Calcium influx through I\textsubscript{f} channels in rat ventricular myocytes

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Abstract

The Hyperpolarization-activated, Cyclic Nucleotide-gated (HCN) channels, or I\textsubscript{f}/I\textsubscript{h} channels, are conventionally considered as monovalent-selective channels. Recently we discovered that Ca\textsuperscript{2+} ions can permeate through HCN4 and I\textsubscript{h} channels in neurons. This raises the possibility of Ca\textsuperscript{2+} permeation in I\textsubscript{f}, the I\textsubscript{h} counterpart in cardiac myocytes, due to their structural homology. We performed simultaneous measurement of fura-2 Ca\textsuperscript{2+} signals and whole-cell currents produced by HCN2 and HCN4 channels (the two cardiac isoforms present in ventricles) expressed in HEK293 cells and by I\textsubscript{f} in rat ventricular myocytes. We observed Ca\textsuperscript{2+} influx when HCN/I\textsubscript{f} channels are activated. Ca\textsuperscript{2+} influx was increased with stronger hyperpolarization or longer pulse duration. Cesium, an I\textsubscript{f} channel blocker, inhibited I\textsubscript{f} and Ca\textsuperscript{2+} influx at the same time. Quantitative analysis revealed that Ca\textsuperscript{2+} flux contributed to about 0.5% of I\textsubscript{HCN2} or I\textsubscript{f}. The associated increase in Ca influx was also observed in spontaneously hypertensive rat (SHR) myocytes in which I\textsubscript{f} current density is higher than that of normotensive rat ventricle. In the absence of EGTA (a Ca\textsuperscript{2+} chelator), pre-activation of I\textsubscript{f} channels significantly reduced the action potential duration and the effect was blocked by another selective I\textsubscript{f} channel blocker, ZD7288. In the presence of EGTA, however, pre-activation of I\textsubscript{f} channels has no effects on action potential duration. Our data extend our previous discovery of Ca\textsuperscript{2+} influx in ih channels in neurons to I\textsubscript{f} channels in cardiac myocytes.

Keywords

Ca\textsuperscript{2+} flux; HCN/I\textsubscript{f} channels

INTRODUCTION

Ca\textsuperscript{2+} entry triggers a variety of essential cellular activities including cardiac muscle contraction (2). The fractional Ca\textsuperscript{2+} current, Pf, is defined as the percentage of current carried by Ca\textsuperscript{2+} in...
the total current through cation channels (32). Pf has been identified in many ion-conducting channels such as nicotinic acetylcholine receptors (nAChRs) (32), glutamate receptors [N-methyl-D-aspartate receptors (NMDA-Rs) (17,24), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA-Rs) (4), cyclic nucleotide-gated (CNG) channels (8), and voltage-dependent Ca$^{2+}$ channels (30). Ca$^{2+}$ influx through these channels contributes to transmitter release, axon guidance, or muscle contraction (2,4,30,32).

In neurons the time- and voltage-dependent inward cation current, $I_h$, is generated by HCN channels (10,14,23). It has been shown that activation of $I_h$ channels in crayfish neurons facilitates secretion (3). However, only monovalent cations was expected to permeate through the $I_h$ channels. Recently, we demonstrated the presence of a fractional Ca$^{2+}$ current through $I_h$ channels in dorsal root ganglion (DRG) neurons (28). We found that Ca$^{2+}$ influx through $I_h$ channels at negative potentials contributes to activity-evoked secretion in DRG neurons (28).

The cardiac counterpart of $I_h$, $I_f$, shares same molecular components. Among four HCN channel isoforms that have been cloned, three of them, HCN1, HCN2, and HCN4, are present in heart (25). Two isoforms, HCN4 and HCN2, are present in the ventricles (25). Our previous finding of Ca$^{2+}$ influx through $I_h$ channel raised the possibility of Ca$^{2+}$ entry through $I_f$ channels in cardiac myocytes and subsequent contribution to cardiac function at negative membrane potentials.

