Mechanisms of spontaneous pacing: SA-nodal cells, neonatal cardiomyocytes, and human Stem cell derived cardiomyocytes.
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Abstract

The sino-atrial node is the primary site from which the mammalian heart is paced, but the mechanisms underlying the pacemaking still remain clouded. It is generally believed that the hyperpolarization-activated current \( I_f \), encoded by Hyperpolarization-activated cyclic nucleotide–gated (HCN) genes, contributes significantly to pacing, which in tandem with inward current generated by efflux of \( \text{Ca}^{2+} \) via the \( \text{Na}^+\text{-Ca}^{2+} \) exchanger (NCX), resulting from the released \( \text{Ca}^{2+} \), mediates the diastolic depolarization. Here we review the data that implicate \( I_f \) as the “pacemaker current” and conclude that there is not only a significant discrepancy between the range of diastolic depolarization potential (-60 to -40 mV) and the activation potential of \( I_f \) (negative to -70 mV), but that also the kinetics of \( I_f \) and its pharmacology are incompatible with the frequency of a heart beat in rodents and man. We propose that \( I_f \) serves as a functional insulator, which protects the SA-nodal cells against the large negative electrical sink of atrial tissue connected to it with connexins. We also evaluate the role of \( I_f \) and calcium signaling in mediating the diastolic depolarization in rat neonatal cardiomyocytes (rN-CM), and human induced pluripotent stem-cell derived cardiomyocytes (hiPSC-CM), and provide evidence for a possible involvement of mitochondrial \( \text{Ca}^{2+} \) in initiating the oscillatory events required for the spontaneous pacing.

Key words

Pacemaking mechanisms; SA-nodal; \( I_f \)/HCN; hiPSC-CM; mitochondrial \( \text{Ca}^{2+} \); Functional insulator; atrial electrical sink
Early studies

The role of the SA-node as the primary electrical oscillator of the heart became established with the advent of microelectrode technology in the 1950s. This advance made it possible to map the precise site of initiation of the electrical signal from the heart and establish the SA-nodal cells as the primary locus from where the cardiac action potential first emerges, spreading then to the atria, the A-V node and ventricles. These early studies also established that the action potentials of SA-nodal cells, unlike the atrial or ventricular muscles, had no stable resting potential, but showed slow periods of diastolic depolarization that cycled from a maximal diastolic potential of about -60 to -40mVs, where it triggered the rapid upstroke phase of the action potential, depolarizing the membrane to overshoot potentials of ~10mVs. The diastolic depolarization phase that slowly depolarizes the membrane from -60 to -40mVs was recognized as the electrophysiological signature of specialized pacing tissues in the heart (SA-node, AV-node, and Purkinje fibers bathed in low K⁺ solutions) (Brooks et al. 1955). SA-nodal cells, in particular, were found to be fairly insensitive to physiological changes in extracellular K⁺ or Na⁺, but the frequency of oscillations of diastolic depolarization was highly sensitive to adrenergic and cholinergic hormones, and to temperature and mechanical stretch (Brooks and Lu 1972). Development of voltage clamp techniques for cardiac tissues in the early to mid-1960s identified that SA-nodal cells had a low expression of inwardly rectifying K⁺ channels (I_{K1}) and TTX-sensitive Na⁺ channels, in sharp contrast to atrial and ventricular cells, but expressed a slowly activating inward current that was identified to have resulted from the turn-off of K⁺ conductance, based on studies on Purkinje fibers (Vassalle 1966).
As is often the case in biological investigations, the cardiac Purkinje fibers, with its large
cells convenient for 2-microelectrodes voltage-clamp technique, became a favorite tissue
model to probe the mechanisms of pacing (Deck et al. 1964). For over a decade, the
experiments reported by Dennis Noble’s team in Oxford, UK and Trautwein’s team in
Heidelberg, Germany confirmed that diastolic depolarization was indeed generated by turn-off
of a $K^+$ conductance in Purkinje fibers and by analogy in SA-nodal cells (Noble and Tsien 1968;
Peper and Trautwein 1969; Tsien and Carpenter 1978). In the late 1970s, using for the first time
$K^+$-selective microelectrodes in intact SA-nodal or in strips of SA-node tissue under single
sucrose-gap voltage clamp technique, we failed to confirm the turn-off of a $K^+$ conductance as
the mechanism for diastolic depolarization (Maylie et al. 1979; Maylie et al. 1981; Weiss et al.
1978). Instead our studies showed that hyperpolarization-activated inward current in rabbit SA-
nodal cells resulted from activation of a slow TTX-insensitive $Na^+$ current, had no reversal
potential around $E_K$, and was blocked by 2mM $Cs^+$ (Maylie and Morad 1984; Maylie et al. 1981;
Noma et al. 1983), in sharp contrast to the findings in Purkinje fibers, where $I_f$ appeared to
reverse at voltages negative to -100mVs, approximating $E_K$.

