Published in final edited form as:

J Mol Cell Cardiol. 2011 January ; 50(1): 66–76. doi:10.1016/j.yjmcc.2010.09.018.

Rhythmic beating of stem cell-derived cardiac cells requires dynamic coupling of electrophysiology and Ca cycling

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Abstract

There is an intense interest in differentiating embryonic stem cells to engineer biological pacemakers as an alternative to electronic pacemakers for patients with cardiac pacemaker function deficiency. Embryonic stem cell-derived cardiocytes (ESCs), however, often exhibit dysrhythmic excitations. Using Ca^{2+} imaging and patch-clamp techniques, we studied requirements for generation of spontaneous rhythmic action potentials (APs) in late-stage mouse ESCs. Sarcoplasmic reticulum (SR) of ESCs generates spontaneous, rhythmic, wavelet-like Local Ca^{2+} Releases (LCRs) (inhibited by ryanodine, tetracaine, or thapsigargin). L-type Ca^{2+} current (I_{CaL}) induces a global Ca^{2+} release (CICR), depleting the Ca^{2+} content SR which resets the phases of LCR oscillators. Following a delay, SR then generates a highly synchronized spontaneous Ca^{2+} release of multiple LCRs throughout the cell. The LCRs generate an inward $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) current (absent in Na^+ -free solution) that ignites the next AP. Interfering with SR Ca^{2+} cycling (ryanodine, caffeine, thapsigargin, cyclopiazonic acid, BAPTA-AM), NCX (Na^+ -free solution), or I_{CaL} (nifedipine) results in dysrhythmic excitations or cessation of automaticity. Inhibition of cAMP/PKA signaling by a specific PKA inhibitor, PKI, decreases SR Ca^{2+} loading, substantially reducing both spontaneous LCRs (number, size, and amplitude) and rhythmic AP firing. In contrast, enhancing PKA signaling by cAMP increases the LCRs (number, size, duration) and converts irregularly beating ESCs to rhythmic “pacemaker-like” cells. SR Ca^{2+} loading and LCR activity could be also increased with a selective activation of SR Ca^{2+} pumping by a phospholamban antibody.

Conclusions—SR Ca^{2+} loading and spontaneous rhythmic LCRs are driven by inherent cAMP/PKA activity. I_{CaL} synchronizes multiple LCR oscillators resulting in strong, partially synchronized diastolic Ca^{2+} release and NCX current. Rhythmic ESC automaticity can be achieved by boosting “coupling” factors, such as cAMP/PKA signaling, that enhance interactions between SR and sarcolemma.

Keywords

Embryonic stem cells; cardiac differentiation; local Calcium release; ion currents; biological pacemaker; cAMP; Protein Kinase A

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Disclosures: None

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Introduction

Sick Sinus Syndrome is a disorder and/or a loss of sinoatrial node pacemaker function that causes symptomatic bradycardia, which can lead to syncope and even sudden death. While electronic pacemakers remain a satisfactory therapy for Sick Sinus Syndrome, they have many limitations and shortcomings, including size (in pediatric patients), need for replacement, possibility of infection, absence of the autonomic rate modulation, etc. (review [1]). Cell therapy is thus emerging as an alternative promising approach for repair of the hearts pacemaker [2]. Since embryonic stem (ES) cells can differentiate into any cell type of the body, including highly differentiated heart cells [3], ES cell-derived cardiocytes (ESCs) have been recently extensively explored as a valuable substrate for creating the biological pacemakers [2].

Since in vitro-grown ESCs generate spontaneous action potentials (APs), and functionally integrate with ventricular myocytes in vitro [4] and with the host myocardium in vivo [5], they can indeed pace hearts after their implantation (as reported in guinea pigs [4] and pigs [6]) and can even overcome complete atrioventricular block in animal models [6]. While these results provide proof-of-concept evidence for these cells to function as biological pacemakers, some fundamental problems of this approach still remain unresolved.

Although spontaneously beating ESCs express major cardiac ion currents [3] and Ca^{2+} cycling proteins [7,8], they are not true adult pacemaker cells. Accordingly, a major problem to directly use the ESCs as biological pacemakers is that they beat irregularly and can cause arrhythmia [9,10]. Presently there is no effective means to improve rhythm characteristics of spontaneously beating ESCs.

