Initiation of Embryonic Cardiac Pacemaker Activity by Inositol 1,4,5-Trisphosphate–dependent Calcium Signaling[□] [▽]

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Submitted October 8, 2004; Revised February 28, 2005; Accepted March 1, 2005
Monitoring Editor: Guido Guidotti

In the adult, the heart rate is driven by spontaneous and repetitive depolarizations of pacemaker cells to generate a firing of action potentials propagating along the conduction system and spreading into the ventricles. In the early embryo before E9.5, the pacemaker ionic channel responsible for the spontaneous depolarization of cells is not yet functional. Thus the mechanisms that initiate early heart rhythm during cardiogenesis are puzzling. In the absence of a functional pacemaker ionic channel, the oscillatory nature of inositol 1,4,5-trisphosphate (InsP₃)-induced intracellular Ca²⁺ signaling could provide an alternative pacemaking mechanism. To test this hypothesis, we have engineered pacemaker cells from embryonic stem (ES) cells, a model that faithfully recapitulates early stages of heart development. We show that InsP₃-dependent shuttle of free Ca²⁺ in and out of the endoplasmic reticulum is essential for a proper generation of pacemaker activity during early cardiogenesis and fetal life.

INTRODUCTION

The heart rate is initiated and driven by dominant pacemaker cells located in the sino-atrial node of adult myocardium. These cells feature at their membrane a functional pacemaker channel, namely the hyperpolarization-activated cyclic nucleotide (HCN)-gated cationic channel, generating the I_f current and setting the spontaneous and rhythmic action potentials of the heart (Accili *et al.*, 2002; Clapham, 1998). The first cell contractions are observed in the murine embryo as early as E7.5 (Porter and Rivkees, 2001), despite the small amplitude of the I_f current recorded before E9.5 (Stieber *et al.*, 2003). Interestingly, mice deficient for HCN4, the predominant HCN message in the sino-atrial node, live without cardiac arrhythmias till embryonic day 9.5–11.5 despite the absence of mature cAMP-regulated pacemaker cells in their heart (Stieber *et al.*, 2003). This suggests that HCN-independent, not yet described mechanisms must be responsible for a pacemaker activity at the early stages of cardiac development. Such a mechanism is crucial because it would allow for contraction of the cardiac tube and of the fully developed embryonic heart to ensure viability of the embryo till midgestation.

The various roles of Ca²⁺ as an intracellular second messenger have been characterized extensively. This cation regulates

such important biological processes as contraction, cell migration, secretion, growth and differentiation (Clapham, 1995; Rizzuto *et al.*, 2002; Berridge *et al.*, 2003; Webb and Miller, 2003). Importantly, the action of intracellular Ca²⁺ begins at very early stages of development affecting pathways involved in fertilization and embryogenesis (Berridge *et al.*, 2000, 2003; Webb and Miller, 2003). Ca²⁺ is released from the endoplasmic reticulum (ER) by inositol 1,4,5-trisphosphate (InsP₃) generated after phospholipase C activation, decoding the signal conveyed by extracellular growth factors, hormones, or neurotransmitters. In turn, Ca²⁺ depletion of the ER triggers a capacitative Ca²⁺ influx (CCI) across the plasma membrane to refill the stores (Putney *et al.*, 2001; Venkatachalam *et al.*, 2002). The oscillatory nature of the InsP₃-induced intracellular Ca²⁺ signal has been long recognized (Hirose *et al.*, 1999; Berridge *et al.*, 2003). We hypothesize that InsP₃-induced intracellular Ca²⁺ oscillations, generating rhythmic repetitions of events over time intervals, may provide a pacing mechanism in embryonic heart.

It is difficult to investigate the molecular basis of cardiac pacemaker activity in the murine embryo. Although knockout or targeted mutations of specific genes might provide valuable information to address this question, the dependence of the murine embryo upon blood flow and thus myocardial function makes this approach and interpretation of the results intricate because any defect in heart function may compromise the survival of the embryo. To overcome this problem, here we used differentiating embryonic stem (ES) cell–derived cardiomyocytes, which recapitulate in vitro the early stages of cardiogenesis (Leahy *et al.*, 1999; Loebel *et al.*, 2003). We engineered pacemaker cells with disrupted intracellular Ca²⁺ signaling by overexpression of calreticulin, an ER Ca²⁺-buffering protein or by knocking down expression of type I InsP₃

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E04-10-0883>) on March 9, 2005.

[□] [▽] The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

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receptor. These genetic approaches were combined with a pharmacological strategy together with cell biology technologies designed to specifically target InsP₃ signaling. We demonstrate that intracellular Ca²⁺ oscillations supported by InsP₃-sensitive stores constitute a pacemaking mechanism in early cardiac development. Furthermore, we uncovered that this intracellular Ca²⁺ network is also essential in mediating pacemaker activity in fetal heart.

MATERIALS AND METHODS

ES Cell Propagation and Differentiation

ES cells (CGR8) were propagated in BHK21 medium supplemented with sodium pyruvate, nonessential amino acids, mercaptoethanol, 7.5% fetal calf serum (FCS; Hyclone, Perbio, France) and leukemia inhibitory factor (LIF)-conditioned medium. Differentiation was carried out using the hanging drop method. Briefly, embryoid bodies (EB) were formed in hanging drops of differentiation medium (BHK21 medium supplemented with Na pyruvate, nonessential amino acids, selenium, β -mercaptoethanol, and 20% FCS, without LIF) for 2 d (D0–2). Then, the embryoid bodies were incubated for 3 d in suspension (D3–5) and plated on gelatin-coated dishes at day 6.

Plasmid Construction, ES Cell Clones, and ES Cell Differentiation

A cDNA encoding rabbit calreticulin was subcloned downstream the α -MHC promoter (GenBank U71441; Nakamura *et al.*, 2001). To generate an InsP₃RI antisense cDNA, a region (420 base pairs, nucleotides 834–1254 from ATG) of the InsP₃ binding domain of the InsP₃RI was amplified by PCR from a brain cDNA library using two primers including an enzyme restriction site, taaagc-cggggggaagtggagga (XmaI) and gctgaaaattggtacctctccct (KpnI). The PCR fragment was then subcloned in a vector in the antisense orientation downstream the α -MHC promoter. The linearized plasmids were electroporated into CGR8 ES cells according to standard protocol. The ES cell clones were selected for 10 d with G418 (250 μ g/ml) and screened by PCR before differentiation using the hanging drop method (Meyer *et al.*, 2000). Two separate ES cell clones from each clone were used in the study. To monitor the expression of the antisense mRNA, total RNA extracted from embryoid bodies was reverse-transcribed using the sense primer (taaagcggggggaagtggagga). The resulting products were then amplified by real-time quantitative PCR using the primers listed above and run on agarose gel.

