Mathematical Model of Mouse Embryonic Cardiomyocyte Excitation–Contraction Coupling

Topi Korhonen, Risto Rapila, and Pasi Tavi
Institute of Biomedicine, Department of Physiology and Biocenter Oulu, University of Oulu, 90014 Oulu, Finland

Excitation–contraction (E–C) coupling is the mechanism that connects the electrical excitation with cardiomyocyte contraction. Embryonic cardiomyocytes are not only capable of generating action potential (AP)-induced Ca\(^{2+}\) signals and contractions (E–C coupling), but they also can induce spontaneous pacemaking activity. The spontaneous activity originates from spontaneous Ca\(^{2+}\) releases from the sarcoplasmic reticulum (SR), which trigger APs via the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX). In the AP-driven mode, an external stimulus triggers an AP and activates voltage-activated Ca\(^{2+}\) in- 
trusion to the cell. These complex and unique features of the embryonic cardiomyocyte pacemaking and E–C coupling have never been assessed with mathematical modeling. Here, we suggest a novel mathematical model explaining how both of these mechanisms can coexist in the same embryonic cardiomyocytes. In addition to experimentally characterized ion currents, the model includes novel heterogeneous cytosolic Ca\(^{2+}\) dynamics and oscillatory SR Ca\(^{2+}\) handling. The model reproduces faithfully the experimentally observed fundamental features of both E–C coupling and pacemaking. We further validate our model by simulating the effect of genetic modifications on the hyperpolarization-activated current, NCX, and the SR Ca\(^{2+}\) buffer protein calreticulin. In these simulations, the model produces a similar functional alteration to that observed previously in the genetically engineered mice, and thus provides mechanistic explanations for the cardiac phenotypes of these animals. In general, this study presents the first model explaining the underlying cellular mechanism for the origin and the regulation of the heartbeat in early embryonic cardiomyocytes.

INTRODUCTION

In this issue (see p. 397), we presented experimental characterization and central functional components of the excitation–contraction (E–C) coupling and pacemaking of embryonic (E9-E11) mouse ventricular cardiomyocytes (Rapila et al., 2008). According to the experiments, these cells are capable of maintaining their activity alone by producing spontaneous cytosolic calcium oscillations upon repetitive calcium releases from the SR as suggested earlier (Sasse et al., 2007). The same cells are also capable of producing action potential (AP)-induced calcium influx and subsequent CICR from the SR upon electrical stimulation. In the developing heart, the APs triggered could conduct from cell to cell, thereby synchronizing the electrical activity and contraction of adjacent cells. In addition to this, we identified the mechanisms behind the spontaneous activity of the SR release. We showed that these calcium oscillations require functional ryanodine receptors (RyRs), inositol-3-phosphate receptors (IP\(_3\)Rs), and SR Ca\(^{2+}\) ATPase (SERCA) (Rapila et al., 2008). Further, we showed that the frequency of the spontaneous oscillations depends on the calcium leak through the IP\(_3\)Rs, which provides a mechanism for the regulat- 
ation of the heart rate of the embryonic heart. Collectively, the detailed experimental characterization of the individual features of E–C coupling and pacemaking in E9-E11 myocytes introduces a view of a rather complicated array of cellular functions. Therefore, to further analyze how these different mechanisms operate in parallel, we built a mathematical model into which we incorporated the experimentally characterized components of calcium signaling and excitability of these cells. Mathematical modeling has been widely used as a tool in explaining and studying E–C coupling in adult cardio- myocytes (Luo and Rudy, 1994; Dokos et al., 1996; Jafri et al., 1998; Pandit et al., 2001; Bondarenko et al., 2004). However, based on our results, the differences between embryonic and adult cardiomyocytes are so dramatic that novel approaches are required to model the embryonic cardiomyocyte E–C coupling and pacemaking. In embryonic cardiomyocytes, the cytosolic Ca\(^{2+}\) signals are more heterogeneous than in adult cardiomyocytes (Rapila et al., 2008). Therefore, instead of the common pool approach used in the adult myocyte models (Luo and Rudy, 1994; Dokos et al., 1996; Jafri et al., 1998; Pandit et al., 2001; Bondarenko et al., 2004), a more detailed description of the cytosolic Ca\(^{2+}\) dynamics was required. In addition, the SRs in adult models (Luo and Rudy,
tion dynamics. A fundamental feature of the model is that in
1999; Wakimoto et al., 2000; Nakamura et al., 2001; and pacemaking, this type of SR dynamics had to be intro-
introduced to the model.

Here, we present a model of E–C coupling and pace-
making in E9-E11 mouse ventricular cardiomyocytes with
the novel features described above. For developing the
model, we also characterized the major ion currents in
the sarcolemmal (SL) membrane of E9-E11 cardiomyo-
cytes. The model is constructed based on this electro-
physiological data and data from our accompanying
paper (Rapila et al., 2008). We use the model to study if
the identified SL ion currents and the SR components
(IP3R, RyR, and SERCA) are sufficient to explain the
function of E9-E11 cardiomyocytes. With the model we
show the following: (1) IP3R, RyR, and SERCA, with obligatory roles for all, can produce spontaneous cytosolic Ca2+
oscillations at the rate of ∼0.2–1.1 Hz; and (2) the identi-
fied SL ion currents together compose an excitable membrane that (a) produces APs when cytosolic Ca2+
oscillates spontaneously, (b) produces APs and CICRs
when exposed to external electrical excitation, and (c)
is quiescent without either of these stimuli. We further
simulate the model with modified hyperpolarization-
activated current (Ih), Na+ /Ca2+ exchanger (NCX), and
SR Ca2+ buffer calreticulin amounts and show that our
simulations are in line with previous experimental stud-
ies with corresponding transgenic animals (Mesaeli et al.,
1999; Wakimoto et al., 2000; Nakamura et al., 2001;
Stieber et al., 2003). These simulations validate our model
further and also provide the underlying mechanism for
the observed E–C coupling and pacemaking alterations
in these transgenic animals.

MATERIALS AND METHODS

Electrophysiological Recordings

The cell isolation and culturing and the electrophysiological
recordings were performed as described in this issue in Rapila et al. (2008). The used solutions and recording methods are summa-
rized in Table S1, which is available at http://www.jgp.org/cgi/content/full/jgp.200809961/DC1. Ion currents were measured
at +25°C. The membrane currents were scaled by dividing them
with the measured cell membrane capacitance.