In this paper, we demonstrated that a fractional Ca$^{2+}$ current is present in currents induced by HCN2 and HCN4 channels, which were ectopically expressed in HEK293 cells, and in $I_f$ of rat ventricular myocytes, designated as Pf ($I_f$). Preliminary results for understanding its potential in cardiac function are shown and the future investigation for establishing its physiological role in cardiac pacemaker cells is discussed.

**MATERIALS AND METHODS**

**Heterologous expression of HCN channels**

The full-length cDNA of mouse HCN2 was subcloned into EcoRI/XbaI sites in pCMS-EGFP vector (Clontech), human HCN4 was a gift from Forschungszentrum Julich. HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. When cells approached confluence, they were seeded into 35 mm dishes, and subsequently transfected with the HCN plasmids using a Ca$^{2+}$ phosphate method (28).

**Cell dissociation**

Adrenal chromaffin cells were isolated from Wistar rats and cultured as previously described (7,31). Cells were used in the experiments after 2-6 days in culture. Single ventricular cardiac myocytes were isolated from adult Sprague-Dawley rats (2-3 months old; weight, 225-300g, from SLACCAS Inc., Shanghai) using a previously described Langendorff method (21). Briefly, the heart was removed, placed in Tyrode's solution containing (in mM): NaCl, 137; MgCl2, 0.5; glucose, 10; KCl, 5.4; CaCl2, 1.8; and HEPES, 11.8 (pH adjusted to 7.4 with NaOH), and squeezed gently to expel the blood. Ventricular myocytes were prepared using a Langendorff perfusion apparatus. Briefly, the hearts were removed and perfused with calcium-free Tyrode's containing (in mM): NaCl, 130; MgSO4, 1.2; KC1, 5.4; KH2PO4, 1.2; HEPES-NaOH, 6 (pH adjusted to 7.2 with NaOH) with collagenase (Liberase Blendzyme 4, 0.1 mg/ml, Roche Molecular Biochemicals) for approximately 9 min. After washing out the collagenase with calcium-free Tyrode's, single cells were dissociated by mincing the ventricle and shaking the tissue in “Kraftbrühe” (KB) solution containing (in mM): KCl, 83; K2HPO4,
30; MgSO₄, 5; Na-pyruvate, 5; Na-β-hydroxybutyrate, 5; creatine, 5; taurine, 20; glucose, 10; EGTA, 0.5; HEPES-KOH, 5; ATP-Na₂, 5 (pH adjusted to 7.2 with KOH). Cells were washed and resuspended in KB.

The use and care of animals in this study complied with the guidelines of the Animal Research Advisory Committee in the Shanghai Institutes of Biological Sciences.

**Whole-cell patch clamp recordings**

Ionic currents were studied in the whole-cell patch-clamp configuration using an EPC-9 amplifier (HEKA Elektronik, Germany). The membrane was held at −40 mV unless otherwise stated. A RCP-2B perfusion system was used for switching external solutions. The system has a fast exchange time (100ms) controlled electronically among 7 channels (Inbio, Wuhan, China (29)).

Experiments on chromaffin and HEK cells were conducted at room temperature (22-24°C). Ventricular myocytes were studied at 32-35°C.

Solutions used in experiments are defined in Table 1. Pipettes with resistances of 2-5 MΩ were used for all three types of cells.

Like our previous work, fluorescence calibration for Pf were performed on chromaffin cells (4,24,28,32). An intracellular solution containing high CsCl (see Table 1) was used to measure voltage-gated Ca²⁺ currents.

DMEM and fetal bovine serum were purchased from Gibco/Invitrogen. Fura-2 salt was from Molecular Probes. All other chemicals were from Sigma.