Cell Microdissection and Combined Approaches

Beating areas of EBs featuring EYFP-expressing cells were dissected with a laser-assisted device (PALM Laser, Bernried, Germany) or a microscalpel and cells were enzymatically isolated using 1 mg/ml collagenase in ADS buffer without Ca²⁺ (containing 20 mM HEPES, 5 mM glucose, 117 mM NaCl, 5.7 mM KCl, 1.2 mM NaH₂PO₄, 4.4 mM NaHCO₃, 1.7 mM MgCl₂). Cells were then plated in differentiation medium on gelatin-coated dishes. Real time PCR was carried out on dissected EYFP-expressing cells after reverse transcription from RNA extracted using a RNA extraction kit (PALM Laser).

Electrophysiology

Electrophysiological recordings were made at room temperature (25 $\pm$ 2°C) using an Axopatch-200 patch clamp amplifier (Axon Instruments, Foster City, CA) and analyzed using the Clampfit 9 software (Axon Instruments). Monitoring of action potentials (AP) in ES cell-derived pacemaker cells was performed as early as 24 h after their isolation and no longer than 48 h after with no change in morphology, or electrical properties during the 2-d culture. AP were recorded with a 30–100 M Ω micropipette filled with 3 M KCl. EYFP-expressing cells were impaled with the microelectrode and electrode capacitance was nulled. Voltage-activated K⁺ currents were recorded in response to 1.5-s voltage steps to potentials between –140 and +50 mV in 10 mV increments, from a holding potential of –70 mV. Recording pipettes contained (mmol/L): 135 KCl, 1 MgCl₂, 10 EGTA, 10 HEPES, and 5 glucose; pH 7.2 (300–310 mosmol/L). The bath solution contained (mmol/L): 117 NaCl, 5.5 KCl, 1.8 CaCl₂, 2 MgCl₂, 10 HEPES, and 10 glucose. pH and osmolality were adjusted to 7.4 and 295–305 mosmol/L, respectively.

Isolation of Embryonic Cardiomyocytes, Cell Transfection, and Microinjection

E9–9.5 murine embryos were dissected, kept in the yolk sac and put in culture medium (DMEM supplemented with 2% FCS) for 24 h before in situ whole heart experiments. Alternatively, the heart was removed from E14.5 mouse embryos. Atria were separated from ventricles and bathed in ADS buffer. Then cells were isolated with pancreatin and collagenase as previously described (Puceat *et al.*, 1994). Cells were plated in the same medium as the one used for EBs. Monitoring of Ca²⁺ spikes in embryonic cardiomyocytes was

performed as early as 24 h after their isolation and no longer than 48 h after. No change in cell morphology and Ca²⁺ homeostasis were observed during the 2-d culture. The day after their isolation, embryonic cardiomyocytes were transfected with Ds Red alone or Ds Red and a plasmid encoding the InsP₃ phosphatase or a pcDNA3.1 plasmid encoding calreticulin, all under control of the CMV promoter. In other experiments, embryonic cardiomyocytes were transfected with the InsP₃RI antisense construct (see above) together with a plasmid encoding myosin light chain 2v (MLC2v) fused to GFP under the control of the cardiac α -actin promoter, in order to track the transfected cells. Transfection was performed with Effectene (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Cell microinjection was performed as previously described (Bony *et al.*, 2001; Jaconi *et al.*, 2000). The pipettes contained 2.5 mM fluo-3 (K salt) and 500 μ g/ml monoclonal 18A10 anti-InsP₃RI antibody or mouse IgGs, or 2.5 mM fluo-3 (K salt) and 3 mg/ml JPW1114.

Imaging of Cytosolic Ca²⁺ Concentration and Membrane Potential

ES cell-derived embryoid bodies, embryonic cardiomyocytes, or whole E9–9.5 mouse embryos were loaded with 5 μ M fluo-4/AM (Molecular Probes, Eugene, OR) for 10–15 min, transferred to the stage of a fast-acquisition epifluorescence confocal microscope and superfused with ADS buffer added with 2 mM Ca²⁺. The field was illuminated at 488 nm (Argon laser). Fluo-4 emission fluorescence was recorded through a dichroic mirror (cutoff 510 nm) and a long pass emission filter (cutoff 520 nm) set on a motorized filter wheel. The images were recorded each 30 or 50 ms using a confocal Perkin Elmer-Cetus Ultraview microscope (Norwalk, CT) coupled to a CCD camera (Cool-snap, Princeton Scientific Instruments, Monmouth Junction, NJ) and digitized on line by a computer (Metamorph software, Universal Imaging, West Chester, PA). To calculate the frequency and synchronization of Ca²⁺ spikes in embryoid bodies, regions of interest were selected in cells within a beating area and the average pixel intensity was plotted as a function of time. All experiments were performed at 35 $\pm$ 2°C and were repeated at least three times. To simultaneously record fluo-3 and JPW1114 fluorescence, cells were bathed in a culture dish set on the stage of a Meta LSM 510 Zeiss microscope (Thornwood, NY) warmed at 37°C. Cells were illuminated at 488 or 514 nm using the Argon laser, and fluorescence was recorded by the photomultipliers after crossing a HFT 488 and NFT 545 dichroic mirrors and two bandpass filters at 505–530 nm (fluo-3) or 620–670 nm (JPW1114). Images were acquired each 50–70 ms by scanning a small region of interest within the cell.

To estimate the release of Ca²⁺ from the ER, 1 μ M thapsigargin was superfused in the absence of Ca²⁺ to fluo-4-loaded spontaneously Ca²⁺ spiking E14 embryonic myocytes overexpressing calreticulin or to mock cells. The amplitude of the Ca²⁺ release was estimated when a plateau was reached. The maximal rate of increase in cytosolic Ca²⁺ concentration ([Ca²⁺]_i) was fitted using a sigmoidal equation after normalization of values to the maximal value of fluorescence and was expressed as the $t_{1/2}$ required to reach the plateau.