Mathematical Model of the Embryonic Ventricular Myocyte

A mathematical model of the E9-E11 embryonic mouse ventricu-
lar myocyte was developed and simulated. The model describes
the function of SR and SL ion channels, membrane voltage dy-
namics, and cytosolic Ca2+, Na+ and K+, and SR Ca2+
concentration dynamics. A fundamental feature of the model is that in
addition to a time coordinate, the cytosolic Ca2+ concentration
is also modeled as a function of a spatial coordinate. All param-
eters of the components and the environmental parameters of
the model were fitted to our own experimental data and experi-
mental conditions (in this and in Rapila et al. [2008]) whenever
possible and necessary. Complete model equations are shown
in the Supplemental text, which is available at http://www.jgp.
org/cgi/content/full/jgp.200809961/DC1.

Structure of the Model. As shown in Fig. 1, the cell and the in-
tracellular structures were assumed to have spherical shapes.
The nucleus is located in the center of the cell and the SR on
the surface of the nucleus, and the cytosol is the space between
the SL and SR. The dimensions of the structure were estimated
from our confocal and light microscope images and from patch-
clamp data by calculating the membrane area and from
the membrane capacitance (Table S2, available at http://www.jgp.
org/cgi/content/full/jgp.200809961/DC1).

Ca2+ Diffusion and Buffering in Cytosol. The spatial differences
of the Ca2+ concentration within the cytosol were modeled using Fick’s
law of diffusion. For the calcium diffusion coefficient Dc, of the
model, we used the value previously reported for calcium in water,
0.79 μm2/ ms (Cüssler, 1997) (Table S5, available at http://www.jgp.
org/cgi/content/full/jgp.200809961/DC1). With this Dc value,
the model reproduced well the delay in Ca2+ signal between cytosol
areas below the SR and SL, which was experimentally defined by
Rapila et al. (2008) (28.5 ± 4.9 ms; n = 12). Due to the shape of the
model cell, the diffusion is modeled using spherical coordinates
and by assuming radial symmetry. The cytosolic Ca2+ buffering of
troponin C and calmodulin is modeled as described previously
(Luo and Rudy, 1994). The free and buffered Ca2+ concentrations
are calculated for all radii values between boundaries. Embryonic
myocytes have a lower Ca2+ binding capacity than adult myocytes,
which is mostly caused by a lower troponin C concentration (Creazzo
et al., 2004). Based on this, we reduced the amount of troponin C
to 30% and calmodulin to 75% of the original values.

SL Ion Channels. The models of the NCX (Luo and Rudy, 1994), Ij
(Pandit et al., 2001), fast Na+ current (I Na ) (Bondarenko et al.,
2004), slowly activated delayed rectifier K+ current (I K1D ) (Dokos
et al., 1996), time-independent background K+ current (I K0 )
(Dokos et al., 1996), L-type Ca2+ current (I CaL ) (Bondarenko et al.,
2004), and T-type Ca2+ current (I CaT ) (Dokos et al., 1996) were fi tted to
our whole cell voltage-clamp data (Fig. 2). The fi tted conductances
were scaled from T = 25°C (in Fig. 2) to T = 34°C with Q 10-NCX = 1.35
(Hart, 1983; Hille, 2001), except Q 10-NCX = 1.6 (Debetto et al., 1990;
Puglisi et al., 1996; Shannon et al., 2004) and Q 10-K0 = 1.8 (Puglisi
et al., 1999; Shannon et al., 2004). The Ij time constant was scaled with
Q 0 = 3 (Hart, 1983; Hille, 2001). As described previously, the
fi ting of the I Na was based partly on the AP upstroke proper-
ties (Bondarenko et al., 2004). The background Ca2+ current is mod-
ed as a linear ohmic current and fi tted to obtain a physiologically
correct diastolic [Ca2+]c (∼0.1–0.2 μM). The model of the NaK-
ATPase (Luo and Rudy, 1994) was fi tted to maintain physiologically
correct intracellular Na+ and K+ concentrations (∼14 and ∼140
mM, respectively). The ion currents change the cytosolic [Ca2+]c
below the SL and the cytosolic common pool [Na+]c and [K+]c.

SR. SR is modeled as a common pool compartment for Ca2+ ions.
In the SR the Ca2+ is buffered with calreticulin using a similar buff-
ering model as in the cytosol. Ca2+ ions move from the SR to
the cytosol via RyR and IP3R and from the cytosol to SR via SERCA.
The models of these channels and the Ca2+ flux via these channels
interact with the cytosolic [Ca2+]c below the SR ( [Ca2+]c,subSR ). The
function of RyR is modeled using a previously published model
(Keizer and Levine, 1996). The function of IP$_R$ is modeled using a model that takes the [IP$_3$] as an input parameter and computes the IP$_R$ Ca$^{2+}$ flux based on the open (O) and active (A) states of the receptor (Sneyd and Dufour, 2002). The results presented here were simulated using [IP$_3$] = 0.075 μM unless stated otherwise. The model of SERCA (Jafri et al., 1998) is used with half-maximal activation by [Ca$^{2+}$]$_{r, m}$ set to 0.25 μM (Frank et al., 2000).

The values for calreticulin concentration, amounts of SR ion channels, and [IP$_3$] were fitted to give a physiologically relevant (Ca$^{2+}$) transient amplitude, a fractional release comparable to our measurements, and spontaneous Ca$^{2+}$ oscillation frequencies that are comparable to the experimentally observed range of oscillation frequencies (0.1–1.3 Hz; Rapila et al. [2008]).