**Theory and measurement of fractional Ca²⁺ currents**

Intracellular Ca²⁺ concentration, [Ca²⁺]ᵢ, was measured by dual-wavelength ratiometric fluorometry. The Fura-2 was excited with light alternating between 340 and 380 nm using a monochromator-based system (TILL Photonics), and the resulting fluorescence signals were measured using a cooled CCD. Relative changes in [Ca²⁺]ᵢ were calculated from the ratio of F₃₄₀ to F₃₈₀, which were sampled at 1 Hz. The image data were transferred and analyzed by Igor software (WaveMetrix) (28).

Fractional Ca²⁺ current, Pf, is defined as the percentage of Ca²⁺ current in the total current passing through the cation channels (Iᵢ_HCΝ in this case). According to the original definition (28,32),

\[ \text{Pf} = \frac{\int I_{\text{HCΝ, Ca}} \, dt}{\int I_{\text{HCΝ}} \, dt} = \frac{\Delta F_d}{f_{\text{max}} \cdot \int I_{\text{HCΝ}} \, dt} \]  

(Eq. 1)

where Iᵢ_HCΝ is the HCN current, and Iᵢ_HCΝ,Ca is the proposed fractional Iᵢ_HCΝ current carried by Ca²⁺.

The change of Fd, ΔFd, is the “modified Ca²⁺-sensitive fura-2 signal” immediately before (Fd’) and after (Fd”) a voltage-pulse induced Ca²⁺ influx (30). Under the condition that all entering Ca²⁺ ions are bound by fura-2, Fd is a measure of Ca²⁺ influx (30). Fd is determined by the difference of fluorescence signals at 340nm and 380nm.

\[ F_d = F_{340} - F_{380} \]  

(Eq. 2)

\[ \Delta F_d = F_{d''} - F_{d'} \]  

(Eq. 3)
f_max is determined by measuring Ca^{2+} influx through voltage-gated Ca^{2+} channels in chromaffin cells under the condition that intracellular fura-2 is sufficiently high (> 0.4 mM, ref. 32).

Under physiological conditions, only Ca^{2+} ions contribute to the Ca^{2+} channels (30), or Pf = 100%. From Eq.1 we have

$$f_{\text{max}} = \frac{\Delta F_d}{\int I_{\text{Ca}} dt}$$

(Eq. 4)

where I_{Ca} is the current through voltage-gated Ca^{2+} channels. Although the calibration of f_max is measured in chromaffin cells, the accuracy of Pf(I_f) determined in myocytes should be safe because f_max is insensitive to cell types (4).

To record the time course of fura-2 dialysis, we used the Ca^{2+}-independent fluorescence signal F360 (32), which can be calculated from F340 and F380.

$$F_{360} = F_{340} + \alpha F_{380}$$

(Eq. 5)

where \(\alpha\) is the “isocoefficient”. According to Eq. 5, \(\alpha\) can be determined by any experimental recording that shows rapid changes in Ca^{2+} concentration. In our setup, \(\alpha = 0.35\). Since F360 is Ca^{2+}-independent, it can be used as an indicator of the intracellular fura-2 concentration, [fura]. After establishing the whole-cell recording configuration, fura-2 was dialyzed into the cell. Dialysis was accompanied by a proportional F360 increase. Once F360 reached a steady-state level, we assumed that [fura] was equal to the fura-2 concentration in the pipette (Fig.2, also see ref 32).

We applied equations 1-4 to determine the Pf of HCN2 and I_f channels by measuring the fura-2 signals.

Data were analysed with IGOR Pro3.12 software (Wavemetrics, Inc., Lake Oswego, OR). Unless otherwise stated, the data were presented as mean ± SD. Statistical significance was tested with Student’s t-test. P < 0.05 was considered statistically significant.