Immunocytochemistry

For immunostaining, embryoid bodies or cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, and labeled with a mouse antisarcomeric α -actinin antibody (Sigma, St. Louis, MO) or with an anti- β MHC antibody and Alexa350– or Alexa546–conjugated anti-mouse IgG (Molecular Probes) or with an anticalreticulin or anti-InsP₃RI antibody (Jaconi *et al.*, 2000) and a secondary Alexa488– or Alexa546–conjugated anti-rabbit IgG (Molecular Probes). In situ immunostained sarcomeres were visualized in 0.3- μ m optically z-sectioned embryoid bodies. To improve resolution and signal-to-noise ratio, images were restored using the Huygens software (Huygens 2.2.1, Scientific Volume Imaging, Hilversum, The Netherlands) and visualized using Imaris (Bitplane, Zurich, Switzerland). Calculations were performed on Dell Precision 450 workstations (Round Rock, TX).

RNA Extraction, Reverse Transcription Reaction, and Real-time Quantitative PCR by SYBR Green Detection

Total RNA was isolated from embryoid bodies using an RNA extraction kit (RNeasy, Qiagen, France). One microgram of RNA was reverse-transcribed using the M-MLV reverse transcriptase (Invitrogen, Cergy, France) and used in real-time PCR.

The nucleotide sequences of the PCR primers used were as follows: MEF2C forward: 5'-AGATACCCACAACACACCACGCGCC-3' and reverse: 5'-ATCCTTCAGAGAGTCGCATGCGCTT-3'; Nkx2.5 forward: 5'-TGCAGAGGCAGTGGAGCTGGACAAGCC-3' and reverse: 5'-TGCACITGTAGCGACGGTTCTGGAAACCAG-3'; β -tubulin forward: 5'-CCGGACAGTGTGGCAACCAGATCGG-3' and reverse: 5'-TGGCCAAAAGGACCTGAGCGGAAACGG-3'; HCN1 forward: 5'-CAAGACAGCCAGAGCACTTCGT-3' and reverse: 5'-AGATACATACACCAGTGGGAAGAG-3'; HCN2 forward: 5'-TCGTCTTCAACGTGGTCTCG-3' and reverse: 5'-ACAACACGGAGATCATCCTGGAC-3' (Yasui *et al.*, 2001); HCN4 forward: 5'-GGATTATCCACCCTACAG-3' and reverse: 5'-GTCTCGCCAAAGTCAATGAGGAAGAAT-3'; EYFP forward: 5-ACGTAAACGGCCACAAGTTC-3' and reverse: 5'-

AAGTCGTGCTGCTTCATGTG-3'; MLC2a forward: 5'-TCTTCTAATGTCT-TCTCAATGTTTCG-3' and reverse: 5'-TCTACTCCTCTTTTCATCC; CCGTG-3'; MLC2v forward: 5'-GCCAAGAAGCGGATAGAAGG-3' and reverse: 5'-CTGTGGTTCAGGGCTCAGTC-3'; α -MHC forward: 5'-CCAAT-GAGTACCGCGGTGAA-3' and reverse: 5'-ACAGTCATGCCGGGATGATG-3'; Cx45 forward: 5'-TGTGTGCAACACAGAGCAGC-3' and reverse: 5'-CC-ATCTCTCGAATTCGTGTCG-3'. Real-time quantitative PCR was performed using a LightCycler rapid thermal cycler (Roche, Meylan, France). Amplification was carried out as recommended by the manufacturer. Twelve-microliter reaction mixture contained 10 μ l of LightCycler-DNA Master SYBR Green I mix (FAST Start KIT, containing *Taq* DNA polymerase, reaction buffer, deoxynucleoside triphosphate mix, and SYBR Green I dye), added with 3 mM $MgCl_2$, 0.5 μ M of appropriate primer mix, and 2 μ l of cDNA. Results were expressed as a function of the level of expression of the gene of interest in first stage of differentiating EBs using a previously described mathematical model (Pfaffl *et al.*, 2002). The data were normalized by PCR analysis of β -tubulin. The amplification program included the initial denaturation step at 95°C for 8 min, and 40 cycles of denaturation at 95°C for 3 s, annealing at 65°C for 10 s, and extension at 72°C for 10 s. The temperature transition rate was 20°C/s. Fluorescence was measured at the end of each extension step. After amplification, a melting curve was acquired by heating the product at 20°C/s to 95°C, cooling it at 20°C/s to 70°C, keeping it at 70°C for 20 s, and then slowly heating it at 0.1°C/s to 95°C. Fluorescence was measured through the slow heating phase. Melting curves were used to determine the specificity of PCR products, which was confirmed using conventional gel electrophoresis.

The Student's *t* test was used to analyze statistical significance. All *p* values corresponded to two-tailed test, and *p* < 0.05 was considered statistically significant.

Western Blotting

For Western blotting, embryoid bodies were lysed using RIPA buffer (20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% Triton X-100, 1% deoxycholic acid, 1 mM EDTA, 0.05% SDS) supplemented with phenylmethylsulfonyl fluoride. Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad, Richmond, CA). Proteins (50 μ g) were fractionated by SDS-PAGE, electroblotted onto a nitrocellulose transfer membrane, and probed with an anti-calreticulin antibody. Antibodies were visualized by addition of goat HRP-conjugated anti-rabbit IgGs and enhanced chemiluminescence reagents (ECL reagent, Amersham Pharmacia Biotech Europe, Freiburg, Germany).

RESULTS

Engineering of Pacemaker Cells from ES Cells

When cultured in high serum medium, ES cells aggregate within an EB, a three-dimensional cell formation that includes the three embryonic layers, and then spontaneously differentiate into myocytes featuring all cardiac phenotypes (i.e., ventricular and atrial cells including sino-atrial cells; Maltsev *et al.*, 1994). To locate pacemaker cells among myocytes differentiating within embryoid bodies (Meyer *et al.*, 2000), we engineered an ES cell clone expressing the EYFP. Expression of EYFP was driven by the cardiac-specific α isoform of myosin heavy-chain (α -MHC) promoter, functional in E7 embryos, and early developing embryonic atria, where differentiation of pacemaker cells originates (Lyons *et al.*, 1990; Moorman and Lamers, 1999; Morkin, 2000; Figure 1A). EYFP-expressing cells were small and displayed mostly a round or elongated shape (Figure 1B, inset). They were dispersed in the embryoid bodies within large fields of cardiomyocytes (Figure 1B). In comparison, ES cells in which the expression of EYFP was driven by the broadly functional cardiac α -actin promoter (Behfar *et al.*, 2002) produced clusters of large myocytes (Figure 1C). This suggests that the activity of the α -MHC promoter is significantly restricted among differentiating cardiac cells at early stages of development of embryoid bodies. Cardiac sarcomeres in the EBs were visualized at high magnification by immunofluorescence, using an antibody against sarcomeric actinin. Figure 1D shows that cells expressing EYFP under the transcriptional control of the α -MHC promoter featured a poor myofibril network. Furthermore, cells expressing EYFP under the transcriptional control of α -MHC did not express the β isoform of myosin heavy chain (β -MHC) while surrounded by contractile β -MHC-positive cells (Figure 1E). Videomicroscopy showed