**Simulation Methods.** The model consists of 31 ordinary differential equations combined with one partial differential equation. The model was simulated in the Matlab 6.5 (The Mathworks) environment using a self-written solver algorithm for combined partial and ordinary differential equation systems based on the forward Euler method (Korhonen and Tavi, 2008). We used a spatial step size of Δr = 0.1 μm and time step sizes of Δt = 1–0.00001 ms to obtain solutions with a relative error of <0.1% at each integration step. The simulations shown here were started from the steady-state initial conditions. The steady-state conditions were obtained by simulating the model until the intracellular ion concentrations were stable. The electrical stimulation of the model cell was simulated by applying a stimulus current of −80 pA/pF (−120 pA/pF in Fig. 5) for 0.5 ms. In some simulations, the models of individual ion channels were driven with a voltage-clamp protocol. The effect of caffeine was simulated by setting the diffusion rate of RyR Ca$^{2+}$ flux to a large constant value (see Supplemental text). The SERCA Ca$^{2+}$ flux was set to zero, as the Ca$^{2+}$ is extruded almost instantaneously. The de-esterification of IP$_3$-AM to IP$_3$ in cytosol is not instant. The de-esterification of IP$_3$-AM to IP$_3$ in cytosol is not instant. The de-esterification of IP$_3$-AM to IP$_3$ in cytosol is not instant. The de-esterification of IP$_3$-AM to IP$_3$ in cytosol is not instant. The de-esterification of IP$_3$-AM to IP$_3$ in cytosol is not instant.

**Data Analysis**

Data analysis was made using the Clampfit 9.2 (MDS Analytical Technologies), Origin 7.5 (OriginLab Corporation), and Matlab 6.5 (The Mathworks) software. Clampfit’s leak resistance subtraction and software filters were used when necessary. Matlab’s (The Mathworks) “nlinfit” function for nonlinear least-squares data fitting by the Gauss-Newton method was used to fit the model components to experimental data. Results are expressed as mean ± SEM.

**Online Supplemental Material**

The online supplemental material includes a table for the used solutions and recording methods in electrophysiological recordings (Table S1), the parameters of the model (Tables S2–S5), complete model equations (Supplemental text), a figure of the simulated effects of RyR, IP$_R$, and SERCA blocks (Fig. S1), and an animation of the simulated spontaneous activity (Video 1). The online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.200809961/DC1.

**RESULTS**

**Electrophysiological Characterization of the Mouse E9-E11 Ventricular Myocytes**

We identified seven major ion current types from E9-E11 mouse ventricular cardiomyocytes (Fig. 2). The I$_{\text{NCX}}$ measured as the Ni$^{2+}$-sensitive current had a density of −0.55 ± 0.19 pA/pF ($n$ = 7) at −100 mV. The theoretical reversal potential for I$_{\text{NCX}}$ in the recording solution (3E$_{\text{Na}}$−2E$_{\text{Ca}}$ = −15 mV) (Despa et al., 2003) agrees with the reversal potential in our experimental I-V relation for I$_{\text{NCX}}$ (−12 mV, Fig. 2 A). As described previously (Yasui et al., 2001), we found an I$_{\text{f}}$ current, which activates slowly (τ ~0.3–3 s) upon hyperpolarization of the cell membrane. The threshold for the activation of I$_{\text{f}}$ was −70 mV (Fig. 2 B). The recorded I$_{\text{f}}$ current had a similar I-V relation shape, as described previously (Doevendans et al., 2000) (Fig. 2 C), and had a large peak current of −92.1 ± 13.8 pA/pF at −20 mV ($n$ = 11). We identified two types of repolarizing K$^+$ currents: an I$_{\text{KDR}}$ and an I$_{\text{KI}}$ (Fig. 2 D). The I$_{\text{KDR}}$ is active at $V_m$ above −30 mV, and it corresponds to the early repolarization of the AP. The I$_{\text{KI}}$ operates at smaller $V_m$ values and contributes to the late repolarization of the AP and to the resting potential (r.p.). Both L- and T-type voltage-activated Ca$^{2+}$ channels are present in embryonic mouse cardiomyocytes (Seisenberger et al., 2000; Gribbs et al., 2001). We recorded the I$_{\text{CaL}}$ with a peak amplitude of −4.5 ± 0.6 pA/pF ($n$ = 11) and I$_{\text{CaT}}$ with a peak amplitude of −0.59 ± 0.21 pA/pF ($n$ = 5) (Fig. 2 E). We included all of these seven types of ion channels in the model with similar voltage-dependent behavior as recorded in our experiments (Fig. 2). In addition to these currents, the NaK-ATPase and background Ca$^{2+}$ leak were included in the SL membrane (Fig. 1).

**Simulation of the Pacemaking and E–C Coupling of an E9-E11 Cardiomyocyte**

In Rapila et al. (2008) in this issue, we showed that the spontaneous Ca$^{2+}$ signals in E9-E11 cardiomyocytes...
require both IP$_3$R and RyR at the SR. We suggested that the Ca$^{2+}$ leak via IP$_3$R increases the cytosolic Ca$^{2+}$ and triggers the Ca$^{2+}$ release from RyR. In 48–72-h cultured early embryonic cardiomyocytes (Sasse et al., 2007) and in stem cell–derived differentiating cardiomyocytes (Mery et al., 2005), IP$_3$Rs are able to produce [Ca$^{2+}$], oscillations alone. These kind of oscillations have been modeled before, and they require either biphasic (activation/inactivation) calcium-dependent modulation of IP$_3$R (Atri et al., 1993) or oscillation of cytosolic [IP$_3$] (Politi et al., 2006). Sensitivity to [IP$_3$] is common to all IP$_3$R isoforms (Ramos-Franco et al., 1998; Hagar and Ehrlich, 2000), whereas calcium-dependent inactivation is characteristic to IP$_3$R-type 1 only (Hagar et al., 1998; Ramos-Franco et al., 1998). In our experiments (Rapila et al., 2008), IP$_3$R activation alone did not produce spontaneous Ca$^{2+}$ oscillations. Activation of IP$_3$Rs produced only an [IP$_3$]-dependent constant Ca$^{2+}$ leak. Consequently, IP$_3$Rs have no Ca$^{2+}$-dependent inactivation with increasing [Ca$^{2+}$]. Because these calcium activation features are characteristic of the type 2 IP$_3$R (Ramos-Franco et al., 1998), the predominant IP$_3$R isoform in ventricular myocytes (Perez et al., 1997), we implemented a model of the type 2 IP$_3$R (Sneyd and Dufour, 2002) to the SR of our mathematical model. Together with modeled RyRs (Keizer and Levine, 1996) and IP$_3$Rs in the simulation of our model with no external stimulus, the model produces spontaneous activity that is initiated by a slowly increasing Ca$^{2+}$ leak via IP$_3$R from SR to cytosol (Fig. 3, A and B, and Video 1, which is available at http://www.jgp.org/cgi/content/full/jgp.200809961/DC1). When a certain threshold in cytosolic [Ca$^{2+}$] near the SR is crossed, the RyRs are triggered and a large Ca$^{2+}$ release occurs from the SR to the cytosol. Altogether, the simulated RyR and IP$_3$R channels have quite different dynamic features; RyRs produce rapid transient openings, whereas the IP$_3$R open probability changes slowly and remains almost constant in the time scale of the pacemaking cycle (Fig. 3 B and Video 1).