Here we demonstrate that rhythmic automaticity of ESCs requires a complex dynamic integration of electrogenic sarcolemmal proteins (ion channels and transporters) and Ca^{2+} cycling proteins (Suppl.Fig.S1), i.e. similar to that recently discovered in adult sinoatrial node cells (SANC), the primary cardiac pacemaker cells (recent reviews [11,12] and numerical studies [13,14]). Specifically, diastolic local Ca^{2+} releases (LCRs) from sarcoplasmic reticulum (SR) activate $\text{Na}^+/\text{Ca}^{2+}$ exchanger current, but L-type Ca^{2+} current during AP synchronizes the phases of LCR oscillators. Based on this mechanism, the present paper suggests a new principle of how to convert dysrhythmic beating of ESCs to rhythmic beating, a characteristic of a robust biological pacemaker, by boosting “coupling” factors, such as cAMP/PKA signaling, that drive spontaneous LCRs and enhance interactions between SR and sarcolemma.

Material and Methods

Mouse ES cells of line R1 were differentiated into cardiocytes using the “hanging drop” technique [3,15,16]. Single ESCs were isolated from in vitro-grown embryoid bodies by microdissection followed by collagenase treatment [15,16] (see Online Supplement for details). Since this study explored the integration of subcellular and sarcolemmal mechanisms of ESC automaticity, we used only late-stage differentiated ESCs (“7+ 9 to 20” days) that reportedly express both major ion channels (I_{CaL} , I_{f} , I_{Na} , I_{to} , I_{K})[3], exchangers (NCX)[17] and Ca^{2+} cycling proteins (RyR2, SERCA2A, calsequestrin and phospholamban)[7,8](Suppl.Fig.S1). The fact that our cells generate high amplitude APs and Ca^{2+} transients in current clamp and spontaneous Ca^{2+} releases under voltage clamp (Results) indicates that these cells express major cardiac ion channels and Ca^{2+} cycling proteins. Details of electrophysiology, confocal Ca^{2+} imaging, data analysis, and chemical skinning of ESCs are described in Online Supplement. Pharmacological tools, used here to target key components of the integrative mechanism of ESC rhythmic automaticity, are also

described in the Online Supplement (Suppl.Fig.S1). Chemicals were purchased from Sigma and Invitrogen. Doses of drugs are shown in the figures. Data are presented as mean $\pm$ SEM. The statistical significance was evaluated by Student's *t* test. The signals were stable for the duration of our experiments (tested in time-matched vehicle controls, Suppl.Fig.S3,S4).

Results

Substantial spread in rhythmicity of AP firing by ESCs

While many ESC exhibit spontaneous activity, the degree of regularity of spontaneous beating among different cells varies from rather regular (rhythmic) to very irregular (dysrhythmic) (Fig.1A,B). The rhythmicity of AP firing was assessed by calculating the cycle length (CL) variability index, also known as coefficient of variation [18], defined as $100\% \times \text{CL standard deviation} / \text{CL mean}$. ESCs exhibit a broad range of CL variability index varying from about 5-12% ("rhythmic" cells) up to 45% ("dysrhythmic" cells) (Fig.1C). Correlation analysis of CL variability vs. basic electrophysiological parameters is presented in Suppl.Fig.S5.

ESCs possess Ca^{2+} clock similar to adult cardiac pacemaker cells

The major distinctive property of natural pacemakers, i.e. adult SANC, is its ability to spontaneously generate rhythmic wavelet-like LCRs (" Ca^{2+} clock") independent of membrane function when activation of surface membrane ion channels is disabled in chemically skinned or voltage-clamped cells [12]. We experimentally tested whether ESCs, similar SANC, also possess the Ca^{2+} clock generating spontaneous LCRs under these conditions. Indeed, at a low bathing $[\text{Ca}^{2+}]$ of 150 nM and 35°C, chemically skinned ESCs produce wavelet-like LCRs (Fig.2A) that are rhythmic (see a power spectrum in Fig.2B) with a dominant frequency ranging from 1 to 6.5 Hz (3.9 ± 0.5 Hz, $n=10$ cells). The LCRs range from 1.8 to 22 μm (5.58 ± 0.12 μm , 747 LCRs, 17 cells) in size and from 20 to 110 ms in duration (49.2 ± 0.9 ms) (Fig.2C,D). LCR size and duration are highly variable for any individual cell, with maximum and minimum differing at least in two times. The LCRs are likely produced by SR ryanodine receptors (RyRs) as they are inhibited by disabling either the RyRs (with ryanodine or tetracaine) or the SR Ca^{2+} pump (with thapsigargin) (Fig.2E-G).

Also, similar to adult cardiac pacemaker cells [19,20], spontaneously beating ESCs generate LCRs (shown by triangles in Fig.3A) during the diastolic period, i.e. between APs or between global transients induced by the APs. The LCRs in intact cells range between 2 and 28 μm (5.94 ± 0.24 μm , $n=354$) in size and 10 to 100 ms (35.98 ± 0.84 ms, $n=354$) in duration (distributions in Fig.3C,D), i.e. similar to that in saponin-skinned ESCs (Fig.2C,D). The LCRs occur primarily in the second half of the cycle, with the occurrence rate increasing (i.e. becoming gradually more synchronous) before next excitation (distribution in Fig.3B for 423 LCRs measured in 21 cells).