In the decade of the 1990s, cloning and molecular techniques identified the
hyperpolarization-activated current to be encoded by the HCN family of genes, which led to the
universal acceptance of the HCN family as the pacemaker channel, and provided the grounds
for $I_f$ to be considered as the pacemaker current responsible for the generation of diastolic
depolarization (Ishii et al. 1999; Vaccari et al. 1999)

The $I_f$ conundrum
In the 1980s, even though $I_f$ was generally recognized as the “pacemaker current,” other transporters such as T-type Ca$^{2+}$ channels and Na$^+$-Ca$^{2+}$ exchanger continued to be proposed to contribute to diastolic depolarization phase of the action potential (Bogdanov et al. 2001; Hagiwara et al. 1988; Ju and Allen 1998). Such inward current generating mechanisms, however, were considered ancillary to the dominant role of $I_f$ in generating the diastolic depolarization and pacemaking. To quantify the role of $I_f$ in diastolic depolarization, together with Professor Irisawa’s group in Japan in 1983, we examined the contribution of $I_f$ to the diastolic depolarization and automaticity. In isolated rabbit SA-nodal tissue using 2-microelectrode voltage clamp technique, we found: 1) significant differences in the range of diastolic depolarization potentials (-60 to -40mVs) and the voltages at which $I_f$ was activated (negative to -70mVs); 2) 2mM Cs$^+$ blocked $I_f$ fully but did not significantly change the frequency of spontaneously developing diastolic depolarization and pacing; 3) the Cs$^+$ block of $I_f$ was not voltage dependent; 4) in Cs$^+$-blocked SA-nodal cells beta-adrenergic agonists increased the slope of the diastolic depolarization and accelerated the frequency of spontaneous pacing without unblocking $I_f$. These findings, published in 1983 under the title “Does the pacemaker current generate the diastolic depolarization in the rabbit SA node cells”, have been fully ignored and forgotten. It took some 25 years for another group (Verkerk et al. 2007) to repeat and confirm our findings in human single SA-nodal cells. Although the data of Verkerk et al. showed that in human SA-node $I_f$ was similarly activated negative to -70mVs and diastolic depolarization oscillated between about -60 and -40mVs, and 2mM Cs$^+$ similarly fully blocked $I_f$ with only a small effect on the rate of diastolic depolarization, they nevertheless concluded that $I_f$ contributed to diastolic depolarization. Figure 1 shows similar data in single rat SA-nodal cells.
where the activation voltages for $I_f$ and $I_{Ca}$ have been quantified at the potential range spanning the diastolic depolarization, panel E. Clearly as the membrane depolarizes from -60 to -40 mV there is little or no $I_f$, but there is significant activation of $I_{Ca}$, which is strongly enhanced by isoproterenol, and is accompanied by about a 10mV shift in $I_f$ (compare the degree of activation of $I_f$ and $I_{Ca}$ in panels A, B, C and E). The shift in activation of $I_f$, often argued to place $I_f$ in the voltage range of diastolic depolarizations, may in fact serve as a compensatory mechanism for the isoproterenol-induced stimulation of Na$^+$/K$^+$-ATPase that causes myocardial hyperpolarization (Kockskamper et al. 2000; Martin and Morad, 1982), or for the profound alterations of cardiac Ca$^{2+}$ signaling. Consistent with the latter idea, optical mapping of the membrane potential and intracellular calcium in intact canine SA-node showed a slow rise in cytosolic Ca$^{2+}$ that preceded or accompanied the development of diastolic depolarization, proposed by the authors to mediate the acceleration of heart rate by isoproterenol through the Ca$^{2+}$ clock mechanism (Joung et al. 2009).