that they were not beating (Supplementary Video) while driving contractions of the neighbor myocytes, suggesting that they might be pacemaker cells. To obtain further insight into the phenotype of EYFP-expressing cells, they were microdissected out of the EBs using a laser-assisted device or a microscalpel followed by real-time RT-PCR analysis as well as electrophysiological recordings. Figure 1F shows that these cells contained a high level of mRNAs encoding connexin 45, as well as HCN4, two well-known markers of mouse sino-atrial cells (Moosmang *et al.*, 2001; Verheijck *et al.*, 2001). No expression of mRNA encoding HCN1 or HCN2 was found in these cells (unpublished data). ES cell-derived EYFP-expressing cells displayed spontaneous action potentials (APD90 = 176 ± 4 ms at 25°C, *n* = 6) starting with a slow diastolic depolarization, typical of sino-atrial cells (Figure 1G). Patch-clamp technique was used to test the presence or absence of functional I_f channels in ES cell-derived EYFP-expressing cardiomyocytes dissected out of EBs at days 9 and 11 of differentiation. Hyperpolarizing pulses from -30 to -140 mV were applied to EYFP-expressing cells, from a holding potential of -70 mV (see inset Figure 1H). At day 9 of differentiation, the presence of a small inward depolarizing K^+ current with electrophysiological properties similar to native I_f current recorded in sino-atrial node cells of adult mouse hearts (Mangoni and Nargeot, 2001) was observed in only two of the eight cells analyzed, albeit of small amplitude. The other six cells only expressed an inward background K^+ conductance with small density (Figure 1H). On the contrary, in cells isolated from day 11 EBs, I_f current was recorded in all EYFP-positive cells. Density and activation time constants of I_f current were 24.9 ± 7.5 pA/pF and 503 ± 63 ms at -140 mV (*n* = 6), respectively. Interestingly, the current/voltage relationships of total inward and outward voltage-gated K^+ currents, obtained at days 9 and 11 of differentiation (Figure 1H), demonstrated no difference in the current density of outward voltage-gated K^+ currents.

Combined, our findings demonstrate that cells expressing EYFP under the transcriptional control of the α -MHC promoter display all essential hallmarks of pacemaker cells. We have thus established a cell model which recapitulates in vitro the formation of pacemaker cells.

Voltage-sensitive Ionic Conductances Do Not Underlie Automaticity of ES Cell-derived Pacemaker Cells

In excitable muscle cells, spontaneous electrical activity is translated into spontaneous Ca^{2+} spiking. Thus, the latter phenomenon can be used as a read-out of pacemaker activity. To further test the involvement of a voltage-sensitive ionic current in pacemaker activity of day 9 whole embryoid bodies, 140 mM KCl was applied to spontaneously Ca^{2+} -spiking cells, within fluo-4-loaded embryoid bodies to abolish membrane potential. A sudden and sustained increase in basal Ca^{2+} was observed upon depolarization of cells. More interestingly, despite the annihilation of the membrane potential, rhythmic Ca^{2+} oscillations were still observed when basal Ca^{2+} reached a plateau (Figure 2A). To more specifically establish a participation, if any, of the I_f current in spontaneously Ca^{2+} -spiking cells, ivabradine or zeneca (ZD7288), two inhibitors of I_f conductance (BoSmith *et al.*, 1993; Bucchi *et al.*, 2002), were applied to day 9 fluo-4-loaded embryoid bodies. Both drugs had no effect on spontaneous Ca^{2+} spiking activity of ES cell-derived cardiomyocytes (Figure 2B) as expected from the lack of functional I_f current in ES cell-derived pacemaker cells in day 9 embryoid bodies (Figure 1H). Similarly, when tested on E9.5 embryonic hearts in situ, both ivabradine and zeneca (ZD7288) had no effect on their spontaneous Ca^{2+} -spiking activity

Figure 1. Cells expressing EYFP under control of the α -MHC promoter are true pacemaker cells. (A) α -MHC expression was measured by real-time quantitative PCR after reverse transcription of mRNA extracted from embryonic ventricles and atria at different stages of development (E7, E9, E14.5, E17.5) or after birth in neonatal mice (Neo). Data are normalized to β -tubulin expression and expressed as fold increase in expression compared with E7 embryonic heart α -MHC mRNA content. CGR8 ES cell clones expressing EYFP under control of the α -MHC promoter (B) or of the cardiac α -actin promoter (C) were allowed to differentiate into cardiomyocytes within embryoid bodies (EBs). EYFP was visualized by confocal microscopy at 10 $\times$ (BC) and 63 $\times$ magnification (inset B). (D) Sarcomeric units of differentiating myocytes including some expressing EYFP under control of the α -MHC promoter were immunostained within EBs with an anti-actinin antibody and anti-mouse secondary antibody coupled to Alexa350 and visualized by confocal microscopy (63 $\times$ magnification). (E) Cardiomyocytes including α -MHC-EYFP-expressing cells were immunostained with an anti- β -MHC antibody and an anti-mouse secondary antibody coupled to Alexa546 and imaged by confocal microscopy (63 $\times$ magnification). (F) Myocytes expressing EYFP under control of the α -MHC promoter were microdissected out of day 8–10 EBs. RNA was extracted from the cells; EYFP, Cx45, and HCN4 were amplified by real-time quantitative PCR. Profiles of melting curves obtained at the end of the PCR run are illustrated; the amplified fragments were further separated on agarose gel to identify the products (inset). (G) Other EYFP-expressing cells were enzymatically dissociated using collagenase and cultured for 24–48 h. Action potentials (G) and the I_f current (H) were recorded in these cells under microelectrode or voltage-clamp configuration, respectively. The figure is representative of 6–8 experiments. *Significantly different from D9 ($p \leq 0.05$)

(Supplementary Figure S1). This indicates that at early stages of cardiogenesis, the I_f current does not play any role in pacemaker activity. In contrast, 2 d later, in day 11 embryoid bodies, a stage at which a functional I_f current was recorded in ES cell-derived pacemaker cells (Figure 1H), both I_f current inhibitors slowed down the rate of spontaneously spiking ES cell-derived cardiac cells and changed the decay of the Ca^{2+} transient (Figure 2C). In line with this result, depolarization of the cells with 140 mM KCl increased basal Ca^{2+} and stopped Ca^{2+} oscillations recorded within embryoid bodies (Figure 2D). We conclude that the I_f current is functional only at day 11 of differentiation of ES cells. Thus, another voltage-insensitive pacemaking mechanism must account for cardiac automaticity during heart formation at early embryonic stages.