LCRs generate spikes of inward NCX current that ignites action potentials

Next we tested the electrophysiological effects of LCRs. When activation of ion channels and APs are both disabled by voltage clamp at -50 mV, spontaneous LCRs occur for at least 5 s (see example in Fig.4A). Similar to the skinned cell configuration (Fig.2), the wavelet-like LCRs under voltage clamp are rhythmic with a dominant frequency of 2.3-5.2 Hz (3.95 ± 0.19 Hz, $n=4$) (Fig.4B). Under voltage clamp, LCRs generate spikes of inward currents (Fig.4A, middle panel). The periodicity of the current spikes is identical to that of the LCR peaks (Fig.4C, and see also arrows connecting 4 first current peaks and 4 first Ca^{2+} signal peaks in Fig.4A). The almost coincident occurrence of LCRs and current spikes indicates a close cause-effect relationship between the two. LCRs likely cause the current

spikes via NCX activation. The y-intercept in Fig.4C is the time delay between the NCX and LCR peaks. The negative value indicates that the LCR peak precedes the NCX peak by 15.4 ms. The delay is likely due to a propagation from the site of confocal measurement of the LCR to the NCX (membrane) location. The LCRs activate NCX current spikes because the high-amplitude current spikes (ranging 10 to 20 pA in control) disappear when NCX function is disabled in Na⁺-free medium (Na⁺ is substituted by Li⁺), and the residual small spikes (if any) become comparable to the current noise (within ~5 pA) under these conditions (tested in 4 cells, see example in Fig.4D).

We performed “a first order estimate” for the total charge movement of one current spike and found that this charge can indeed substantially depolarize the cell membrane. The current spike shown in Fig 4D has a magnitude of about $I=10$ pA and lasts about $t=100$ ms, yielding a total charge movement of $Q=I \cdot t=10\text{pA} \cdot 0.1\text{s}=1\text{pC}$. For a typical ESC with electrical membrane capacitance of $C=50$ pF, the respective voltage change ΔV is estimated to $\Delta V=Q/C=1\text{pC}/50\text{pF}=0.02\text{V}=20\text{mV}$.

To test the importance of these LCR-activated currents for spontaneous beating, we shortly (for ~1 s) applied a Na⁺-free solution (“Na⁺-free spritz”) to spontaneously beating cells under zero-current perforated patch clamp and simultaneously imaged intracellular Ca²⁺ by confocal microscopy (Fig.4E). This Na⁺-free spritz resulted in acute, transient, partial inhibition of NCX (Na⁺ removal is incomplete during such short time). Under these conditions rhythmic LCRs continue (similarly to that in our voltage clamp experiments, Fig. 4A) and produce partial depolarizations, insufficient to trigger APs.

We interpret this result to indicate that the LCR-initiated, NCX-mediated current spikes couple LCRs and the APs. Indeed, the excitation failure occurs because the cell is unable to depolarize to the threshold for firing of Ca²⁺ current and/or Na⁺ current (many ESCs express Na⁺ current [3]). Of note, since Na⁺ channels are highly permeable to Li⁺ (ionic permeability ratio $P_{\text{Li}^+}/P_{\text{Na}^+}=0.93$ [21], Na⁺ channel currents are expected to remain almost unchanged under these conditions. Importantly, as current spikes disappear in the Na⁺-free, Li⁺ solution, there was no remarkable change in the base current (i.e. the current offset vs. 0 level marked by arrow in Fig.4D), indicating that the net activity of all Na⁺-sensitive electrogenic mechanisms, other than NCX, is affected insignificantly by Li⁺ substitution for Na⁺.

Another possible effect of Na⁺ replacement might be related to I_f channel, which has a smaller conductance for Li⁺ vs. Na⁺ (although both minor vs. that for K⁺: $P_{\text{Na}^+}/P_{\text{K}^+}=0.11$ and $P_{\text{Li}^+}/P_{\text{K}^+}=0.03$ [22]). To exclude the possibility that excitation failure in Fig.4E is linked to I_f, and also to test the importance of I_f in spontaneous beating of ESCs, we also examined effects of I_f inhibition. Specific blockade of I_f by 2 mM Cs²⁺ or by 7 μM UL-FS-49 (zatebradine) did not stop the spontaneous beating of ESCs, but moderately and reversibly reduced their beating rate (by ~24 and 22%, $n=16$ and 15 cells, respectively, Suppl.Fig.S7,A-D). Our separate voltage clamp experiments insured that Cs⁺ and zatebradine indeed effectively suppressed I_f in ESCs (Suppl.Fig.S7,E,F).