Considering these findings, it is somewhat puzzling that $I_f$ continues to be regarded as the mechanism underlying the diastolic depolarization. Similarly, little attention has been paid as to how a voltage-gated channel activating only at potentials negative to the maximum diastolic potential with time constants of 3-5 seconds contributes to membrane oscillations positive to -60mVs -especially enough to generate rhythmic frequencies of 6 per second in rodents and 1-2 per second in human hearts.

**Spontaneous pacing in hiPS-CM and rat neonatal cardiomyocytes and the role of $I_f$**
Human fibroblast-derived cardiomyocytes (hiPSC-CM) and rat neonatal cardiomyocytes (rN-CM) beat spontaneously at frequencies ranging from 80-120 beats/minute at room temperatures, but express either low levels (hiPSC-CM) or no $I_f$ (rN-CM) (Zhang et al. 2015). Surprisingly, spontaneous pacing continued with little or no change in frequency when either cell-type was voltage clamped at holding potentials of -40 or -50 mV for minutes, where no significant activation of ionic channels occurs (Zhang et al. 2015, and Fig. 2). The frequency of pacing in such cells was neither suppressed by $I_f$ channel blocker, nor by $Ca^{2+}$ channel blockers, but was inhibited by tetracaine (RyR2 inhibitor), thapsigargin (SERCA inhibitor that depletes eventually the $Ca^{2+}$ stores) and unexpectedly by small (50nM) concentrations of mitochondrial uncoupler, FCCP, (Zhang et al. 2015) thus suggesting a critical role for both mitochondria and the SR in initiating the spontaneous pacing in these developing cells. To distinguish between whether membrane depolarization or $Ca^{2+}$ release events were responsible for the slow phase of diastolic depolarization, we compared the profiles of rise of $Ca^{2+}$ in both current and voltage clamped cells with the time course of membrane depolarization. Fig. 3 shows that the rise of $Ca^{2+}$ (Fluo-4 signal) preceded the diastolic depolarization phase and upstroke of the action potential, generally in central regions of the cell (traces a, red and orange color-coded ROI), while the triggered action potential that followed caused additional rise of $Ca^{2+}$ especially in the peripheral regions of the cell (green and blue ROI). This is consistent with the idea that the release of $Ca^{2+}$ was responsible for the diastolic depolarization phase. Similarly, in voltage clamp mode the rise of $Ca^{2+}$ in the central cellular region preceded the activation of $Na^+$/Ca$^{2+}$ exchanger current, traces B. Note also that the pacing frequency was similar irrespective
whether the cell was current or voltage clamped (Fig. 3 A and B), suggesting that the pacing frequency was independent of ionic channel activation in the developing cardiomyocytes.

In current clamped and spontaneously pacing myocytes, where the GCaMP6-FKBP probe was used to virally target RyR2, 2mM Cd\textsuperscript{2+}, known to block both Ca\textsuperscript{2+} channels and I\textsubscript{NCX}, blocked only the triggered action potentials but not the spontaneously triggered Ca\textsuperscript{2+} oscillations (Fig.4A). FCCP at 50nM, on the other hand, effectively blocked both the spontaneously triggered Ca\textsuperscript{2+} oscillations and the action potentials (Fig.4B), suggesting that spontaneous pacing is triggered by mechanisms independent of membrane depolarizations, but regulated, possibly by the mitochondrial activity.

To probe the possible involvement of mitochondrial Ca\textsuperscript{2+} signaling in spontaneous pacing, we engineered genetically encoded Ca\textsuperscript{2+} biosensors targeted to the mitochondrial matrix, using an inverse pericamp viral mitycam-E31Q (Haviland et al. 2014; Kettlewell et al. 2009). Figure 5 shows a focal rise and fall of Ca\textsuperscript{2+} in individual mitochondria, associated with spontaneous cellular pacing (color-coded ROI, panel B), as indicated by activation of NCX current (black traces, panel A) in the patch-clamped beating hiPSC-CMs. We were intrigued to find diversity in the release and uptake of Ca\textsuperscript{2+} in different mitochondrial populations, such that while some mitochondria cyclically took up Ca\textsuperscript{2+}, others released Ca\textsuperscript{2+} simultaneously during the spontaneous pacing activity (Fig. 5B). This finding and the rapid reversible block of pacing by small, non-depolarizing concentrations of FCCP (Brennan et al. 2006; Zhang et al. 2015) suggest that the mitochondrial Ca\textsuperscript{2+} oscillations are critical in generating the global cellular Ca\textsuperscript{2+} oscillations that underlie pacing.
Cellular Mechanism required for pacing and possible role for \( I_f \)