Overexpression of Calreticulin in Pacemaker Cells Impairs Their Spontaneous Activity

Intracellular Ca^{2+} signaling is disrupted by increased levels of calreticulin in the ER (Krause and Michalak, 1997). Elevated expression of calreticulin leads to increased concentrations of Ca^{2+} in the lumen of the ER in nonexcitable cells (Mery *et al.*, 1996) and to a concomitant inhibition of capacitative Ca^{2+} influx (Mery *et al.*, 1996; Fasolato *et al.*, 1998) as well as inhibition of SERCA (John *et al.*, 1998). This limits Ca^{2+} entry into the ER, and in turn impairs Ca^{2+} efflux from the ER (Xu *et al.*, 2000). Besides, buffering ER luminal Ca^{2+} revealed that the filling state of the ER regulates the extent of InsP_3 -triggered Ca^{2+} release through a mechanism independent from cytosolic Ca^{2+} or refilling by the sarco-ER Ca^{2+}

Figure 2. Pacemaker activity of day 9 embryoid bodies (EBs) does not depend upon a voltage-sensitive ionic conductance. Fluo-4-loaded day 9 EBs were challenged with 140 mM KCl (KCl replaced NaCl) (A) or with 10 μ M ivabradine or 1 μ M zeneca (B). Fluo-4-loaded day 11 EBs were challenged with 10 μ M ivabradine or 1 μ M zeneca (C) or with 140 mM KCl (D). Recordings show Ca^{2+} spikes within selected regions of interest (ROI) and are expressed as $\Delta F/F_0$, where F_0 is the lowest basal fluorescence recorded at the beginning of the experiment. The figure is representative of at least five experiments.

ATPase (SERCA; Caroppo *et al.*, 2003). Overexpression of calreticulin may thus decrease free intraluminal Ca^{2+} and reduce InsP_3 -triggered Ca^{2+} release beyond what can be expected from a simple reduction of ER free Ca^{2+} content. We also observed that calreticulin overexpressed in E14 spontaneously Ca^{2+} spiking embryonic cardiomyocytes tended to slow down thapsigargin-induced Ca^{2+} release from the ER ($t_{1/2}$ of increase in cytosolic Ca^{2+} : median [range]: 67.5 s [38.7134.5], $n = 6$) in control cells and 41.6 s [21.1;82.5], $n = 6$, in CRT-overexpressing cells) and significantly decreased the total amount of released Ca^{2+} : in arbitrary units, 0.82 [0.36;1.22] in control cells and 0.35 [0.1;0.65] in CRT-overexpressing cells. These results and the documented effects of calreticulin on intracellular Ca^{2+} signaling prompted us to manipulate its intracellular expression in ES cell-derived pacemaker cells to investigate the role of InsP_3 -sensitive Ca^{2+} stores in triggering embryonic cardiac excitability. We generated an ES cell line in which calreticulin is expressed under the transcriptional control of the α -MHC promoter (designed as α -MHC-CRT). As described above, this

Figure 3. Overexpression of calreticulin in ES cell-derived pacemaker cells impairs excitability and contractility of embryoid bodies (EBs). (A) Wild-type CGR8 ES cells or cells expressing calreticulin under control of the α -MHC promoter were allowed to differentiate within EBs and beating activity of EBs was monitored daily from day 7. The figure is a mean of three separate differentiation experiments. The inset shows an anti-calreticulin Western blot of proteins extracted from wild-type CGR8 or α -MHC-calreticulin EBs. Wild-type CGR8 or α -MHC-calreticulin EBs were loaded with fluo-4 and Ca^{2+} transients were monitored in ES cell-derived cardiomyocytes using fast acquisition confocal microscopy (B). Recordings show Ca^{2+} spikes within selected regions of interest (ROI) and are expressed as $\Delta F/F_0$, where F_0 is the lowest basal fluorescence recorded at the beginning of the experiment. The figure is representative of at least five experiments.

effectively targets overexpression of calreticulin to pacemaker cells. α -MHC-CRT ES cells were allowed to differentiate within EBs into cardiomyocytes including pacemaker cells (inset in Figure 3). Figure 3 shows that the embryoid bodies derived from α -MHC-CRT ES cells exhibited a dramatic reduction in the beating activity of cardiomyocytes differentiating within the mesoderm. Wild-type CGR8 ES cells differentiated into cardiomyocytes within 9–12 d, as shown by a high percentage ($67 \pm 16\%$) of spontaneously contracting EBs. In contrast, in a population of EBs containing pacemaker cells that overexpressed calreticulin, only $23 \pm 4\%$ were beating at day 12 (Figure 3A). However, buffering Ca^{2+} by calreticulin in the ER of pacemaker cells did not affect expression of mRNAs encoding cardiac-specific transcription factors (i.e., Nkx2.5 and

Mef2C; Supplementary Figure S2). Similarly, myofibrillogenesis as assessed by anti- α -actinin immunostaining was normal in EBs containing pacemaker cells that overexpressed calreticulin. Furthermore, both atrial and ventricular myosin light chains were expressed normally in EBs that overexpressed calreticulin (Supplementary Figure S3).

To further delineate the phenotype of pacemaker cells overexpressing calreticulin, Ca²⁺-spiking activity was imaged in day 8–10 fluo-4-loaded EBs, before appearance of a functional I_f current (Figure 2). Cardiomyocytes in the wild-type EBs showed synchronous and vigorous Ca²⁺ spikes, with a rate (1 Hz at 35°C) similar to the one of the action potentials of ES cell-derived pacemaker cells (0.8 Hz at 25°C, Figure 1), whereas those in the EBs with pacemaker cells that overexpressed calreticulin displayed weak and asynchronous Ca²⁺ transients (Figure 3B). Thus, buffering intraluminal ER Ca²⁺ and limiting capacitative Ca²⁺ influx by high expression of calreticulin in pacemaker cells have an inhibitory effect indicating that homeostasis of ER Ca²⁺ stores plays a crucial role in generation of early pacemaker activity in developing embryoid bodies.