The SR Ca$^{2+}$ release and NCX have been shown to play a role in embryonic pacemaking (Sasse et al., 2007) and adult pacemaker cells (Bogdanov et al., 2001; Vinogradova et al., 2005). In our accompanying paper in this issue, we showed that the spontaneously released Ca$^{2+}$ diffuses in the cytosol from SR to SL, where it triggers an AP (Rapila et al., 2008). The triggering of the AP was shown to depend on the $I_{\text{NCX}}$. E9-E11 cardiomyocytes have a relatively
long diffusion distance from SR to SL, which is implemented into the model. Long distance results in a time delay between the near-SR and near-SL Ca\(^{2+}\) increment during simulated spontaneous SR Ca\(^{2+}\) release and cytosolic Ca\(^{2+}\) diffusion (Fig. 3 A and Video 1). The increment of the near-SL Ca\(^{2+}\) activates a depolarizing \(I_{\text{NCX}}\) current when NCX extrudes Ca\(^{2+}\) (Fig. 3 C and Video 1). The \(V_m\) depolarizes slowly until the threshold for \(I_{\text{Na}}\) activation is crossed and the AP is initiated. The activated \(I_{\text{Na}}\) causes rapid depolarization of the cell membrane and subsequently also activates the voltage-activated Ca\(^{2+}\) channels, \(I_{\text{CaL}}\) and \(I_{\text{CaT}}\) (Fig. 3 C and Video 1).

Even though the E9-E11 cardiomyocytes are capable of producing their cytosolic Ca\(^{2+}\) transients and subsequent APs spontaneously, the activity can also be synchronized with an external stimulus (Rapila et al., 2008). The cells can thus maintain their activity alone but also synchronize their activity with other cells to form coordinated contraction. In line with the experiments, the simulations showed that the pacemaking and E–C coupling mode can coexist in the same cell. We simulated the effect of an external electrical excitation during spontaneous activity of the model cell. The membrane voltage and intracellular Ca\(^{2+}\) oscillations were synchronized to the frequency of the stimulus, and the intracellular Ca\(^{2+}\) gradients were reversed compared with the spontaneous activity (Fig. 4 A). During external pacing, the Ca\(^{2+}\) increases first at the SL side when AP-activated Ca\(^{2+}\) intrudes to the cytosol (Fig. 4 A). This Ca\(^{2+}\) diffuses through the cytosol to the sub-SR region and activates CICR via RyR. The overlay of the \(I_{\text{NCX}}\) during spontaneous and externally evoked activity reveals a depolarizing “hump” in the \(I_{\text{NCX}}\) curve that activates the AP during spontaneous Ca\(^{2+}\) oscillations, but which is not naturally present in the \(I_{\text{NCX}}\) curve during external pacing (Fig. 4 B).

The difference between spontaneous and paced activity can also be seen from the net SL and SR fluxes (Fig. 4 C). This naturally results from the fact that during the spontaneous activity, the Ca\(^{2+}\) is first released from the SR side, whereas during the paced activity the Ca\(^{2+}\) release occurs first at the SL side. The released amounts of Ca\(^{2+}\) from both sides of the cytosol are taken up during relaxation. This produces net integrals of zero for the SR and SL Ca\(^{2+}\) fluxes over one cycle of activity, and thus the Ca\(^{2+}\) homeostasis is maintained in the model (Fig. 4 C). During spontaneous activity, the net SL Ca\(^{2+}\) flux extrudes 1.1 µM of Ca\(^{2+}\) when NCX triggers the AP. The voltage-activated Ca\(^{2+}\) channels \(I_{\text{CaL}}\) along with the minor contribution of \(I_{\text{CaT}}, I_{\text{Cab}},\) and \(I_{\text{NCX}}\) reverse mode (Ca\(^{2+}\) intrusion mode) together provide sufficient Ca\(^{2+}\) intrusion, compensating for the Ca\(^{2+}\) extrusion by NCX during activation of the AP. This compensation prevents the depletion of SR Ca\(^{2+}\) and cessation of the spontaneous activity in E9-E11 cardiomyocytes as we described in Rapila et al. (2008).

Simulations with our model produce [Ca\(^{2+}\)]\(_i\), [Na\(^{+}\)]\(_i\), and [K\(^{+}\)]\(_i\) values that are physiologically coherent (Table I) (Bers, 2001), and the r.p. and AP amplitude values are also comparable to experimental data (Table I) (Rapila et al., 2008). In the experiments, we also estimated the fractional Ca\(^{2+}\) release by comparing the amplitude of the twitch Ca\(^{2+}\) transient, which equals the sum of calcium fluxes from the SR and the SL to cytosol, to the amplitude of the caffeine-induced Ca\(^{2+}\) transient, which represents the whole SR Ca\(^{2+}\) content. By comparing the twitch Ca\(^{2+}\) transient to the caffeine-induced transient, it was calculated that during a spontaneous calcium
Model of Embryonic Cardiomyocyte E–C Coupling

Table I, which is ~14% of that in the adult mouse ventricular myocyte (142 ± 9 μM) (Maier et al., 2003). To make a reasonable comparison, the Ca²⁺ concentrations above are in the cytosol volume of the corresponding cell type to normalize the effect of different cell size between adult and embryonic cardiomyocytes.

The mode for spontaneous Ca²⁺ oscillations and the mode for AP-induced CICR can be separated on the basis of their resting potential boundaries. We simulated the spontaneous and externally paced activity of the model cell while adjusting the membrane voltage with constant current injection. We found that spontaneous calcium release can operate at relatively depolarized membrane potentials between −88 to −27 mV, whereas AP-induced CICR is recruited between −107 to −38 mV (Fig. 5). The spontaneous activity cannot operate at r.p. values below −88 mV because hyperpolarization increases Ca²⁺ extrusion via NCX and thereby depletes the SR Ca²⁺ content. On the other hand, the externally triggered AP-driven mode cannot operate at r.p. values above −38 mV because depolarization inactivates the voltage-activated Ca²⁺ channels in the SL.