For cardiac cells to serve as pacemaker of the heart they must express: 1) molecular mechanisms that cyclically depolarize and repolarize their cellular membranes, and 2) low resistance electrical connections to each other and to the atrial cells. In the mammalian heart the SA-nodal region expresses such electrical oscillatory cells that are connected to each other and to atrial cells with connexins that facilitate the transmission of their cyclically generated diastolic depolarizations. It is interesting to note that these oscillating SA-nodal cells have also distinctly different morphology than either the atrial or ventricular cells. They are generally small (20-30pF) flat cells, some spindle-shaped, others spider-shaped, with neuronal-like dendritic processes (Verheijck et al. 1998; Wu et al. 2001). Perhaps more critically, SA-nodal cells have surface to volume ratios larger than one in sharp contrast to the atrial or ventricular cells with surface to volumes ratios less than one (Table 1). Based on the cellular morphology alone, it appears that cells with larger surface to volume ratios show proclivity for spontaneous pacing (see Table 1).

The two membrane depolarizing mechanisms proposed, thus far, for SA-nodal pacing: a) activation of \( I_f \) (membrane clock) and b) SR Ca\(^{2+}\) release, (Ca\(^{2+}\) clock) do not consider the cellular morphology of pacing cells, in particular, their surface/volume ratios as contributing factor to their pacing capabilities. Clearly, the \( I_f \) system is a membrane delimited system unaffected by the cellular cytosolic volume. The Ca\(^{2+}\) clock system, on the other hand, because it requires release of SR Ca\(^{2+}\) and depolarization of membrane via activation of NCX, is critically dependent on the cellular volume in which calcium is released and thereby highly dependent on the surface to volume ratio of the cell. Although there is some discord as to the fractional
contribution of the membrane-clock vs. the Ca$^{2+}$ clock to pacing, it is now assumed that the two systems work in tandem to generate the diastolic depolarization phase of the action potential that sets the rhythm of the heart (Lakatta et al. 2010; Lakatta et al. 2006; Joung et al. 2009). It should be pointed out that the $I_f$ system, which was considered in the 1970s and 1980s as the “pacemaker current” (Accili et al. 2002; DiFrancesco 1984; DiFrancesco 1993), has activation voltage too negative (-70mVs) and kinetics too slow (tau in seconds) to contribute significantly to diastolic depolarization that operates between -60 to -40mV (Brown and Difrancesco 1980; Maylie and Morad 1984; Verkerk et al. 2007). Considering these limitations, it is likely that the Ca$^{2+}$ clock mechanism is the dominant mechanism responsible for pacing. So, it is fair to ask, what role does $I_f$ system plays in the SA-nodal pacing, and why is it expressed in the SA-nodal cells?

Ultimately the spontaneously generated oscillatory electrical signals, generated in the SA-node, must be transmitted along the syncytial myocardium to drive the atrial and ventricular tissues. This requires electrical coupling between the SA-nodal cells that oscillate between -65 and -40mVs and the atrial cells that have resting potentials of about -90mVs. Considering the much larger mass of atrial tissue compared to SA-nodal cells and the cable properties of the myocardium, it is intriguing that the SA-nodal cells are able to maintain their oscillatory voltages between -60 to -40mVs while connected to electrical sink of atrium at ~ -90mVs. It is likely that the electrical drag of the atrial tissue on much smaller SA-nodal tissue is counteracted by the expression of hyperpolarization-activated channels (HCN4, $I_f$) in the SA-nodal cells that provides them with a slowly activating inward current that counteracts the atrial hyperpolarizing sink effect. This mechanism serves to functionally insulate the SA-nodal
cells from large negative voltages of atrium and effectively insulate them without blocking the
conduction of the electrical wave emerging from the SA-node. Thus, $I_f$ acting as a functional
insulator needs only to activate slowly and to variable degrees depending on the
hyperpolarizing potentials that it encounters in different regions of the SA-nodal/atrial network.
Consistent with this idea Boyett et al. 2000 found a larger expression of $I_f$ in the junctional SA-
atrial regions than at the central SA-nodal cells. The expression of $I_f$ in the SA-nodal cells is
therefore critical in protecting the SA-nodal cells from being hyperpolarized by the atrial tissue
and allows them to oscillate by the calcium clock mechanism.