InsP₃-induced Ca²⁺ Release Is Required for Pacemaker Activity

Because the excitability of cardiomyocytes in EBs was severely compromised when the pacemaker cells overexpressed calreticulin, we hypothesized that repetitive InsP₃-induced Ca²⁺ release from the ER plays an essential role in early cardiac automaticity. To get more insight into the role of the InsP₃-dependent pathway in mediating pacemaker activity, we tested an alternative genetic approach to disrupt Ca²⁺ cycling within the pacemaker cell. An ES cell line was generated to express specifically in the pacemaker cells an InsP₃ receptor antisense cDNA under the transcriptional control of the α -MHC promoter (Figure 4), an approach previously reported as successful to disrupt InsP₃ signaling in T-cells (Jayaraman *et al.*, 1995). The antisense dramatically decreased the number of type I InsP₃ receptors, the major InsP₃ receptor isoform expressed in these cells (unpublished data) as shown by immunostaining embryonic cardiac cells expressing the antisense with a specific anti-type I InsP₃ receptors antibody (Figure 4A). Differentiation of the genetically modified ES cells (InsP₃R antisense) within embryoid bodies recapitulated the phenotype of the embryoid bodies with pacemaker cells overexpressing calreticulin. Beating activity was severely impaired (Figure 4B). In line with the weak contractility, spontaneous Ca²⁺ spiking within embryoid bodies in which pacemaker cells expressed the InsP₃R antisense was weak and an infrequent event (Figure 4C). Differentiation per se of ES cells into cardiomyocytes was not affected as revealed by the presence of abundant actinin-positive cardiac cells within the embryoid bodies (Supplementary Figure S4).

To further address the role of InsP₃ receptors, we used xestospongin C, the most specific InsP₃ receptor antagonist (Gafni *et al.*, 1997). In the presence of xestospongin C at a low concentration (5 μ M), which does not affect Ca²⁺ reuptake by the ER (De Smet *et al.*, 1999), spontaneous Ca²⁺ transients recorded within day 8–10 EBs were stopped within 5 min (Figure 4D). Similarly, applied to whole E9.5 mouse embryos, the InsP₃R antagonist desynchronized and abolished Ca²⁺ spiking in both the atrial and ventricular primordia of embryonic heart. Washing the drug out allowed a slow recovery of fast Ca²⁺ transients (Figure 4E).

InsP₃ Regulates Pacemaker Activity of E14.5 Embryonic Pacemaker Cells

Embryonic (E14.5) atrial cells in which α -MHC is transiently expressed (Figure 1A) include a population of pacemaker cells. We wanted to address whether InsP₃-induced Ca²⁺ cycling also underlies pacemaker activity of these cells at a stage of differentiation far more advanced than ES cell-derived pacemaker cells in D8–10 EBs. To induce a fast hydrolysis of InsP₃, we used InsP₃ phosphatase expressed in atrial cells isolated from E14.5 embryonic hearts and plated at low density. Spontaneous Ca²⁺ spiking in isolated cells was used as a criterion to track pacemaker cells among the atrial cells. Such pacemaker cells represented a population of 6% of E14.5 atrial cells and featured a developed ER as shown by the presence of high levels of calreticulin and InsP₃RI (Figure 5A). Automatic activity (i.e., Ca²⁺ spiking) was scored in InsP₃ phosphatase- and Ds Red-coexpressing cells versus cells expressing only Ds Red. Figure 5B shows that hydrolysis of InsP₃ by the phosphatase prevented automaticity in 77% of pacemaker atrial cells.

Next, we used a specific anti-InsP₃RI antibody (clone 18A10) to block receptor function (Nakade *et al.*, 1991). The antibody or mouse IgGs as a control were microinjected into atrial embryonic pacemaker cells together with fluo-3. The antibody prevented Ca²⁺ spiking in 65% of atrial pacemaker cells (Figure 5C). In line with the previous findings, xestospongin C blunted both spontaneous Ca²⁺-spiking and firing of action potential simultaneously monitored in fluo-3 and JPW1114 injected atrial pacemaker cells (Figure 5D). Altogether, these findings demonstrate that InsP₃ must be a key messenger to mediate pacemaker activity in embryonic hearts.

DISCUSSION

The molecular and subcellular keystones of pacemaker activity in early stages of cardiogenesis, before appearance of the I_f pacemaker current, have not been understood at the subcellular level. Here, we uncovered that intracellular Ca²⁺ signaling emanating from the ER in embryonic pacemaker cells is essential for cardiac cell automaticity. We demonstrate that depolarization of pacemaker cells in early embryonic pacemaker cells is triggered by InsP₃-sensitive intracellular Ca²⁺ stores. Furthermore, this early pacemaker mechanism is preserved throughout murine development and works together with the I_f current-based mechanism in fetal pacemaker cells.

Engineering of ES Cell-derived Pacemaker Cells

Using the α -MHC promoter to drive expression of EYFP in CGR8 ES cells, we established a model of early cardiac pacemaker cells. The ES cell-derived pacemaker cells featured a poor myofibrillar network and did not beat while driving contraction of surrounding cells. This suggests that the cells generated and propagated an electrical signal. Indeed, the membrane potential of the pacemaker cells was unstable, triggering spontaneous and rhythmic firing of action potentials. The depolarizing phase of the action potential was composed of a slow and then a very fast upstroke, a time course typical of pacemaker cells of the sino-atrial node (Mangoni and Nargeot, 2001). These cells also expressed connexin 45, a specific isoform of connexin whose expression is restricted to nodal cells (Verheijck *et al.*, 2001) and HCN4, although not yet functional at early stages of cell differentiation but becoming operative in later stages.