Below −70 mV, 1 Hz pacing is not possible because longer diastole is required to refill the SR due to the higher Ca²⁺ extrusion via NCX. On the other hand, above −70 mV the cell cannot be synchronized to pacing frequencies 0.25 and 0.5 Hz because the frequency of the spontaneous activity is higher. This is in line with our suggestion (Rapila et al., 2008) that the activity in tissue is synchronized to the cell with the highest frequency.

It has been reported that embryonic cardiomyocytes have a smaller cytosolic Ca²⁺ buffering capacity per cytosol volume than adult cardiomyocytes (Creazzo et al., 2004). Taking this into account in the model, smaller Ca²⁺ cycling per cytosol volume between the cytosol and Ca²⁺ sources (SR and the extracellular space) is required to produce contraction and relaxation. In the embryonic cardiomyocyte, the amount of cycled Ca²⁺ (i.e., the net Ca²⁺ release to cytosol) during a spontaneous twitch in cytosol volume is 9.6 – 1.1 + 5.0 = 13.5 μM (Fig. 4 C), which is 35% of that in the adult mouse ventricular myocyte (39 ± 3 μM) (Maier et al., 2003). Furthermore, the diastolic SR Ca²⁺ content in cytosol volume in the spontaneously beating embryonic cardiomyocyte is 19.3 μM
transients. In contrast to this, the simulated effect of the applied IP3-AM stimulating the IP3 R with RyR block produces only an increasing Ca2+ leak via IP3 R, which slowly increases the diastolic [Ca2+]i (Fig. 7 B). Both of these simulations are in line with our experiments (Rapila et al., 2008) and with our previous conclusions that the RyR is the only possible route for transient Ca2+ release in these cells, but the IP3 R plays a role as a stimulator of RyR.

To support our conclusion that IP3 R Ca2+ leak stimulates RyR openings, we showed that stimulation of IP3 Rs with IP3-AM increases the frequency of spontaneous activity (Rapila et al., 2008). This same regulatory mechanism is reproduced in our model (Fig. 7 C). In the simulations, the oscillation frequency showed sigmoidal dependence on the [IP3]. The possible oscillation frequencies achieved with [IP3] regulation in the model cell were in the range of 0.2 to 1.1 Hz. In the experiments (Rapila et al., 2008), the frequencies of the spontaneous activities (global cytosolic Ca2+ transients and APs) were all within the range of 0.1 to 1.3 Hz (Fig. 7 D). This supports the idea that the dynamics and the structure of SR in the model are comparable to those in real E9-E11 cardiomyocytes. Modeling also supports the conclusions drawn from the experiments and demonstrates that in theory [IP3] could act as a major regulator of the embryonic heart beat frequency.

Model Predictions of the Embryonic Pacemaking and E–C Coupling Defects
During the early embryonic cardiac development, genetic disruption of the ion channel proteins regulating excitability (Stieber et al., 2003) or calcium influx (Weissgerber et al., 2006) as well as proteins involved in cardiomyocyte calcium handling (Takeshima et al., 1998; Wakimoto et al., 2000) lead to drastic changes in calcium signaling and heart rate, with a consequent increase in embryonic lethality. We have shown (Figs. 3, 4, 6, and 7) that our model reproduces faithfully the normal features of the Ca2+ signaling and excitability of E9-E11 myocytes. Modeling also supports the conclusions drawn from the experiments and demonstrates that in theory [IP3] could act as a major regulator of the embryonic heart beat frequency.

Figure 5. The resting potential windows favoring spontaneous Ca2+ activity and Ca2+ activity generated by electrical pacing. A constant current from 4 to −2.5 pA/pF with 0.25 pA/pF intervals was applied to the model cell to adjust the r.p. of the model cell to values between −107 and −27 mV. At each r.p. value, the spontaneous and paced (0.25, 0.5, and 1 Hz) cytosolic Ca2+ activity was observed for 10 s after 40 s of stabilization of the model. The figure shows the average amplitudes spontaneously evoked and electrically stimulated cytosolic [Ca2+] transients at given frequency.

and experiments (Rapila et al., 2008) to 42.3 versus 32.5 ± 2.3% (average cytosolic Ca2+ transient), respectively, as no CICR occurred. Just like in the experiments (Rapila et al., 2008), the block of either SERCA or IP3 R both stopped the spontaneous activity while the electrical excitability was maintained (Fig. S1, available at http://www.jgp.org/cgi/content/full/jgp.200809961/DC1). Based on these simulations, the ion current types and densities recorded from E9-E11 myocytes (Fig. 2) are incapable of producing Vm oscillations without an intracellular Ca2+ stimulus or external electrical excitation. For spontaneous Ca2+ oscillations, all three SR Ca2+ handling components are required. Without SERCA the SR Ca2+ stores are depleted, without RyRs the IP3 Rs cannot produce transient Ca2+ signals, and without IP3 Rs the RyRs are not activated without a sufficient cytosolic Ca2+ trigger.

To further validate the function of the SR and the dynamics of RyRs and IP3 Rs, we simulated the cytosolic Ca2+ under pharmacological stimulation of these receptors as in our experiments (Rapila et al., 2008). In the presence of IP3 R block, the simulated effect of caffeine, which rapidly opens the RyRs, produces a rapidly activating large Ca2+ transient (Fig. 7 A). The amplitudes of the sub-SR and sub-SL caffeine-induced Ca2+ transients are ~1.5-fold compared with normal spontaneous Ca2+ transients. In contrast to this, the simulated effect of the applied IP3-AM stimulating the IP3 R with RyR block produces only an increasing Ca2+ leak via IP3 R, which slowly increases the diastolic [Ca2+]i (Fig. 7 B). Both of these simulations are in line with our experiments (Rapila et al., 2008) and with our previous conclusions that the RyR is the only possible route for transient Ca2+ release in these cells, but the IP3 R plays a role as a stimulator of RyR.