This scheme of spontaneous pacing is also supported by the findings and mechanisms
proposed for spontaneous pacing of neonatal cardiomyocytes and hiPSC-CMs (Zhang et al.
2015), where $I_f$ was found to be either absent or expressed at very low levels, respectively (Fig.
6A). It was also noted that at holding potentials of -60 to -30 the voltage-clamped cells continue
to beat at the same frequencies as those of control unclamped cells even when their ionic
channels were prevented from activating or when their $I_f$ and $I_{ca}$ were blocked by ivabradine or
nifedipine, respectively (Fig. 6B-D). These findings confirm that spontaneous pacing in
developing cardiomyocytes does not require the activation of $I_f$ but can be initiated by release
and uptake of Ca$^{2+}$.

The “Master Ca$^{2+}$ Oscillator” and the role of Mitochondria

Since the advent of the Ca$^{2+}$ hypothesis as the possible SA-nodal mechanism for pacing,
(Bogdanov et al. 2001), it has remained somewhat of a puzzle as to what sets the rhythm of the
calcium oscillator. Considering the recently evolving role of mitochondria in beat-to-beat Ca$^{2+}$
signaling, we attempted to examine the possible role of mitochondria in spontaneous pacing of rat neonatal cardiomyocytes and hiPSC-CMs. To obtain a reliable Ca\textsuperscript{2+} signal specific to mitochondrial matrix, we constructed and used genetically encoded Ca\textsuperscript{2+} biosensors targeted to mitochondrial matrix (Haviland et al. 2014; Zhang et al. 2015). Using targeted probes (mito-GCaMP6, or mito-GCaMP3, or Mitycam-E31Q) with K\textsubscript{d}s for Ca\textsuperscript{2+}, ranging from ~0.2 to 3 micro-molar, we found significant Ca\textsuperscript{2+} uptake and release from mitochondria during the spontaneous beating of either rat neonatal cardiomyocytes or hiPSC-CMs. Unexpectedly, however, we found that while peripheral mitochondrial population sequestered Ca\textsuperscript{2+}, the central population released Ca\textsuperscript{2+} almost simultaneously (Zhang et al. 2015). This diversity of Ca\textsuperscript{2+} oscillatory behavior of mitochondria could provide the inherent negative feedback required for a pacemaking mechanism, which may also coordinate the cyclic production of ATP. Figure 7A shows spontaneously activated Ca\textsuperscript{2+} release and uptake signals measured in different populations of mitochondria (Panel C) in a current clamped hiPSC-CM, infected with mito-GCaMP6 probe targeted to mitochondrial matrix. Note that while some mitochondria take up calcium during the time course of the action potential others release Ca\textsuperscript{2+} simultaneously. Panel B shows that spontaneously triggered pacing, but not the action potentials, is completely suppressed by mitochondrial uncoupler FCCP (50nM). These finding are consistent with the idea that mitochondrial Ca\textsuperscript{2+} signaling maybe critically involved in spontaneous pacing activity of developing cardiomyocytes. Whether a similar mechanism is also at play in the adult pacemaker cells of SA-node remains to be explored.

Conclusions and future vistas of exploration
Recent data in spontaneously pacing developing rat or human cardiomyocytes suggest that automaticity is critically regulated by calcium signaling and requires little or no contribution from the HCN/\(I_f\) system. Whether adult SA-nodal cells also depend exclusively on \(Ca^{2+}\) signaling for their spontaneous activity is not fully determined. Critically, for the released \(Ca^{2+}\) to be effective in activating a significant depolarizing current, the cellular volume must be comparably small as compared to its cellular surface area. The development of a flat cellular morphology in developing cardiomyocytes and in the SA-nodal cells is consistent with this idea and satisfies the \(Ca^{2+}\) release requirements to efficiently activate \(I_{\text{NCX}}\). It remains yet to be determined what in fact is the fractional contribution of different \(Ca^{2+}\) cellular pools to this process, and what gives the system its oscillatory properties. The intriguing finding that different populations of mitochondria could simultaneously release or sequester \(Ca^{2+}\) provides a possible negative feedback mechanism required for oscillatory mechanisms. As genetically encoded fluorescent biosensors targeted either to mitochondrial matrix or \(Ca^{2+}\) release sites of SR become available, the possibility of directly monitoring the cross-talk between mitochondria and SR becomes realizable, and may provide direct clues as to the role of this signaling pathway in initiation and regulation of pacemaking.

