control extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{o}\)) conditions as well as of EDS cardiomyocytes preincubated for 5 min in solution containing 10 mM Ca\(^{2+}\) before the addition of caffeine. Note that [Ca\(^{2+}\)]\(_{i}\) spiking continued in EDS cardiomyocytes in the continuous presence of caffeine, whereas spiking was absent in LDS cardiomyocytes. *P < 0.05, significantly different from untreated EDS cardiomyocytes.

induced [Ca\(^{2+}\)]\(_{i}\) transient to basal [Ca\(^{2+}\)]\(_{i}\), despite the continuous presence of caffeine, whereas in the majority of LDS cardiomyocytes [Ca\(^{2+}\)]\(_{i}\) spiking was abolished.

To further investigate the role of the filling state of the SR on the characteristics of Ca\(^{2+}\) sparks, EDS cardiomyocytes were superfused with E1 buffer containing 10 mM Ca\(^{2+}\), which should augment the SR Ca\(^{2+}\) load (Figs. 9 and 10) (28). This treatment resulted in an elevation of basal [Ca\(^{2+}\)]\(_{i}\) as well as an increase of the frequency of Ca\(^{2+}\) spiking from 32 ± 1 to 37 ± 1 per second (n = 14) (see Fig. 9A). An evaluation of the characteristics of Ca\(^{2+}\) sparks revealed a significant increase of the spark frequency from 4 ± 0.4 to 7.5 ± 1 s\(^{-1}\), and a significant increase in the spark amplitude F/F\(_0\) from 1.5 ± 0.04 to 1.8 ± 0.1 (see Fig. 9, B and C, and Fig. 10), which was not significantly different from the values for the spark frequency and amplitude obtained in LDS cardiomyocytes. Furthermore, the half-time to rise was significantly increased to 33.7 ± 8 ms on incubation in high extracellular [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_{o}\)) conditions, whereas no change in the half-time to decay and the spatial spark diameter was observed (data not shown) (n = 12). In LDS cardiomyocytes superfusion with E1 buffer containing 10 mM Ca\(^{2+}\) did not significantly alter spark characteristics compared with the control. Under high [Ca\(^{2+}\)]\(_{i}\) conditions, the spark frequency, the spark amplitude F/F\(_0\), the spatial spark diameter as well as the half-time to rise and to decay amounted to 8.6 ± 0.9 s\(^{-1}\), 1.7 ± 0.1 and 3.4 ± 0.6 \(\mu\)m, and 22.2 ± 4.6 and 42.3 ± 7.5 ms, respectively (n = 12).

**DISCUSSION**

The present study reports for the first time on events of elementary Ca\(^{2+}\) release in cardiomyocytes differentiated from pluripotent ES cells. This in vitro system allows the investigation of early steps of cardiomyogenesis. Our data demonstrate that Ca\(^{2+}\) sparks displaying comparable characteristics with sparks investigated in cardiac cells of different mammalian species (4, 20, 21, 28, 34) occurred in EDS and LDS cardiomyocytes differentiated from murine ES cells. During the time course of cardiomyogenesis, a prominent increase in the number of cells displaying typical spatially restricted Ca\(^{2+}\) sparks was observed.

**Absence of Ca\(^{2+}\) sparks in VEDS cardiac precursor cells.** Sparks were totally absent in VEDS cardiac precursor cells despite the obvious presence of voltage-dependent Ca\(^{2+}\) channels and the presence of caffeine-sensitive Ca\(^{2+}\) stores. These cardiac precursor cells did not show any signs of spontaneous contractions under light microscopy inspection. In clusters of cardiac precursor cells, global [Ca\(^{2+}\)]\(_{i}\) fluctuations with large variations in amplitude and frequency occurred which may indicate that cells of different developmental stages are present in close association. The [Ca\(^{2+}\)]\(_{i}\) fluctuations in cardiac precursor cells may be driven by the d-myoinositol 1,4,5-trisphosphate [Ins(1,4,5)\(_{3}\)] pathway and/or mitochondria, because they have been previously shown to persist in high-K\(^{+}\) solution and in the presence of blockers of voltage-dependent Ca\(^{2+}\) channels (32). However, we never observed Ca\(^{2+}\) puffs and blibs, which have been previously associated to Ca\(^{2+}\) release via Ins(1,4,5)\(_{3}\)-sensitive Ca\(^{2+}\) stores (3). The presence of Ins(1,4,5)\(_{3}\)-sensitive Ca\(^{2+}\) stores in ES cells has been recently demonstrated (19) and is likewise evident in adult cardiomyocytes, where Ins(1,4,5)\(_{3}\) plays a role in the regulation of cardiac autonomic [Ca\(^{2+}\)]\(_{i}\), spiking (15).