To support our conclusion that IP3 R Ca2+ leak stimulates RyR openings, we showed that stimulation of IP3 Rs with IP3-AM increases the frequency of spontaneous activity (Rapila et al., 2008). This same regulatory mechanism is reproduced in our model (Fig. 7 C). In the simulations, the oscillation frequency showed sigmoidal dependence on the [IP3]. The possible oscillation frequencies achieved with [IP3] regulation in the model cell were in the range of ~0.2 to 1.1 Hz. In the experiments (Rapila et al., 2008), the frequencies of the spontaneous activities (global cytosolic Ca2+ transients and APs) were all within the range of 0.1 to 1.3 Hz (Fig. 7 D). This supports the idea that the dynamics and the structure of SR in the model are comparable to those in real E9-E11 cardiomyocytes. Modeling also supports the conclusions drawn from the experiments and demonstrates that in theory [IP3] could act as a major regulator of the embryonic heart beat frequency.

Figure 6. Simulated effect of RyR block. The near-SL and near-SR cytosolic [Ca2+] and APs are shown from simulations of (A) spontaneous and (B) electrically excited activity. The RyR block was applied after three control E–C coupling cycles.
The hyperpolarization-activated cardiac “pacemaker” current $I_f$ is encoded by three genes, HCN1, HCN2, and HCN4 (Yasui et al., 2001). It has been suggested that $I_f$ is responsible for generating the spontaneous activity in embryonic ventricular cardiomyocytes (Yasui et al., 2001) as well as in specialized pacemaker cells (Stieber et al., 2003). Genetic ablation of the HCN4 reduces $I_f$ in E10.5 mouse cardiomyocytes by ~75%, with concomitant slowing of the heart rate by ~35%, leading to embryonic lethality without structural anomalies between E9.5 to 11.5 (Stieber et al., 2003). In the model, reduction of the $I_f$ from its the normal, measured density slows down the frequency of the spontaneous Ca$^{2+}$ signals and APs, in line with the results from HCN4$^{-/-}$ cardiomyocytes (Stieber et al., 2003) (Fig. 8, A and B). On the other hand, up to twice an increase of $I_f$ has very little effect on the spontaneous rate of the individual myocyte, although it suppresses the amplitude of the APs (Fig. 8, A and B). Our experiments (Fig. 2 B) (Rapila et al., 2008) and modeling (Figs. 6 and 8 A) indicate that neither $I_f$ current density nor the voltage activation range ($\sim -70$ mV threshold) of embryonic $I_f$ is sufficient for initiating spontaneous $V_m$ oscillations and APs at the resting potential ($\sim -57.2 \pm 0.9$ mV) (Rapila et al., 2008) of E9-E11 myocytes. However, the current density affects the excitability of the membrane by two additional mechanisms (Fig. 8, B and C), which might contribute to the phenotype of HCN4$^{-/-}$ mice hearts. First, $I_f$ current is partly carried by Na$^+$ ions; therefore, the $I_f$ current density will effect the cytosolic [Na$^+$] and consequently modulate the NCX current, which will be augmented at lower $I_f$ densities (lower [Na$^+$]), and suppressed at higher $I_f$ densities (higher [Na$^+$]) (Fig. 8 B). Second, because $I_f$ is one of the depolarizing currents in embryonic myocytes, reduction of the current density will affect the excitability and the resting potential of the membrane (Fig. 8 C). Even though our experiments and modeling do not suggest a central role for $I_f$ in developing E9-E11 ventricular myocytes, these results do not rule out the possibility that when the true pacemaker cells develop, the $I_f$ current would have a central role in regulating the embryonic heartbeat. Interestingly, it has been suggested that also in fully developed pacemaker cells this SR-originated activity could contribute to the spontaneous activity (Bogdanov et al., 2001).

According to our experiments (Rapila et al., 2008), the NCX has a crucial role in embryonic cardiomyocytes in linking the spontaneous calcium activity with the membrane voltage. Therefore, it is not a surprise that ablation of the cardiac NCX isoform NCX1 results in embryonic lethality before E10, severe cardiomyocyte malformations, and lack of spontaneous heartbeats (Wakimoto et al., 2000). In line with the function of E9.5 NCX1$^{-/-}$ in mouse cardiac myocytes, our model predicts that lack of NCX activity prevents triggering of the AP and decreases the spontaneous activity due to cytosolic calcium accumulation, incapacitating the calcium release. Although mouse embryos with targeted inactivation of NCX1 lack spontaneous heartbeats, cardiomyocytes isolated from NCX1-null embryos are still excitable and generate Ca$^{2+}$ signals when electrically stimulated (Koushik et al., 2001). In line with this, model cells with reduced NCX current density generate cytosolic Ca$^{2+}$ transients when stimulated, but during pacing calcium accumulates in the cytosol because of the reduced calcium extrusion capacity (Fig. 9). On the other hand, overexpression of NCX also inhibits spontaneous activity by depleting the calcium stores, but additionally leads to a hyperexcitable membrane and arrhythmias during external pacing (Fig. 9).

One of the few genetic models highlighting the role of the intracellular calcium stores during cardiac development is the mouse model lacking calreticulin (Mesiel et al., 1999), a ubiquitous SR Ca$^{2+}$ buffer expressed in cardiac myocytes during embryonic development.
shaped curve (Fig. 10 B). With both reduced and increased calreticulin content, membrane excitability and voltage-activated calcium influx are retained. However, with calreticulin overexpression, CICR is enhanced due to increased SR calcium content and release, which leads to arrhythmic spontaneous calcium releases and APs between stimulated APs.

**DISCUSSION**

Here, we present a mathematical model of E–C coupling and pacemaking of E9-E11 mouse ventricular cardiomyocytes. The model explains how these two separate modes for producing Ca\(^2+\) signals, the spontaneous activity originating from the SR triggering APs via NCX and the externally initiated AP-driven activity triggering Ablation of the calreticulin gene is lethal before E18 due to severe cardiac malformation and impaired function (Mesaeli et al., 1999). In our model, the simulated reduction of calreticulin reduces the SR Ca\(^2+\) buffering and release, eventually stopping the spontaneous activity (Fig. 10 A). Overexpression of calreticulin has been reported to cause lower heart rate during embryonic development and cardiac failure and sudden cardiac death during postnatal development (Nakamura et al., 2001). In our model, the simulated overexpression results similarly in a reduced frequency of the spontaneous calcium releases with increases in released and SR Ca\(^2+\) (Fig. 10 A). These experimental findings (Mesaeli et al., 1999; Nakamura et al., 2001) and our simulations predict that the relationship between the amount of calreticulin and the spontaneous beating rate is a bellow-shaped curve (Fig. 10 B). With both reduced and increased calreticulin content, membrane excitability and voltage-activated calcium influx are retained. However, with calreticulin overexpression, CICR is enhanced due to increased SR calcium content and release, which leads to arrhythmic spontaneous calcium releases and APs between stimulated APs.