What is also clear from our studies is that the role of \(I_f\) as the “pacemaker current” in SA-nodal cells has been greatly overestimated. The activation voltage and the kinetics of \(I_f/HCN\) system are much too negative and far too slow for it to contribute significantly to diastolic depolarization. Nevertheless, without the expression of HCN family of proteins in the small SA-nodal cells, the highly negative electrical sink of surrounding atrial tissue would hyperpolarize and suppress pacing of the SA-nodal cells, as evidenced by gene deletion experiments in mice.
where knockout of HCN4 or HCN2 produces bradycardia under control conditions, but appears to have little effect on adrenergically stimulated rates leading to the suggestion that \( I_f \) serves as “depolarization reserve” (Harzheim et al. 2008; Herrmann et al. 2007). Genetic manipulation of HCN family of genes has complicated consequences, as deletion of a member of this family may enhance the activity of another member of the family or lead to activation of other compensatory mechanisms, and as such the conclusions based upon HCN family knockout maybe somewhat misleading. The critical principle to remember is that whatever channel or mechanism is proposed to contribute to diastolic depolarization, it must activate at critical voltages of -60 to -40 mVs, where diastolic depolarization takes place.

We suggest that expression of \( I_f \) counteracts the atrial-sink induced hyperpolarizations by activating a large slowly developing inward current, which serves to functionally insulate the SA-nodal cells from being hyperpolarized by the atrial electrical sink, and cause pauses often observed in genetic deletion experiments. Therefore, the ability of \( I_f \) to insulate the electrically oscillating cells of SA-node from the atrial electrical sink gives \( I_f \) a central and critical role in allowing pacemaking to continue in the functional syncytium of the heart.

Supported by: NIH, RO1HL016152

**Conflict of interest and disclosure**

None of the authors have any actual or potential conflict of interest.


**Table 1. Proclivity for spontaneous pacing**

<table>
<thead>
<tr>
<th>Stage</th>
<th>S/V</th>
<th>Channels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic......Neonatal</td>
<td>S/V&gt;1</td>
<td>RyR/NCX/Ip3</td>
</tr>
<tr>
<td>Spontaneous Ca(^{2+}) oscillations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neonatal........Juvenile</td>
<td>S/V&gt;1</td>
<td>RyR/NCX/Ip3</td>
</tr>
<tr>
<td>Spontaneous beating</td>
<td></td>
<td>CICR ++</td>
</tr>
<tr>
<td>Juvenile........Adult</td>
<td>S/V&lt;1</td>
<td>DHPR/RyR</td>
</tr>
<tr>
<td>Spontaneous beating stops</td>
<td></td>
<td>CICR++++++</td>
</tr>
</tbody>
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Figure legends

Fig.1 Representative $I_{Ca}$ and $I_f$ currents, activated from a holding potential of -50mVs, in a single rat SA-nodal cell using perforated patch clamp technique. A: $I_{Ca}$ recorded at -30mV and -40mV before and after exposure to 100 nM Iso. B: $I_f$ recorded from holding potential of -40mVs at voltages of -50 to -70mV, and following exposure to 100nM Iso (red traces) shows a 10mVs shift in its voltage dependence (see panel E). C: Sample traces of $I_f$ currents before and after Iso during 2s long hyperpolarization pulses from -40mV to potentials ranging from -50 to -120mV, in 10mV steps, in control (black traces) and following exposure to Iso (red traces). D: Block of $I_f$ by 2mM Cs+, and its slight enhancement by 1mM Ba$^{2+}$. E: I-V relations for $I_f$ and $I_{Ca}$ before and after exposure to 100nM Iso. The horizontal arrow indicates the diastolic depolarization (DD) voltage range (-65mV to -40mVs). Timescales apply to each trace within a panel.