Figure 4. InsP₃RIs are required to initiate pacemaker activity in embryoid bodies (EBs). (A) Two clones of ES cells, IP₃Ras13 and IP₃Ras14, were selected for their level of expression of the InsP₃RI antisense cDNA. Left panel, an agarose gel electrophoresis of real time quantitative PCR products amplified after reverse transcription of the antisense mRNA from total RNA of day 12 EB. Wild-type CGR8 d 12 EBs (CGR8) and no DNA in PCR reaction (–) served as negative controls. The α -MHC-InsP₃RI antisense plasmid (+) served as a positive control. Right panel, two embryonic cardiomyocytes cotransfected with a plasmid (α -MHC-InsP₃RI) encoding the InsP₃RI antisense together with a plasmid encoding a fusion protein myosin light chain 2v-GFP (MLC2vGFP) to track the transfected cells and their neighbor nontransfected cardiomyocyte. Cells were stained 72 h after transfection with an anti-InsP₃RI antibody. The white arrows show the absence of InsP₃RIs in the nuclear area of the antisense-transfected cells ($n = 2$ experiments, 55 scored cells), whereas the yellow arrow shows the InsP₃RIs located in the nuclear envelope and emanating ER of the nontransfected cell. (B) ES cells expressing the InsP₃RI antisense cDNA under control of the α -MHC promoter were allowed to differentiate within EBs and beating activity of EBs was monitored daily from day 7. The figure is a mean of two separate differentiation experiments. (C) Day 9–10 CGR8 or IP₃Ras EBs were loaded with fluo-4 and Ca²⁺ transients were monitored in ES cell-derived cardiomyocytes using fast acquisition confocal microscopy. (D) Xestospongine (5 μ M) was applied to wild-type fluo-4-loaded EBs. Right panel, Ca²⁺ spiking was recorded after 2-min exposure to the inhibitor; in the same running experiments, Ca²⁺ spikes were fully abolished after 5 min superfusion of the drug. Recordings show Ca²⁺ spikes within selected regions of interest (ROIs) and are expressed as $\Delta F/F_0$, where F_0 is the lowest basal fluorescence recorded at the beginning of the experiment. The figure is representative of 3–5 separate experiments. (E) Xestospongine (5 μ M) was applied to E9–9.5 mouse embryonic heart loaded with fluo-4 and observed at 10 $\times$ magnification. Ca²⁺ spiking was recorded after 2-min exposure to the inhibitor; in the same running experiments, Ca²⁺ spikes were fully abolished after 5-min superfusion of the drug. Recordings show Ca²⁺ spikes within selected regions of interest (ROIs) located in the atrium (a) or ventricle (v) of the heart tube and are expressed as $\Delta F/F_0$, where F_0 is the lowest basal fluorescence recorded at the beginning of the experiment. The figure is representative of three separate experiments.

Figure 5. InsP₃-induced Ca²⁺ mobilization regulates pacemaker activity of E14.5 embryonic pacemaker atrial cells. (A) Cells were isolated from atria of E14.5 embryos and cultured for 24–48 h. ER of embryonic cardiomyocytes was immunostained with anticalreticulin and anti-InsP₃RI antibodies and visualized by confocal microscopy. Scale bar, 10 μ m. (B) Cells were transfected with a vector encoding Ds Red alone or with both Ds Red and a pcDNA vector encoding InsP₃ phosphatase. After loading with fluo-4, Ca²⁺ transients were recorded in isolated cells. The figure is a mean of three separate experiments including 507 cells expressing Ds Red only and 511 cells expressing both Ds Red and the InsP₃ phosphatase. (C) Cells were microinjected with micropipettes containing 2.5 mM fluo-3 and 500 μ g/ml 18A10 monoclonal anti-InsP₃RI antibody or mouse IgGs. Ca²⁺ transients were recorded in 37 isolated cells microinjected with control IgGs and 47 isolated cells microinjected with the 18A10 antibody. (D) Cells were microinjected with micropipettes containing 2.5 mM fluo-3 and 3 mg/ml JPW1114. Ca²⁺ transients, and action potential were simultaneously recorded in isolated cells before and after 2 or 5 min of xestospongine application (5 μ M). The figure is representative of six experiments.

Impaired InsP₃-dependent Ca²⁺ Signaling Prevents Pacemaker Activity But Not Cardiac Cell Differentiation in Early Cardiogenesis

We disrupted the intracellular Ca²⁺ signaling network by buffering ER Ca²⁺ and preventing ER refilling by calreticulin or altered expression of InsP₃RI by using an antisense cDNA. In both cases, the expression of the transgene was driven by the α -MHC promoter to target the Ca²⁺ binding protein or the InsP₃RI antisense cDNA to CGR8 ES cell-

derived pacemaker cells. Ca²⁺ spiking activity and in turn contractility of cardiomyocytes within EBs were severely impaired. Buffering ER Ca²⁺ and limiting ER refilling by calreticulin or inhibiting Ca²⁺ release by an InsP₃RI antisense in pacemaker cells did not, however, prevent differentiation per se, because the cardiomyocytes showed normal expression of cardiac-specific transcription factors (i.e., Nkx2.5 and Mef2C) and normal myofibrillogenesis. Although a role of calreticulin has been suggested in the retinoic acid receptor signaling pathway (Shago *et al.*, 1997), a key pathway in cardiac cell specification (Xavier-Neto *et al.*, 1999; Harvey, 2002), both atrial and ventricular myosin light chains were expressed normally in EBs that overexpressed calreticulin. This demonstrates that atrio-ventricular specification did occur. This was expected from the fact that calreticulin overexpression was restricted to noncontractile pacemaker cells. We conclude that pacing is not required for cardiogenesis from ES cells as previously observed (Yang *et al.*, 2002). However, the excitability of cardiomyocytes was severely compromised in EBs that contained pacemaker cells overexpressing calreticulin or featuring an altered InsP₃RI expression.

The phenotype of pacemaker cells with disrupted InsP₃-dependent Ca²⁺ signaling contrasts with the one of ryanodine-deficient ES cell-derived cardiomyocytes that show no inhibition of beating activity or amplitude of the Ca²⁺ transients, but only modifications in their beating frequency (Yang *et al.*, 2002). This demonstrates that Ca²⁺ release via InsP₃ receptors, and not via ryanodine receptors, plays a key role in the excitability of early pacemaker cells. This might have been expected from a negligible expression of calsequestrin and a not yet developed T-tubule system in ES cell-derived cardiomyocytes within day 9–12 EBs, as assessed by PCR and immunofluorescence (unpublished data).

Early Pacemaker Activity Is Triggered by Ca²⁺ Cycling In and Out of the ER through InsP₃Rs

During early cardiac cell differentiation of ES cells, spontaneous action potentials do not depend on membrane potential but rather on intracellular Ca²⁺ stores. It has been suggested that intracellular Ca²⁺ oscillations activate a Ca²⁺-dependent voltage-insensitive nonspecific cationic conductance and/or of Na⁺/Ca²⁺ exchange, generating depolarizing currents and in turn action potentials (Viatchenko-Karpinski *et al.*, 1999). Although the molecular and subcellular components of this mechanism remain unclear, it applies to pacemaker cells of the gastrointestinal tract (Ward *et al.*, 2000) and is functional in the E9 mouse embryonic heart (Koushik *et al.*, 2001) before the pacemaker I_f current becomes predominant (Yasui *et al.*, 2001; Stieber *et al.*, 2003).