**Figure 8.** The simulated effect of genetic modification of the I\(_{f}\). (A) Simulated effects of ablation (0.25×) and overexpression (2×) of I\(_{f}\). The near-SL and near-SR cytosolic [Ca\(^{2+}\)] and APs are shown during spontaneous activity, which is followed by an application of SR Ca\(^{2+}\) flux block and 1-Hz electrical excitation. (B) Relative changes in [Na\(^{+}\)], and spontaneous beating frequency by altered I\(_{f}\) expression. Insert shows the I\(_{\text{NCX}}\) with different I\(_{f}\) expression levels (0.25×, 1×, and 2×). (C) 3-D plot of the effect of varying I\(_{f}\) current density on the excitability and AP amplitude at different resting potentials.

**Figure 9.** Effects of varying I\(_{\text{NCX}}\) density on the simulated calcium signals and membrane voltage of embryonic cardiomyocytes. Decreased I\(_{\text{NCX}}\) (0.25× control) causes cytosolic calcium accumulation and stops the spontaneous activity (middle) by altering calcium flux through RyRs (J\(_{\text{RyR}}\), insert). Upon simulated external electrical stimulation, the model produces cytosolic calcium transients and APs, where calcium transients rely on calcium flux through voltage-activated calcium channels. Increased I\(_{\text{NCX}}\) (3× control) enhances calcium extrusion, depleting the SR calcium stores (insert), thus stopping the spontaneous activity. The model cell retains its excitability, but due to lower [Ca\(^{2+}\)], levels facing RyRs, calcium influx can only induce sporadic SR calcium releases, thus causing irregular activity (right panels).
function of Ca\textsuperscript{2+} transporting ion channels would need to be calculated separately in all points of the SL and SR surface grid. This would increase the computational cost exponentially and severely limit the practical usability of the model. Our model is capable of explaining the embryonic E–C coupling and pacemaking mechanisms and underlying mechanisms of several genetically engineered embryonic mice. The spherical shape is thus a fair compromise between computational efficiency and the validity of our model.

The Simulated Ca\textsuperscript{2+} Oscillations in E9-E11 Cardiomyocytes
The spontaneous Ca\textsuperscript{2+} oscillations between cytosol and SR/ER have been modeled previously in other cell types than cardiac myocytes (Deyoung and Keizer, 1992; Keizer and Levine, 1996; Sneyd et al., 2003). The models have included the Ca\textsuperscript{2+}-ATPase pump and IP\textsubscript{3} R (Deyoung and Keizer, 1992) or RyR (Keizer and Levine, 1996) or both of these receptors (Sneyd et al., 2003), as Ca\textsuperscript{2+} routes between the SR/ER and cytosol. These models produce cytosolic Ca\textsuperscript{2+} oscillations at the rate of \(0.1-0.01\) Hz (Deyoung and Keizer, 1992; Keizer and Levine, 1996; Sneyd et al., 2003), whereas the spontaneous Ca\textsuperscript{2+} oscillations in the E9-E11 cardiomyocytes had a frequency of \(0.1-1.3\) Hz (Rapila et al., 2008). Here, we showed with modeling that this rapid oscillation (\(\sim 1\) Hz) can also be theoretically produced by the combined activity of RyRs, IP\textsubscript{3} Rs, and SERCA (Fig. 3). The basis of this activity is the \([\text{IP}_3]\)-dependent IP\textsubscript{3} R Ca\textsuperscript{2+} leak, which triggers the large Ca\textsuperscript{2+} releases via RyR (Figs. 3 and 7) and thereby increases the endogenously low spontaneous opening rate of RyRs (Keizer and Levine, 1996). Thus, as in the experiments (Rapila et al., 2008), the magnitude of the IP\textsubscript{3} R Ca\textsuperscript{2+} leak regulates the frequency of the Ca\textsuperscript{2+} oscillations and APs triggered by this oscillation.

In line with our experiments (Rapila et al., 2008), the RyR, IP\textsubscript{3} R, and SERCA all play obligatory roles in producing oscillations (Fig. 6 and Fig. S1). However, as long as all of these components are present to some extent, the oscillation seems to be robust, as it was maintained even with \(\pm 25\%\) change in the amount of RyR, IP\textsubscript{3} R, or SERCA (unpublished data). The cardiac myocytes undergo dramatic changes during development; therefore, the SR-originated oscillation might be a stable backup that guarantees the cell’s capability to maintain its activity in these varying conditions.

Electrophysiology of the E9-E11 Cardiomyocytes
Here, we presented the first complete characterization of membrane ion currents in E9-E11 mouse ventricular cardiomyocytes (Fig. 2). Based on our simulations and experiments (Rapila et al., 2008), the NCX is the most crucial ion current regulating the activity of the embryonic cardiomyocytes. In E9-E11 cardiomyocytes, the I\textsubscript{NCX} triggers membrane depolarization during spontaneous activity and regulates the r.p. and intracellular CICR, coexist in individual cardiomyocytes. In addition to normal myocyte function, our mathematical model elucidates the E–C coupling and underlying mechanisms of the phenotypes of genetically engineered embryonic mouse models. Based on this validation, our mathematical model is the first comprehensive description of how the embryonic heartbeats are generated and regulated in E9-E11 cardiomyocytes.