RESULTS

Hyperpolarization-induced Ca^{2+} influx was present in HCN-expressing HEK cells

In a non-transfected HEK cell held at −70mV, a hyperpolarizing pulse to −120mV induced neither a time-dependent inward I_{HCN} (lower) nor Ca^{2+} flux (upper in the left panel of Fig. 1B). On the other hand, in response to the same hyperpolarizing pulse a HCN2-transfected cell exhibits a typical I_{HCN2} (lower right panel in Fig. 1B) and Ca^{2+} flux (upper part of the right panel in Fig.1B) at the same time, suggesting that the Ca^{2+} signals induced by hyperpolarization are due to the activation of the HCN channels. In both experiments, the cells were killed at the end (see arrows) so that Ca^{2+} flux could be measured by the fura-2 signals (ΔF_d, see ref. 30). Similar results were observed in 5 non-transfected cells and 5 HCN2-transfected cells. In addition, we discovered the requirement of extracellular Ca^{2+} for hyperpolarization-induced Ca^{2+} flux. In response to the same pulse shown in Fig.1B, no Ca^{2+} flux could be detected in the absence of extracellular Ca^{2+} (arrow 2). However, the Ca^{2+} flux appeared in the presence of 2mM Ca^{2+} (arrows 1 and 3). These data support the hypothesis that the extracellular Ca^{2+} and open HCN channels are required to induce Ca^{2+} flux.

Fractional Ca^{2+} current through HCN2 and HCN4 channels in HEK293 cells

If Ca^{2+} ion indeed pass through the HCN channels, the changes in fura-2 Ca^{2+} signals should be directly associated with the time- and voltage-dependent properties of HCN channels. To test this hypothesis, I_{HCN2} was elicited by a step to −120 mV for 3s (#1), 10s (#2), and 20s
(#3) (protocol shown in the insets of Fig. 2B) from a holding potential of −40 mV. Measurement of Ca$^{2+}$ fluorescence (Figs. 2A, 2C) showed a rise in $[\text{Ca}^{2+}]_i$ (arrows #1 in Fig. 2A and 2C), and this rise was increased with longer pulse durations of 10s (arrow #2) and 20s (arrow #3). These data demonstrate a correlation of increasing Ca$^{2+}$ influx with the prolonged (time-dependent) activation of HCN2 channels.

Gating of HCN channels is also voltage dependent. A hyperpolarizing step to −70 mV for 3 seconds did not activate HCN4 channels (middle in Fig. 3B) and induced no Ca$^{2+}$ signal (Fig. 3A, upper in Fig. 3B), whereas a step to −120 mV for 3 seconds activated the channels (middle in Fig. 3B) and simultaneously induced calcium influx (Fig. 3A, upper in Fig. 3B). The pulse protocol is shown in the lower panel of figure 3B. In figure 3A, the peaks between −120 mV/3s and −70 mV/3s marks correspond to hyperpolarizing steps to −120 mV, −110 mV, −100 mV, −90 mV, and −80 mV. Decreasing amplitudes of these peaks at various potentials suggest a voltage dependent change in calcium influx, which simultaneously accompanies the voltage dependent activation of the channels. Fig. 3C elaborates the relationship between normalized ΔFd and total ion inflow (Q) for HCN4 channels at tested pulses. The superimposed traces indicates a correlation between the HCN4 currents and the Ca$^{2+}$ influx through HCN4 channels.