LCRs are synchronized by prior I_{CaL}-triggered Ca²⁺-induced Ca²⁺ release (CICR)

To record simultaneous activity of several local Ca²⁺ oscillators generating LCRs, the confocal line was extended over almost entire cell length (~70 μm). It is important to note that this setting is different to that in our experiments in Fig.4, where the observation was limited to only one such Ca²⁺ oscillator within a span of 15-30 μm. Our voltage-clamp protocol included 5 preconditioning voltage pulses of 100 ms duration applied from -40 mV to 0 mV (1Hz rate). This pulse train was applied each time before a 200 ms testing pulse

(Fig.5) in order to attain a standardized SR Ca^{2+} load (after the train) via the consecutive activation of I_{CaL} (and Ca^{2+} influx).

I_{CaL} time course during the testing pulse had two peaks: the first peak activated by depolarization and the second peak (after 200 ms) in the form of the tail current (upper red trace in Fig.5). Thus, each testing voltage pulse activated CICR two times: first by the depolarization-induced I_{CaL} peak and then, 200 ms later, by the I_{CaL} current tail. (Of note, CICR via I_{CaL} tail in cardiac cell under voltage clamp is well-known phenomenon and has been recently demonstrated also in ESCs [23]). Therefore about 300 ms after the I_{CaL} tail-mediated CICR, the truly spontaneous releases (marked as “Synchronized spontaneous Ca^{2+} release” in the figure) emerge almost synchronously (i.e. largely overlapping in time), with the “fast” oscillator firing first, the “medium” one-next, and the “slow” one-the last (Fig.5). It is important to note that I_{CaL} truly synchronizes spontaneous LCRs because LCRs before the testing pulse appeared to be random, i.e. not synchronized (Online suppl.Fig.S8). Such I_{CaL} -synchronized LCRs' emergence results in a strong spatially averaged Ca^{2+} release (Fig. 5, bottom trace). In the absence of a subsequent synchronizing event, i.e. I_{CaL} -activated CICR, the second average spontaneous Ca^{2+} release has a larger spread in time and smaller amplitude (spontaneous LCRs emerge less synchronously). Since intrinsic rates of LCR oscillators are different (e.g. slow, medium, and fast in Fig.5), LCRs eventually become out of phase (i.e. desynchronized), with their spatial average becoming noisy. This pattern of I_{CaL} -induced LCRs' synchronization was highly reproducible (tested in 8 cells), with the number (2 to 8) of partially synchronized LCR firings depending on the difference of the intrinsic rates of the local Ca^{2+} oscillators.

If I_{CaL} is required for resetting and synchronization of LCRs (Fig.5), the rhythmic pacemaking activity of ESCs must be sensitive to the blockade of I_{CaL} . Indeed, suppression of spontaneous beating produced in the presence of partial pharmacological blockade of I_{CaL} by 200 nM of nifedipine was preceded by a temporal phase (of about 1 min duration) with substantial CL variability (tested in 6 cells, see an example in Suppl.Fig.S9).

Ca^{2+} clock is driven by cAMP/PKA activity and is required for rhythmic AP firing

In order to demonstrate that generation of rhythmic APs requires SR-generated LCRs, we interfered to SR function and inhibit LCRs by buffering intracellular Ca^{2+} with BAPTA-AM, or by disabling RyR function with ryanodine, or by inhibiting SERCA with thapsigargin or cyclopiazonic acid (CPA) ($n=5-8$ cells for each intervention, see examples in Fig.6). Importantly, as LCRs decreased (in frequency and amplitude), spontaneous AP firing and Ca^{2+} transients became irregular with CL variability index significantly ($p<0.05$) increased from 12.7 ± 2.62 to 60.3 ± 13.02 , from 11.78 ± 1.66 to 57.4 ± 9.26 , and from 14.2 ± 3.68 to 47.8 ± 9.3 , from 12.4 ± 1.52 to $48.3\pm11.9\%$ after application of the aforementioned drugs, respectively, culminating in cessation of automaticity or in extremely rare beating. A representative example of a detailed time-course for development of CPA-induced irregular beating, followed by a washout, is shown in Suppl.Fig.10. The time course of the ryanodine effect was, in fact, biphasic, i.e. the phase of rate decrease and irregular beating was preceded by a temporal initial increase in beating rate (see an example in Suppl.Fig.11). Such biphasic pattern is likely related to known characteristics of ryanodine to effect increased SR Ca^{2+} leakage immediately following its application, followed by diminished leakage or release due to luminal SR depletion. Caffeine, which decreases the threshold of RyR activation by cytosolic Ca^{2+} , also induced irregular beating of ESCs culminating in cessation of automaticity (also likely due to luminal SR depletion) (Suppl.Fig.S12).