Limitations of the Model
The delay from SR Ca\textsuperscript{2+} release to AP upstroke is larger in our model than in the experiments (Rapila et al., 2008). This is due to the approximation of the shape of the model cell to a sphere with radial symmetry. The dimensions of real cells are more similar to a flat ellipsoid. Consequently, there is variability in the diffusion distance between SL and SR and in the diffusion restriction in different parts of the cell. The local Ca\textsuperscript{2+} concentrations and consequently I\textsubscript{NCX} are thus larger in parts of real cells, resulting in faster activation of the AP than in our model. In a more realistic ellipsoid-shaped model cell, the description of the diffusion would require three coordinates. The [Ca\textsuperscript{2+}] would have spatial differences in directions parallel to the SL and SR surfaces, and the...
It has been suggested that I_f generates the spontaneous activity in embryonic cardiomyocytes (Stieber et al., 2003). Although we found a significant amount of I_f from E9-E11 myocytes, results in this and our accompanying paper in this issue suggest that the I_f does not initiate the spontaneous activity in E9-E11 cardiomyocytes. The pharmacological inhibition of the I_f in the experiments (Rapila et al., 2008) and the simulated overexpression and down-regulation of the I_f did not stop the rhythmic Ca^{2+} activity triggering the contraction and APs via NCX. The I_f in E9-E11 cardiomyocytes shows a significant active current only at membrane voltages lower than r.p. (Fig. 2 B); thus, even a doubled amount of current in E9-E11 myocytes cannot generate spontaneous APs (Fig. 8 A). Similarly to I_f, we found a small amount of functional T-type Ca^{2+} channel current in E9-E11 cardiomyocytes (Fig. 2). The specific method to measure T-type current would have been recording it as an Ni^{2+}-sensitive current (Furukawa et al., 1992). However, the T-type current was separated sufficiently from the L-type current based on voltage clamps from different holding potentials as described previously (Niwa et al., 2004).

According to model simulations, the modes for spontaneous Ca^{2+} oscillations and AP-induced Ca^{2+} transients can be separated on the basis of their resting potential boundaries (Fig. 5). We found that spontaneous calcium release can operate effectively at relatively depolarized membrane potentials, whereas AP-induced CICR is favored when the membrane potential is more hyperpolarized. This difference between the excitation modes in respect to their r.p. boundaries may represent the order of recruitment of these modes during development because the cardiomyocyte resting potential hyperpolarizes during the progress of fetal development (Reppel et al., 2007). During cell migration in early development the cells are isolated from others (Buckingham et al., 2005), and even with depolarized r.p., their spontaneous activity guarantees that their activity is maintained. Later during development, the cells with more negative r.p. values form a stable tissue wherein the phenotype is shaped toward that of non-spontaneously active adult ventricular cardiomyocytes, which form coordinated contraction induced by an external stimulus (Kamino, 1991). Also, it is energetically cheaper not to provide the AP-driven mode until it is required for efficient and more coordinated contraction because based on our simulations, maintaining a hyperpolarized r.p. requires larger NaK-ATPase activity and therefore induces a higher ATP consumption rate (unpublished data).

**Regulation of the Embryonic E–C Coupling and Heartbeat**

The physical structure of E9-E11 cells sets boundary conditions for the E–C coupling and pacemaking. The lack of T tubules in E9-E11 myocytes results in loose coupling between SR and SL ion channels and other components. This limits the possible heartbeat frequency because a certain time is required for Ca^{2+} diffusion between SR and SL. Furthermore, any alterations in the shape and the size of the cytosol affect the dynamics of the cytosolic Ca^{2+} signals, further modulating the heartbeat frequency. Due to the lack of T tubules and loose spatial coupling between I_{CaL} and RyR, a relatively large SL Ca^{2+} intrusion is required for a strong enough Ca^{2+} signal to diffuse through the cytosol and initiate CICR from SR. In the adult mouse ventricular myocyte, with tight I_{CaL} and RyR coupling, only ~10% of total cytosolic Ca^{2+} intrusion originates from SL and ~90% from SR during CICR (Maier et al., 2003). Our simulations suggest that during AP-induced CICR, ~42% of cytosolic Ca^{2+} intrusion occurs from the SL and ~58% from the SR (Fig. 4 C). In addition, our results suggest that a strong SR function is essential for spontaneous activity in embryonic cardiomyocytes as well as for synchronizing the contractile activity via the AP-driven mode.

On the basis of our results, several contributing mechanisms for the regulation of the beating rate of the embryonic hearts can be suggested. In general, because the spontaneous activity originates solely from the single cell and it is not controlled by any outside triggering mechanism, several parameters within the cell may affect the rate of activity. A dynamic regulation in the time scale of seconds could be addressed via regulation of the activity of IP_3Rs, RyRs, and SERCA. One example of the direct modulation would be the [IP_3], which was shown here to strongly regulate the cardiomyocyte beating frequency (Fig. 7). The upper boundary of the [IP_3]-dependent beating frequency is limited because the increment of [IP_3] also depletes the SR Ca^{2+} required for spontaneous releases. Subsequently, the relationship between cardiomyocyte beating frequency and [IP_3] is a bell-shaped curve. The activity of SERCA could modulate heart rate because altering the refilling velocity of the SR alters the kinetics of the relaxation phase of the Ca^{2+} oscillation as well as Ca^{2+} leak through IP_3Rs. Based on this, it is possible in theory that the dynamic regulatory mechanisms directly or indirectly affecting SERCA function, such as the β-adrenergic pathway, will regulate heart rate by modulating the SR Ca^{2+} uptake. In a slower time scale, the heartbeat could be obviously regulated by altering the expression levels of RyR, IP_3R, and SERCA as well as calreticulin (Fig. 10 B). The SL ion channels also affect the heart rate. They may contribute directly, such as I_{CaL} and I_{NCX}, or indirectly, such as the I_f (Fig. 8), to the kinetics of Ca^{2+} fluxes at the SL and furthermore to the SR Ca^{2+} content and dynamics.
Here, we have presented a characterization and a mathematical model of E–C coupling and pacemaking in early embryonic (E9-E11) mouse ventricular cardiomyocytes. To develop the model, we presented novel approaches in cardiomyocyte modeling. The model includes an implementation of a noncommon pool cytosol and oscillatory SR dynamics incorporated with SL ion current dynamics as characterized in our experiments. The model explains the underlying mechanisms behind the functional dualism of these cells (Rapila et al., 2008). The model reproduces the SR-driven spontaneous activity and the AP-driven externally evoked activity. The model also elucidates the mechanisms behind the altered E–C coupling and pacemaking in genetically modified embryos. The current understanding of the embryonic cardiomyocyte E–C coupling and pacemaking is shaped by the suggestions of the underlying mechanisms based on experimental results, which so far have been controversial. This study shows by mathematical modeling how several of the suggested mechanisms can operate in parallel to control the initiation and regulation of the embryonic heartbeat.

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