Fig.2 Simultaneous measurements of $I_{NCX}$ current and Fluo-4 Ca$^{2+}$-signals in hiPS-CM and rN-CM voltage-clamped at a holding potential of -50 mV. A: Spontaneous pacing in hiPS-CM indicated by Ca$^{2+}$ transients (upper traces) and $I_{NCX}$ transients (lower traces), and caffeine induced Ca$^{2+}$ releases and accompanying $I_{NCX}$ (right upper and lower traces). B: $I_{NCX}$ and Ca$^{2+}$ transients during spontaneous beating (left) and exposure to 3 mM caffeine in a rNCM (right). Modified from Zhang et al. 2015

Fig.3. rNCM beat spontaneously with the similar rate whether generating action potentials under current-clamp conditions or activating $I_{NCX}$ voltage-clamped at -50mVs holding potential. A and B, Time course of color-coded regional Ca$^{2+}$ signals ($\Delta F/F_0$), membrane current ($I_m$)(A),...
and membrane potential (Vm)(B). a and b, enlarged from the gray area of A and B. F0: Baseline fluorescence. ROI: Color-coded regions of interest. Modified from Zhang et al. 2015

Fig.4. Effect of Cd\(^{2+}\) and FCCP on spontaneously generated action potentials and GCaMP6-FKBP fluorescence signals in a current-clamped rNCM. rNCM were infected with GCaMP6-FKBP Ad-virus targeted to ryanodine receptors. A, 2mM Cd\(^{2+}\) blocked the activation of spontaneous action potentials without inhibiting the GCaMP6-FKBP signals. B, 50nM FCCP suppressed both of the GCaMP6-FKBP signal and action potentials.

Fig.5 Confocal images of the spontaneous mitochondrial Ca\(^{2+}\) oscillations in a hiPS-CM voltage clamped at -50mV. Cells were infected with a targeted mitochondrial Ca\(^{2+}\) probe (mitycam-E31Q). The traces show regional color-coded mitochondrial Ca\(^{2+}\) signals (top) and membrane I\(_{\text{NCX}}\) (bottom). Images show regions of interest (ROI) and sample frames correlated to beat 1 and 2, suggesting release of Ca\(^{2+}\) from mitochondrial in red region. Modified from Zhang et al. 2015

Fig.6 I\(_f\) and I\(_{\text{Ca}}\) blockers do not suppress spontaneous pacing at -50mVs holding potentials in hiPS-CM and rNCM. A, Sample traces of I\(_f\) current recorded during 2 s hyperpolarization pulse from -40 mV to potentials from -60 to -120 mV, I\(_f\) has different levels of expression in hiPS-CM and rNCM. B, Addition of 10 μM ivabradine to suppress I\(_f\) had no effect on oscillations of I\(_{\text{NCX}}\) in hiPS-CM or rN-CM. C and D, The Ca\(^{2+}\) channel blocker nifedipine has only a minor effect on spontaneous oscillations of I\(_{\text{NCX}}\) and [Ca2+]i. Modified from Zhang et al. 2015
Fig. 7 Suppression of mitochondrial Ca\(^{2+}\) oscillations by FCCP in GCaMP6-mito probe infected and current clamped hiPS-CM. A and B, traces show changes in regional mitochondrial Ca\(^{2+}\) signals (top) and action potentials (bottom) before and 30 s after exposure to 50 nM FCCP. C, Fluorescence images (measured at the times indicated along the traces) showing repeatable mitochondrial Ca\(^{2+}\) signals before FCCP application (1, 2, 3) and smaller, more localized responses after (4, 5).
Figure 1

A

Control

Iso 100nM

-50mV

-40mV

-30mV

50ms

D

-120mV

-40mV

2s

2mM Cs⁺

10pA/pF

Con

1mM Ba²⁺

B

-50mV

-40mV

-70mV

3pA/pF

1s

C

Control

-80 mV

-120mV

1s

-120mV

E

mV

-120

-100

-80

-60

-40

-20

0

20

40

60

-12

-10

-8

-6

-4

-2

0

-14

10pA/pF

I_Ca

I_Ca

I_Ca

DD Voltage range

I_Ca, Con

I_Ca, Iso

I_Ca, Con

I_Ca, Iso
Figure 2

A

hiPS-CM

Fluo-4
0.03

Caff

Fluo-4
0.05

I_{NCX}
10pA

1s

B

rN-CM

Fluo-4
0.05

Caff

Fluo-4
0.05

I_{NCX}
10pA

1s

I_{NCX}
15pA

1s
Figure 3
504 Figure 4
505
Figure 5

(A) 

$\frac{\Delta F}{F_0} = 0.2$

20 pA

1 s

(B) 

ROI

1

2
Figure 6

A  human iPSC  rNCM

100pA  50pA  1s

C  human iPSC

Fluo-4
0.3
1min

10μM Nifedipine

2s

D  rNCM

Fluo-4
0.5
1min

5μM Nifedipine

2s
Figure 7

A. Control

B. FCCP

C. Masked images