To quantify the Ca$^{2+}$ influx through HCN channels, we employed a widely used calibrating approach: quantitating Ca$^{2+}$ flux through the voltage-dependent Ca$^{2+}$ channel expressed in rat adrenal chromaffin cells, which passes 100% Ca$^{2+}$ (ref 28, also see eq. 4 in Methods for details) as calibration for fura-2 signals. A depolarizing step to 0 mV for 500 ms from a holding potential of −70 mV (lower panel, Fig. 4A) activated a voltage-dependent Ca$^{2+}$ current ($\text{I}_{\text{Ca}}$) and simultaneously induced an increase in Fd (ΔFd2, Fig. 4B, upper panel). The total ion influx charge was calculated from the time integral of $\text{I}_{\text{Ca}}$ current trace (Fig. 4B, middle panel, shaded region). In Fig. 4A we show that in a HEK293 cell expressing HCN2 channels, a hyperpolarizing step to −120 mV for 10 s (inset) activated the HCN2 current (middle panel) and simultaneously induced an increase in Fd (ΔFd1, upper panel). The time integral of ion flux through HCN2 channels was calculated from the current trace (Fig. 4A, middle panel, shaded area). The relationship between total ion influx and the corresponding increase in Fd (ΔFd) obtained with different durations of stimulation (Fig. 4C) was best fitted by a linear equation, indicating a correlation of the increased ΔFd with the increased ion flux through voltage-dependent calcium channels (Fig. 4B) and HCN2 channels (Fig. 4A). Using equation 1 in Methods, we determined Pf for HCN2 to be 0.47 ± 0.02% (n=6) (Fig. 4D).

**Ca$^{2+}$ influx through $I_f$ channels in ventricular myocytes**

Given HCN2 and HCN4 as the two isoforms that encode $I_f$ channels in rat ventricle (25), we hypothesized similar Ca$^{2+}$ flux through $I_f$ channels in rat ventricular myocytes. In response to hyperpolarizing pulses ranging from −70 mV to −150 mV (inset of Fig. 5A) the $I_f$ current traces were shown in fig. 5A. In this cell, $I_f$ began to activate around −80 mV, close to the previously reported values (5,21).

To examine the fractional Ca$^{2+}$ current through $I_f$ channels, we applied a 3 s hyperpolarizing pulse to −120 mV from a holding potential of −40 mV, and detected a rise of Ca$^{2+}$ signal (arrow #1 in Fig. 5B, 5D) concomitant with activation of $I_f$ (Fig. 5C, #1). When $I_f$ was blocked by 2 mM cesium (trace #2 in Fig. 5C), the increased Ca$^{2+}$ signal was blocked as well (mark #2 in Figs. 5B and 5D). These results indicate that Ca$^{2+}$ indeed passes through $I_f$ channels in rat ventricular myocytes, which is consistent with Ca$^{2+}$ influx through HCN2 and HCN4 channels (Figs. 2 and 3). Quantitative analysis revealed a Pf of 0.6 ± 0.1% (n = 3) for $I_f$ channels, similar to HCN2 and HCN4 channels expressed in HEK293 cells (Figs. 2 and 4, ref. 28).
**Ca\(^{2+}\) influx through I\(_f\) channels in SHR ventricular myocytes**

Although we have demonstrated the Ca\(^{2+}\) flux through HCN2 and HCN4 channels in HEK293 cells and I\(_f\) channels in rat ventricular myocytes, we thought the evidence supporting calcium permeation through I\(_f\) channels would be stronger if we can find the altered change in Ca\(^{2+}\) flux at membrane hyperpolarization in an animal model in which I\(_f\) is naturally altered. In spontaneously hypertensive rat (SHR) ventricle I\(_f\) current density is significantly increased (5). Fig. 6 shows a typical example in that in response to a 10s pulse to \(-150\text{mV}\) from the holding potential of \(-70\text{mV}\), both I\(_f\) (upper panel) and Ca\(^{2+}\) influx (lower panel) through I\(_f\) channels are significantly larger in SHR myocytes than in normal rat myocytes (Fig. 6A). The averaged I\(_f\) current density at \(-150\text{mV}\) is increased by 55% in SHR compared to the control (Fig. 6B). The increase in I\(_f\) is associated with an increase in Ca\(^{2+}\) influx (69%) (Fig. 6C).