We tested whether basal cAMP-dependent PKA activation contributes to generation of spontaneous LCRs in ESCs. In saponin-skinned ESCs a specific peptide inhibitor of PKA (PKI) substantially decreases the number LCR oscillators (in 6 of 6 tested cells, see an

example in Fig.7A and autocorrelation analysis in Suppl.Fig.S17A), with the residual LCRs (if any) becoming significantly less rhythmic, less frequent, and smaller in size and amplitude (for average data see Suppl.Fig.S13). (Of note, “less frequent” means that less number of events was observed within the 100 μ m of scan line per unit of time.)

In contrast, application of cAMP to skinned cells (in 5 of 6 cells tested) resulted in the emergence of new local Ca^{2+} oscillators in many cell locations, which were “silent” before the cAMP boost (see an example in Fig.7B and autocorrelation analysis in Suppl.Fig.S17B). Two of 5 cAMP-responding cells, generated global, full-cell-length waves, i.e. their local Ca oscillators (including new oscillators) merge in one global oscillator. Analysis of LCR characteristics in 4 cells (of 6 tested cells) without global waves revealed that the LCRs become more rhythmic, more frequent, and larger in size (but not amplitude) (average data in Suppl.Fig.S14),

The mechanism of enhanced Ca^{2+} cycling by the SR resulting in LCRs in the basal state is likely linked to basal SR Ca^{2+} loading driven by the intrinsic cAMP/PKA-signaling, because the amplitudes of caffeine-induced Ca^{2+} transients were significantly decreased (by ~29%) when cells were pretreated with PKI inhibiting PKA signaling (Fig.7C,D,E).

We further explored the mechanism of LCR generation using an anti-phospholamban (PLB) antibody 2D12 [24]. The antibody prevents association of PLB with SR Ca^{2+} pump (SERCA), thereby selectively accelerating SR Ca^{2+} pumping, but (in contrast to cAMP) not affecting the release channel (RyR) properties. Thus applying the antibody simulates the case of selective SR Ca^{2+} pumping activation when PLB is almost fully phosphorylated. The PLB-antibody increased the LCR signal mass via larger (spatiotemporal) size and amplitude, but the LCR rate remained almost unchanged (Suppl.Figs S16). In half of cells tested (4 from 8), PLB-antibody produced long-lasting (persistent) LCRs (Suppl.Figs S15A,B), similar to those observed by Zima et al. [25] at low tetracaine concentrations in skinned ventricular myocytes (i.e. when SR Ca^{2+} pumping is relatively large vs. Ca^{2+} release). Caffeine transients were increased by PLB-antibody (e.g. Suppl.Figs S15C) but not by cAMP (average data in Fig.7E).

In spontaneously beating ESCs PKA inhibition by PKI (tested in 8 cells, Fig.8A) resulted in irregular APs (CL variability index significantly increased from 12.45 ± 2.68 to $40.77 \pm 6.35\%$, $p < 0.05$). In contrast, enhancing cAMP/PKA signaling by application of a membrane-permeable cAMP analogue (CPT-cAMP) converted irregularly beating ESCs to rhythmically beating cells (Fig.8B). In 5 cells with irregular beating, CPT-cAMP clearly increased the spontaneous rate and simultaneously significantly ($P < 0.05$) improved CL variability index from $23.98 \pm 4.82\%$ (dysrhythmic AP firing) to $11.23 \pm 2.68\%$ (rhythmic AP firing), i.e. converted all of these “dysrhythmic” cells into “rhythmic”, “pacemaker-like” cells.

Discussion

Prior studies considered an essentially sarcolemma-driven ESC automaticity, such as an interplay of Ca^{2+} and K^+ currents [3], classical I_f mechanism [26], I_{CaT} [27], and I_{Na} window current [28]. Other studies considered basically one-way effect of Ca^{2+} release on membrane function [16,29-32]. The present study, for the first time, explored the integrated function of SR and sarcolemma in late-stage ESCs to generate rhythmic APs. Multiple types of experimental evidence presented here indicate that the rhythmic automaticity of these in vitro-derived cells requires dynamically coupled function of Ca^{2+} cycling and membrane ion current activation (summarized in Suppl.Fig.1), i.e. similar to that recently discovered in adult cardiac pacemaker cells [11,12].