**Occurrence of Ca\(^{2+}\) sparks at low frequency and amplitude in EDS cardiomyocytes.** Typical Ca\(^{2+}\) sparks that occurred at low frequency were obvious in EDS cardiomyocytes, which contracted visibly as evaluated by transmission light microscopy. In beating areas of whole mount EDS embryoid bodies displaying spontaneous contractions, immunohistochemical staining of α-actinin showed a distinct sarcomeric organization, although a random organization of the myofibrils prevailed. With prolonged culture time, the frequency...
of the contractions as well as the frequency and the amplitude of the Ca$^{2+}$ sparks increased, whereas no significant difference in the spatial diameter and the half-time of spark rise and decay was observed. The changes in the characteristics of Ca$^{2+}$ sparks may be owing to several causes. These include the maturation of the contractile apparatus and the SR during cardiac cell differentiation, as well as the number of voltage-dependent Ca$^{2+}$ channels and the density of RyRs. Comparable results have been recently achieved with neonatal rabbit ventricular cells, which displayed a significantly reduced frequency of Ca$^{2+}$ sparks compared with adult ventricular cells despite the presence of caffeine releasable Ca$^{2+}$ stores (9). In the latter study, it was discussed that a paucity or immaturity of T-tubular diadic junctions between L-type Ca$^{2+}$ channels and SR Ca$^{2+}$ release channels in immature cells could functionally isolate sarcolemmal Ca$^{2+}$ entry from triggering SR Ca$^{2+}$ release (9). Ca$^{2+}$ sparks have been demonstrated (25, 30) to occur at sites associated with T-tubules, which are spaced at regular intervals along the length of the cells. It has been shown that most neonatal mammalian cardiomyocytes do not develop T-tubules until 8–10 days of age, which may at least partially account for the absence of localized Ca$^{2+}$ sparks in VEDS cardiac precursor cells observed in the present study. Additionally, the spatial association of dihydropyridine receptors and RyRs may be a key factor for CICR and has been recently shown to augment during the differentiation of rabbit neonatal heart cells (29). However, it should be mentioned that in adult atrial myocytes, which lack T tubules, Ca$^{2+}$ sparks occur in cellular regions that are occupied by nonjunctional SR, i.e., from SR, which contains RyRs but is not closely associated with the L-type Ca$^{2+}$
channels of the plasma membrane (2, 13). This points towards the notion that the absence of detectable Ca\(^{2+}\) sparks in VEDS cardiomyocytes may be the result of the lower densities of RyRs and the possibility that RyRs are not clustered in the same fashion as in LDS cardiomyocytes and/or that spontaneous Ca\(^{2+}\) release events may indeed occur but escape confocal imaging because of their small magnitude.

**Expression of RyRs in ES cell-derived cardiomyocytes.** The data of the present study demonstrate an increase in BODIPY FL-X ryanodine staining in beating areas of whole mount LDS cardiomyocytes, suggesting an increased expression of RyRs during the time course of cardiac cell differentiation. Furthermore, RyR immunohistochemistry revealed a pronounced increase in the volume of the SR positively labeled for RyRs during cardiomyogenesis of ES cells. This rise in the number of RyRs parallels the increase in the L-type Ca\(^{2+}\) current density, which has been previously reported (23). In contrast to the increasing expression of RyRs, the expression of Ins(1,4,5)P\(_3\) receptors was significantly downregulated (data not shown), which may indicate a switch from the prevalence of Ins(1,4,5)P\(_3\)-mediated Ca\(^{2+}\) signaling pathways in early stages to CICR pathways in late stages of cardiomyogenesis.