**Shortening of action potential by Ca\(^{2+}\) influx through I\(_f\) channels**

As an initial effort to investigate the functional role of Ca\(^{2+}\) flux through I\(_f\) channels in cardiac myocytes, we took the advantage of the established role of Ca\(^{2+}\) in cardiac action potential. Upon membrane depolarization, L-type calcium channels are activated, allowing Ca\(^{2+}\) to enter the cell, which provides a major inward current contributing to the plateau phase of the action potential in ventricular myocytes (2). It is well documented that Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels causes the channel inactivation (11,12). On the other hand, we were wondering if Ca\(^{2+}\) can enter the cell through I\(_f\) channels upon hyperpolarization, they should also be able to inhibit the subsequent gating of L-type Ca\(^{2+}\) channels which produces less inward current and, in turn, would shorten the action potential duration.

To investigate the effects of pre-activating I\(_f\) on the action potential duration, we compared the action potential duration measured at the 15% of amplitude with and without pre-activating I\(_f\) (Fig. 7A). A hyperpolarizing pulse to \(-120\text{mV}\) for 5s was applied to open I\(_f\) channels prior to initiating an action potential (Fig. 7B). Comparing to the control (without a preceding hyperpolarizing pulse that opens I\(_f\) channels), the action potential duration was shortened by 32 ± 7% (n=5, p<0.05) and returned to control when the hyperpolarizing pulse was removed (Fig. 7A, 7C). In addition, if the intracellular Ca\(^{2+}\) was buffered by adding 10mM EGTA to the pipette solution, the effect of pre-activation of I\(_f\) on the shortening of action potential duration was eliminated (99 ± 3%, n=8, Fig. 7C). This indicates that Ca\(^{2+}\) influx through I\(_f\) channels was functionally involved in the shortening of action potential duration. This conclusion was further supported by two additional experiments. In the first experiment, when the cell was stimulated by a 50 ms depolarization from \(-40\text{mV}\) to 0 mV to activate L-type Ca\(^{2+}\) channels (leading to a Ca\(^{2+}\) influx similar to that of 1 nA I\(_f\) for 5 s), the action potential duration was shortened to a similar degree (27 ± 4%, n=4, p<0.05, Fig. 7C). In the second experiment, ZD7288 (30 \(\mu\)M), which is a specific antagonist of I\(_f\) channels (28), was able to eliminate the shortening effect of I\(_f\) activation on action potential duration (91 ± 5%, n=3, Fig. 7C). Taken together, all these data support the conclusion that Ca\(^{2+}\) influx through I\(_f\) channels can contribute to the action potential duration.

**DISCUSSION**

In this study, we have provided several lines of evidence to demonstrate the permeation of Ca\(^{2+}\) in I\(_f\) channels in rat ventricular myocytes. First, we used HEK293 cells expressing HCN2 and HCN4 channels, the two HCN channel isoforms encoding I\(_f\) channels. Using HEK293 cells allow us to avoid potential contamination of Ca\(^{2+}\) flux measurement since P(f) is small under our experimental conditions. There are no endogenous HCN channels in HEK293 cells (Fig. 1). Activation of HCN2 and HCN4 is accompanied by Ca\(^{2+}\) influx (Figs. 1,2 and 4). Second, longer (Fig. 2) and stronger (Fig. 3) hyperpolarizing pulses enhanced Ca\(^{2+}\) influx.
Third, Ca\(^{2+}\) influx cannot be observed either at less hyperpolarization at which HCN channels are closed (Fig. 3B) or in the presence of Cs\(^{+}\) (Fig. 5). Finally, in HEK293 cells that were not transfected with HCN channels or in the absence of extracellular Ca\(^{2+}\), no Ca\(^{2+}\) influx was detected (Fig. 1). All these evidence point to the direction that the fractional Ca\(^{2+}\) current may present in I\(_{\text{f}}\) in cardiac myocytes.