Specific mechanisms of rhythmic beating include biophysical (I_{CaL} →synchronized LCRs→NCX → depolarization→ activation of $I_{CaL}+I_{Na}$) and biochemical (intrinsic cAMP/PKA activation) coupled function of Ca^{2+} clock and membrane clock (i.e. the ensemble of voltage-gated ion channels). Pharmacological inhibition of LCRs or uncoupling of LCRs from membrane function results in dysrhythmic beating, culminating in cessation of automaticity or extremely rare (i.e. non-physiological) beating (Fig.6, Fig.4E, Suppl.Figs S9-S12). Rhythmic LCRs (Power spectrum in Fig.4B) interact with membrane clock via activation of NCX to ignite rhythmic APs in ESCs (Fig.4). This LCR→NCX coupling mechanism of rhythmic ESC automaticity is similar to that found in adult SANC [20] and embryonic cardiomyocytes [33,34].

I_{CaL} , in turn, orchestrates the function of local Ca^{2+} oscillators. Simultaneous measurements of I_{CaL} and LCRs in the present study directly show that the phases of the local Ca^{2+} oscillators are reset by CICR triggered by prior I_{CaL} and the synchronized LCR emergence result in a strong average Ca^{2+} signal (Fig.5, “Synchronized spontaneous Ca^{2+} release”). The synchronized spontaneous Ca^{2+} releases occur with a delay of about 200-300 ms after peak I_{CaL} and the Ca^{2+} transient it triggers (Fig.5). This delay has been referred to as LCR period in studies of SANC and the average LCR period determines the cycle length of spontaneous AP firing (reviews [11,35]). Without synchronization by I_{CaL} , local Ca^{2+} oscillators eventually go out phase yielding a noisy spatial average (Fig.5 low panel, “LCRs are not synchronized”). The importance of such synchronization is evidenced by our finding that partial blockade of I_{CaL} by nifedipine (Suppl. Fig.S9) results in dysrhythmic beating before the cell becomes inexcitable. On the contrary, only one LCR oscillator (even rhythmic one) may not be powerful enough (i.e. insufficient) to drive the rhythmic beating of the entire cell. Figure 1B shows an example when a rather rhythmic LCR oscillator is operating in a dysrhythmic cell: the Ca^{2+} oscillator in the higher part of the scanning line makes 10 rhythmic cycles between beats 3 and 4, but these Ca^{2+} oscillations are uncoupled from the AP occurrences, so that the cell beats irregularly.

Prior detailed pharmacological studies of I_{CaL} regulation in ESCs (using okadaic acid, IBMX, milrinone, etc.[36,37]) suggested that “ESCs are characterized by a high intrinsic adenylyl cyclase activity, which is counterbalanced by high intrinsic activity of phosphodiesterases and phosphatases” (cited from [36]). Although the specific levels of cAMP in ESCs have yet to be measured, the present study demonstrates that such inherent cAMP/PKA activity is critical for integrative rhythmic automaticity of ESCs, as it enhances not only basal I_{CaL} [36,37], I_f [26], I_{Na} [38], and perhaps I_K , but also basal Ca^{2+} cycling resulting in abundant spontaneous LCRs (Figs 7,⁸,Suppl.Fig.1). The critical importance of basal PKA activity for LCRs and cardiac pacemaker function has been also recently discovered in rabbit SANC [39]. A novel finding of the present study is that an intrinsic PKA-activation maintains Ca^{2+} SR loading (Fig.7C-E) and thereby likely causes the spontaneous Ca^{2+} releases (e.g. via activation by luminal Ca^{2+} [40]). Our result that caffeine transients are increased by PLB-antibody but not by cAMP (Fig.7E) can be interpreted to indicate that selective activation of SR Ca pumping indeed increases SR Ca^{2+} loading in ESCs but the cAMP-induced increase of basal LCR activity is not associated with an increase of SR Ca^{2+} load per se, but likely involves enhanced and matched pumping-release (i.e. PLB-SERCA-RyR) function. Indeed cAMP naturally enhances and couples both pumping and release because cAMP-dependent PKA phosphorylates both PLB and RyRs as we previously showed in sinoatrial node cells [39].

Interestingly, local Ca^{2+} oscillators of the mouse ESCs persist for at least for 4 seconds under voltage clamp at the maximum diastolic potential (Fig.4A). This means that the mouse ESCs likely have a mechanism to protect against NCX-mediated cell Ca^{2+} depletion. One

possibility might be a link to store-operated Ca^{2+} influx, as recently discovered in mouse sinoatrial node [41].