We focused our investigation on the intracellular signaling components that could periodically generate cytosolic Ca²⁺ increase to activate the depolarizing voltage-independent conductances. We now report that shuttle of Ca²⁺ in and out of the ER provides an oscillatory membrane-depolarizing mechanism, which triggers the pacemaker activity. Indeed, overexpression of calreticulin in nonexcitable cells has been shown to limit Ca²⁺ entry into the cell (Mery *et al.*, 1996; Fasolato *et al.*, 1998) and in turn to impair Ca²⁺ mobilization from the ER (Xu *et al.*, 2000). Calreticulin also features a chaperoning function (Michalak *et al.*, 1999) and its overexpression may affect the processing and/or folding of other membrane-associated proteins involved in excitation of a cardiomyocyte. Although such an effect cannot be excluded, we found that calreticulin overexpression in excitable spontaneously spiking embryonic cardiomyocytes

Ca²⁺ cycle within the pacemaker cell

Figure 6. Model of InsP₃-induced spontaneous activity in embryonic pacemaker cells. Ca²⁺ released upon InsP₃ binding to its receptor activates a depolarizing conductance (Na⁺/Ca²⁺ exchanger and/or a nonspecific cationic and depolarizing conductance) and thus triggers action potential firing. CCI allows for refilling the ER. This Ca²⁺ cycling acting in a rhythmic and perpetual manner is a predominant pacemaking mechanism in early cardiogenesis and regulates or is required together with the I_f current as a basis of fetal pacemaking mechanism.

slows down thapsigargin-induced Ca²⁺-release. It further dampens down the total amount of free Ca²⁺ released by the SERCA inhibitor, possibly after inhibition of the capacitative Ca²⁺ influx on a spike to spike basis and/or inhibition of SERCA (John *et al.*, 1998). Therefore, both a Ca²⁺ buffering effect in the ER and a limited pool of releasable Ca²⁺ might contribute to decrease the rate of Ca²⁺ oscillations. Although the exact mechanism by which calreticulin inhibits Ca²⁺ efflux from the ER requires further investigation in excitable cells, overexpression of the protein prevents Ca²⁺-dependent generation of depolarizing currents in ES cell-derived cardiac pacemaker cells. Similarly, disturbed expression of InsP₃Rs in ES cell-derived pacemaker cells by the antisense cDNA limits Ca²⁺ release from the stores and thus activation of the Ca²⁺-sensitive depolarizing membrane conductance. Altogether, the results obtained using the genetically modified ES cells (calreticulin overexpressing- and InsP₃RI antisense-expressing pacemaker cells) point to a key role of Ca²⁺ cycling in and out of the ER for the initiation of pacemaker activity in early cardiogenesis (Figure 6). Indeed, a Ca²⁺ spike and in turn rhythmic oscillations of Ca²⁺ and membrane potential can be initiated only if the cell Ca²⁺ load including free ER Ca²⁺, buffered Ca²⁺ and cytosolic Ca²⁺ is above a threshold. If the Ca²⁺ load drops below threshold because of disrupted InsP₃ signaling (inhibition by xestospongin C, InsP₃RI antisense) or impaired CCI or SERCA activity (calreticulin overexpression), leading to a limitation of free ER Ca²⁺ to provide sufficient driving force

for efflux from the ER, the cell is unable to trigger a Ca²⁺ spike (Sneyd *et al.*, 2004). As a result, there can be no activation of the Ca²⁺-sensitive depolarizing conductance responsible for spontaneous action potentials firing, as further shown by inhibition of the later phenomenon by xestospongin C (Figure 5D).

The InsP₃-based pacemaking mechanism is conserved throughout murine development. In E14.5 embryonic atrial cells, accelerating the hydrolysis of InsP₃ by overexpressing InsP₃ phosphatase (Jaconi *et al.*, 2000) or directly blocking the InsP₃ receptor by microinjection of a specific antibody (Nakade *et al.*, 1991; Miyazaki *et al.*, 1992) dramatically impaired spontaneous Ca²⁺-spiking activity of isolated pacemaker cells. Xestospongin C also blunted spontaneous Ca²⁺-spiking activity in E9.5 embryonic heart. Thus, the intracellular Ca²⁺ signaling network conserved the ability to regulate the cardiac pacemaker throughout development. Besides ionic conductance (i.e., I_f, Ca²⁺ currents), our findings reveal in the fetal life a second cardiac pacemaking mechanism mediated by intracellular Ca²⁺ cycling. Whether this Ca²⁺-dependent mechanism is a regulator or is required together with the I_f current-dependent pacemaking activity and maintained in the adulthood, a stage at which InsP₃Rs are confined to the conduction system (Gorza *et al.*, 1993), remains to be elucidated.

In this study, we provide direct evidence that ER-mediated Ca²⁺ dynamics are important in excitable cells at both embryonic and fetal stages. Movement of Ca²⁺ in and out of the ER plays a crucial role in the excitability of embryonic cardiomyocytes. Overstimulation of the InsP₃-dependent pathway (Jaconi *et al.*, 2000) or perturbation of Ca²⁺ homeostasis in pacemaker cells during cardiac development may then be at the origin of rhythm-related cardiac diseases at the adult stage. Indeed, taking into account the refractory period of the conductive cells, a change in pulse frequency and/or rhythm after alteration of ER Ca²⁺ cycling in pacemaker cells is likely to lead to a conduction block, as observed in mice overexpressing calreticulin in the heart (Nakamura *et al.*, 2001).

ACKNOWLEDGMENTS

We thank Dr. P. Travo (Centre National de la Recherche Scientifique [CNRS], Montpellier) for skillful help in cell imaging, Dr. P. Charnet (CNRS, Montpellier) for help in electrophysiology and Dr. A. Terzic (Mayo Foundation, Rochester, MN) for critical reading of the manuscript. We are also grateful to Prof. M. Berridge for his encouraging advice in this study and to Dr. M. D. Bootman for fruitful discussions. We thank Dr. C. Erneux (Brussels, Belgium) for the gift of the InsP₃ phosphatase cDNA, Dr. Matteo Mangoni (IGH, CNRS, Montpellier) for advices in monitoring I_f current in cells and critical review of the manuscript, and the Institut de Recherches Servier (Suresnes, France) for generously providing ivabradine. We thank Chantal Jacquet and Eric Jouffre in the animal house of the IGMM for breeding the mice (CNRS, Montpellier). This study was funded by the Association Française contre les Myopathies (AFM grant No. 8524 to M.P.). A.M. is a fellow from the Ministère de la Recherche et de la Technologie. F.A. is a fellow from the Fondation de la Recherche Médicale. C.M. was a fellow from the Groupement de Réflexion sur la Recherche Cardiovasculaire. M.M. is a Canadian Institutes for Health Research Senior Investigator and AHFMR Scientist. M.P. is an INSERM established investigator.

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