To strengthen the link between the Ca\(^{2+}\) flux and I\(_{\text{f}}\) channel activation at very negative potential, we need cardiac cells that natively express either higher or no I\(_{\text{f}}\) channels. We chose spontaneously hypertensive rat (SHR) ventricular myocytes in which I\(_{\text{f}}\) channel expression is significantly higher compared to the normal rat ventricle (5). Using SHR cells allows us to compare the Ca\(^{2+}\) flux under two native conditions in the same species. The results shown in fig. 6 provide additional evidence supporting our hypothesis that calcium indeed permeate I\(_{\text{f}}\) channels, although at −150mV we cannot exclude the possible contribution of other ionic mechanisms such as Na/Ca exchanger current and Ca\(^{2+}\) release from sarcoplasmic reticulum. The higher percent increase in Ca influx (69%) than that in I\(_{\text{f}}\) current (55%) may also reflect the possibility of involving other ionic mechanism. Nonetheless, these data point to a potential role of the fractional Ca\(^{2+}\) influx through I\(_{\text{f}}\) channels during diastole in pathophysiologic ventricles where I\(_{\text{f}}\) channel expression is significantly increased (6,9,13).

It is well understood that the plateau phase of a ventricular action potential is maintained by a fine balance of outward and inward currents. The major time-dependent inward current that determines the duration of the plateau phase is the L-type calcium current, generated by calcium influx through L-type channels upon membrane depolarization. The contribution of this calcium inward current to the action potential duration is limited by the inaction of L-type calcium channels partially caused by calcium influx (2). Within every heartbeat, the amount of calcium enters the cell during depolarization will have to get out of cell when the membrane repolarizes to the resting potential via Na/Ca exchanger and calcium pump (2). That means at negative membrane potentials, what we have learned is the mechanisms that extrude intracellular calcium to set the heart at relax (diastolic) stage ready for next action potential. The calcium influx through I\(_{\text{f}}\) channels that are open at negative potentials raised the possibility that calcium can still “leak” into the cell at resting or diastolic stage.

Compared with the fractional Ca\(^{2+}\) currents of other cation channels, such as nAChR (2.5%) (32), glutamate channels (10% for NMDA (17), 0.5-5% for AMPA/kainate receptors (4,24), CNG channels (10-80%) (8), and voltage-dependent calcium channels (100%) (30), the Pf of HCN/I\(_{\text{f}}\) channels is relatively small (0.47% in HCN2 and 0.6% in I\(_{\text{f}}\) channels). However, given the nature of local calcium signaling, this small Ca\(^{2+}\) flux through I\(_{\text{f}}\) channels may be sufficient to increase the local calcium concentration near the intracellular side of L-type calcium channels, effectively accelerates I\(_{\text{Ca}}\) inactivation which, in turn, shortens the action potential duration.

Although we have demonstrated the permeation of Ca\(^{2+}\) through I\(_{\text{f}}\) channels in rat ventricular myocytes, more experiments are needed to illustrate the physiological role of fractional calcium current via I\(_{\text{f}}\) channels in cardiac myocytes. Such evidence can only be achieved in a pacing tissue in which I\(_{\text{f}}\) is activated in the physiological voltage range. For example, I\(_{\text{f}}\) appears around −50mV in the cardiac pacemaker sinoatrial (25) and atrial myocytes (18) and −70mV in the neonatal rat ventricular cells (21). In addition, recent studies have shown that under dynamic conditions the activation of HCN1 and HCN2 channels can be dramatically shifted to rather positive voltages (1,16). Under those conditions, many mechanistic aspects and physiological implications of Ca\(^{2+}\) permeation in I\(_{\text{f}}\) channels would be tested. This can finally establish the physiological relevance of fractional Ca\(^{2+}\) current through HCN.
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HCN channels induced Ca influx in HEK293 cells
(A). Extracellular Ca^{2+} is required for hyperpolarization-induced Ca^{2+} influx in HEK 293 cells expressing HCN4. In response to a hyperpolarizing pulse to $-120 \text{ mV}$ for 5 s (arrows 1 and 3) from the holding potential of $-70 \text{ mV}$, calcium influx (arrow 1) was abolished when Ca^{2+} was removed from the bath (arrow 2), and re-appeared after Ca^{2+} was added back to the bath (arrow 3). Similar results were observed in 10 cells.