One possibility to improve the rhythm of ESCs might be to decrease I_{K1} , resulting in decreased hyperpolarization (smaller MDP in Fig.1D) that will likely facilitate coupling of Ca^{2+} clock and membrane clock, shifting ESCs in their relations towards a more rhythmic behavior (to the left, Fig.1D). The idea of targeting I_{K1} has been indeed previously suggested for engineering biological pacemakers from cardiomyocytes [42]. However, stabilization of the resting potential via developmentally increasing expression of I_{K1} [3,43] is likely not the single factor that interferes with rhythmic beating of ESCs. Our results with cAMP and PKI suggest that the reasons for irregular and rare beating of a majority of late-stage ESCs likely also include a decreased activity of Ca^{2+} cycling proteins (e.g. due to a developmental decrease in intracellular cAMP level [44] and/or an increased phospholamban expression [8]). Previous studies have demonstrated that the rate and rhythm of spontaneous beating of ESCs with RyR2 knock-out is substantially impaired (38 vs. 143 bpm, “KO” vs. wild type ESCs, respectively)[16]. Thus, the present results show (Figs7,⁸ and Suppl.Fig.S1) that the rhythm of ESCs can be improved by boosting Ca^{2+} cycling (i.e. LCRs) and coupling of electrophysiological and Ca^{2+} cycling mechanisms via targeting regulatory proteins, e.g. to maintain the high level of basal cAMP/PKA activity (as in adult pacemaker cells [39]). Indeed in the present study exposure to a membrane-permeable cAMP analogue was sufficient to convert “dysrhythmic” and/or rarely beating ESCs into “rhythmic” ESCs (Fig.8B). A critically important factor in rhythmic beating of ESCs, however, is not just increased cAMP level per se but likely cAMP-dependent PKA-mediated phosphorylation, because “rhythmic” cells became “dysrhythmic” or ceased beating when their PKA activity was inhibited (Fig.8A).

This new principle to enhance rhythmic beating of ESCs via boosting cAMP/PKA signaling that couples electrophysiological and Ca^{2+} cycling mechanisms, can be also relevant to human ESCs and, therefore, to prospective designs of biological pacemakers for patients. Indeed, high expression of major Ca^{2+} cycling proteins, such as L-type Ca^{2+} channels, NCX, SERCA2, IP3 receptors, and RyRs that generate both Ca^{2+} transients and wavelet-like, diastolic LCRs have been observed not only in mouse ESCs [3,7,8,16,17,31], but also in human ESCs [32,45]. Furthermore, local control of Ca^{2+} releases by L-type Ca^{2+} channels in human ESCs [23] might be also important in synchronizing Ca^{2+} clocks (termed “local Ca^{2+} events” in these human cells[32]) to generate strong diastolic Ca^{2+} signals similarly to that shown in Figure 5.

In summary, the complex nature of cardiac pacemaker function and regulation has been the subject of debate and intensive experimental studies for long time. Evidence for the strongly coupled function of electrophysiology and intracellular Ca^{2+} cycling as a novel fundamental principle of normal cardiac impulse initiation and regulation in the sinoatrial node, the hearts natural pacemaker, is now emerging (recent reviews [11,12] and numerical studies [13,14]). The present study shows that this principle also underlies rhythmic automaticity of ESCs. Thus, genetic engineering of the ESC-based biological pacemakers to boost this cAMP/PKA-dependent integrative mechanism (Suppl.Fig.S1) would be of natural choice, i.e. evolution-selected and, therefore, highly robust and responsive.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the Intramural Research Program of the National Institutes of Health, National Institute on Aging. We thank Larry Jones from Indiana University for providing anti-phospholamban antibody.

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Fig.1.

Broad variations of ESC intrinsic rhythm. A,B: Examples of Ca^{2+} transients in rhythmically and irregularly beating ESCs. C: Histogram of Cycle Length (CL) variability index ($100\% \cdot \text{SD}/\text{mean}$) measured in 21 ESCs.

Fig.2.

SR Ca^{2+} clock in "skinned" ES cell-derived cardiocytes. ESCs possess an intracellular, SR-based Ca^{2+} clock: spontaneous LCRs in saponin-"skinned" ESCs are rhythmic and linked to SR function (RyRs and SERCA). A: Confocal line-scan image of a representative "skinned" ESC. B, power spectrum of a continuous 27s-long recording of LCRs (for the average signal in a cell fragment between triangles in panel A). C,D: Histograms of LCR size and duration (747 LCRs, 17 cells). E-G: Confocal line-scan images of representative ESCs before (left) and after (right) superfusion with ryanodine, tetracaine, or thapsigargin (of 9, 9, and 5 cells tested, respectively). LCR spatial size was indexed as the full width at the half maximum amplitude (FWHM), and its duration characterized as the full duration at half maximum amplitude (FDHM).

Fig.3.