**Evidence for a lower Ca\(^{2+}\) load in the SR of EDS compared with LDS cardiomyocytes.** The Ca\(^{2+}\) load of the SR is an additional feature that may influence the characteristics of Ca\(^{2+}\) sparks. In the present study, the Ca\(^{2+}\) load was evaluated by investigation of the caffeine-releasable Ca\(^{2+}\) pool. Interestingly, it was observed that Ca\(^{2+}\) spiking returned after Ca\(^{2+}\) release after treatment of EDS cardiomyocytes with caffeine, whereas spiking was abolished in LDS cardiomyocytes. This may either indicate that additional Ca\(^{2+}\) stores, which are not caffeine sensitive, are present in EDS cardiomyocytes, or further Ca\(^{2+}\) transport pathways, e.g., the Na\(^+\)/Ca\(^{2+}\) exchange (9), are involved in Ca\(^{2+}\) spiking. The data of the present study clearly demonstrate by Ca\(^{2+}\) release experiments using the SR store agonist caffeine that the filling state of the SR in LDS cardiomyocytes is significantly increased compared with EDS cardiomyocytes. An influence of the SR Ca\(^{2+}\) load on the frequency as well as the amplitude of Ca\(^{2+}\) sparks has been reported (6, 28, 31) and has been interpreted as an increased open probability of SR Ca\(^{2+}\) release channels owing to the elevated SR Ca\(^{2+}\) content and/or an increased sensitivity of the channels to activation by cytosolic Ca\(^{2+}\) (28). Furthermore, it has been suggested that increased SR Ca\(^{2+}\) load augments the effectiveness of a given Ca\(^{2+}\) current to activate SR Ca\(^{2+}\) release (1, 10, 16). The data of the present study demonstrate a lower expression level of RyRs and a smaller extension of the SR in EDS compared with LDS cardiomyocytes, which correlates with a reduced L-type Ca\(^{2+}\) current density in EDS cardiomyocytes that has been previously described (23). Hence, our data corroborate observations in fetal rat ventricular cardiomyocytes, which displayed fewer RyRs compared with adult cardiac cells (26). The elevation of the SR Ca\(^{2+}\) load by superfusion with 10 mM external Ca\(^{2+}\) increased spark frequency and amplitude to the level of LDS cardiomyocytes. This may suggest that the density of RyRs and voltage-dependent Ca\(^{2+}\) channels or possible differences in the microarchitecture between the RyR and the L-type Ca\(^{2+}\) channel in EDS compared with LDS cardiomyocytes play a secondary role for the observed differences in the spark characteristics. However, elevation of [Ca\(^{2+}\)]\(_i\), results in a larger unitary current through L-type Ca\(^{2+}\) channels due to the higher driving force for Ca\(^{2+}\), which may cause a larger rise in [Ca\(^{2+}\)]\(_i\) in the diadic cleft. Elevated [Ca\(^{2+}\)]\(_i\) in the diadic cleft could compensate for potential differences in the microarchitecture of the RyR-dihydropyridine receptor arrangement and thereby affect properties of the Ca\(^{2+}\) sparks. Furthermore, the elevated cytosolic [Ca\(^{2+}\)]\(_i\), that occurs after incubation of EDS cardiomyocytes in high-[Ca\(^{2+}\)]\(_o\), solution may directly trigger the opening of RyR through CICR. This then results (see Ref. 24) in the occurrence of sites that generate sparks at high frequency. Frequent spark sites were observed in the present study when EDS cardiomyocytes were incubated in 10 mM external Ca\(^{2+}\).

**Mechanisms of E-C coupling.** According to the local control theory of E-C coupling in adult cardiac cells, CICR is locally initiated by Ca\(^{2+}\) influx through a single L-type Ca\(^{2+}\) channel that activates a group of SR Ca\(^{2+}\) release channels at T tubule SR junctions, which, during the action potential, equal a global [Ca\(^{2+}\)]\(_i\), transient. In newborn rabbit ventricular myocytes, the absence of the T tubule network has been evidenced, which led to the assumption that the immature SR may play a minor role in E-C coupling. On the basis of the observations in newborn cardiomyocytes that the myofibrils are located subsarcolemmally, the cytosolic Ca\(^{2+}\) buffering is lower, and Na\(^+\)/Ca\(^{2+}\) exchange activity is increased, it was suggested that the Na\(^+\)/Ca\(^{2+}\) exchange pathway is the predominant pathway for contraction and relaxation in newborn rabbit ventricular myocytes. This assumption can be verified by using a mathematical model (9). Compelling evidence has been recently provided that mechanisms different from the classical model of E-C coupling occur likewise in early cardiac cells differentiated from ES cells (32). In these EDS cardiac cells, spontaneous [Ca\(^{2+}\)]\(_i\) oscillations drive the cell contractions, which led to the assumption that in these cells the voltage-dependent Ca\(^{2+}\) current may primarily serve for store refilling. The absence of Ca\(^{2+}\) sparks in noncontracting VEDS cardiac precur- sor cells and the age-dependent increase in spontaneously contracting EDS cells displaying typical sparks support these previous investigations and envision [Ca\(^{2+}\)]\(_i\) release mechanisms distinct from the known CICR mechanism as primary rhythm generator in VEDS and EDS cardiac cells. This primary rhythm generator is then gradually replaced during the later stages of cardiac cell differentiation by the CICR mechanism, which is characterized by distinct spatially localized Ca\(^{2+}\) spark events.
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