Local Ca²⁺ releases occur more frequently before excitations. LCRs emerge during diastole, with the rate of occurrence increasing with time, peaking just prior to the beginning of the next AP. A: Simultaneous recording of APs (blue line) and Ca²⁺ signals (confocal line-scan image) in an intact single spontaneously beating ESC. LCRs are shown with arrowheads. Also shown is the time course of the normalized fluorescence (F/F₀) averaged along the scanline for the image above. B-D: Histograms of LCR phase, size, and duration (423 LCRs of 21 cells). The frequency of LCR occurrence within spontaneous cycles in panel B was measured by numerical comparison (in %) of each LCR period (a period of time from AP-induced Ca transient peak to the LCR peak) with the length of the spontaneous cycle, in which each LCR occurs.

Fig.4.

Rhythmic, membrane-independent LCRs produce spikes of inward NCX current that ignite rhythmic APs. A: Simultaneous recordings of membrane potential or current and confocal line-scan image of Ca^{2+} release in a representative spontaneously beating ESC before and during voltage clamp to -50 mV. B: Power spectrum of LCRs during voltage clamp. C: Correlation plot between timing of LCRs and current spikes under voltage clamp. D: Inward currents in an ESC clamped at -50 mV (control) disappear after cell exposure to a rapid, brief Na^+ -free (Li^+ -containing) solution that disables NCX function. The arrowhead indicates the zero current level. E: Simultaneous recording of Ca^{2+} signals and APs in a spontaneously-active ESC which was exposed to a rapid and brief superfusion with a Na^+ -

free solution (bar “Na⁺-free”) to inhibit NCX function. SR Ca²⁺ clock continues to generate LCRs, but the uncoupled LCR signals fail to ignite APs, until Na⁺ is restored and coupling re-established (“Coupled LCR signals”).

Fig.5.

I_{CaL} triggers CICR and thereby synchronizes phases of LCR oscillators. Shown is an example of simultaneous recordings of I_{CaL} (voltage clamp) and Ca^{2+} dynamics (confocal line scan imaging over the entire cell length). I_{CaL} was induced by a short voltage clamp pulse to 20 mV from a -50 mV holding potential. See text for details. To identify LCRs, our original confocal images were smoothed by spatiotemporal Gaussian filtering of 20×20 pixels ($27.9\text{ms} \times 4.76\mu\text{m}$).

Fig.6.

Ca^{2+} clock is crucial for rhythmic AP firing: Strong Ca^{2+} buffering (A) or interfering with Ca^{2+} release (B) or Ca^{2+} pumping (C,D) result in irregular beating, often culminating in cessation of automaticity. Shown are recordings of spontaneous APs and confocal line scan images of Ca^{2+} signals of representative ESCs (different cells for APs and Ca^{2+}) prior to and during exposure of the drugs (including an intermediate stage with irregular beating). Ca^{2+} images are shown along with their spatial average of the Ca^{2+} signal. The values for variability index of spontaneous APs are shown for each drug in control, in transition, at a steady state. The effects of ryanodine, thapsigargin and BAPTA-AM are not washable. A complete experiment with CPA effect, including washout, is provided in Online Suppl.Fig.S10. The effect of the drugs (except ryanodine) reached their steady-state within about 3 min. The effect of ryanodine was complex (biphasic, see Suppl. Fig.S11).

Fig.7.

Rhythmic local Ca^{2+} releases are driven by intrinsic cAMP/PKA activity that maintains SR Ca^{2+} loading. A, B: Confocal line-scan images of a representative skinned ESC before (top) and after (bottom) superfusion with a specific PKA inhibitor (PKI), or stimulation with cAMP. PKI decreases, but cAMP increases the number of local Ca^{2+} oscillators (demonstrated by autocorrelation analysis in Suppl. Fig. S17 for cell locations shown by red arrows). C,D: Representative examples of confocal line-scan images and their respective Ca^{2+} transients (spatial average) induced by rapid application of caffeine to skinned ESC in control and after incubation with PKI. E: Modulation of average amplitude (F/F_0) peak of caffeine-induced Ca^{2+} transients by PKI, cAMP, and PLB antibodies.

Fig.8.

Intrinsic cAMP/PKA activity is required for ESCs to exhibit rhythmic automaticity (pacemaker-like cells). Typical examples of APs and Ca^{2+} releases recorded in spontaneously beating ESCs (different cells) before and during superfusion with PKI (panel A) or with CPT-cAMP (panel B). While inhibition of PKA activity by PKI deteriorates ESC automaticity, cell stimulation with cAMP converts dysrhythmic ESCs into pacemaker-like cells. The values for variability index of spontaneous APs are shown in control, in transition, at a steady state, and after washout. The drugs reached their steady-state effects within about 3 min.