(B). Requirement of HCN channels for hyperpolarization-induced Ca^{2+} influx. Left panel: there were no time-dependent inward HCN current (lower left) and Ca influx signals (upper left) in response to a 50 s hyperpolarization pulse (inset shows the pulse protocol) in a non-

Figure 1.
transfected HEK293 cell. Right panel: in a HEK293 cell transfected with GFP-HCN2, a 10 s hyperpolarization pulse induced a time-dependent inward HCN2 current (lower inset) and Ca influx (upper right) simultaneously.
Figure 2.
Ca\(^{2+}\) influx through HCN2 channels in HEK293 cells.
(A). Ca\(^{2+}\) signals in response to a 3 s (arrow #1), 10 s (arrow #2), and 20 s (arrow #3) hyperpolarizing pulse (see protocol in panel B). Fluorescence signals during fura-2 loading (0.1 mM in the pipette) at 360 nm (upper trace F360, Ca\(^{2+}\)-insensitive fluorescence, indicating the process of fura-2 entry into the cell); at 380 nm (second trace F380, Ca\(^{2+}\)-sensitive fluorescence, indicating Ca\(^{2+}\) influx), Fd = F340 - F380 (third trace Fd, modified signal indicating Ca\(^{2+}\) influx) are shown together with intracellular free Ca\(^{2+}\) concentration (lower trace \([\text{Ca}^{2+}]_i\)). The cell was hyperpolarized to −120 mV for 3 s twice, for 10 s twice, and for 20 s once. Arrow #1 indicates the rise of Ca\(^{2+}\) influx corresponding to the second 3s pulse,
arrow #2 the second 10s pulse, and arrow #3 the 20s pulse. Similar results were observed in 14 cells.

(B). HCN2 currents at −120 mV for 3 s, 10 s, and 20 s. The voltage protocol is shown in the lower panel.

(C). Enlarged Fd signals corresponding to arrows #1, #2 and #3 in panel A.
Figure 3.
Ca\textsuperscript{2+} influx through HCN4 channels in HEK293 cells
(A). Calcium signals in response to different hyperpolarizing pulses. Arrows indicate changes in Fd in response to steps to −120 mV and −70 mV for 3s, respectively. The peaks between arrows represent calcium signals in response to −120 mV, −110 mV, −100 mV, −90 mV, and −80 mV for 3 seconds. The last peak is the calcium signal in response to −120 mV for 10 seconds.
(B). Comparison of enlarged calcium signals (upper traces) and HCN4 currents (middle traces) at −70 mV and −120 mV for 3 seconds. Lower panel is the voltage protocol. A leak current of −80 pA was subtracted for optimal comparison of two current traces.
(C). Voltage dependence of normalized total ion inflow (Q) and Ca\textsuperscript{2+} influx (ΔFd) through HCN4 channels.
Figure 4.
Protocol to determine fractional Ca\(^{2+}\) current through HCN2 channels.

(A). Ca\(^{2+}\) signal changes in response to a hyperpolarizing pulse to \(-120\) mV for 10 s (lower trace) in a HEK293 cell expressing HCN2 channels. The change in fluorescence induced by the stimulus is shown as \(\Delta F_{d1}\) (upper trace). Dashed line of middle trace represents zero current. The shaded area indicates total ion influx charge through the HCN2 channels.

(B). Fluorescence changes in response to a depolarizing pulse (lower panel) in a rat adrenal chromaffin cell. The change in fluorescence induced by the stimulus is shown as \(\Delta F_{d2}\) (upper trace). Dashed line of middle trace represents zero current. The shaded area indicates total ion influx charge through voltage-dependent calcium channels (VDCC).

(C) & (D). Quantitative determination of the fractional Ca\(^{2+}\) current through HCN2 channels. In C, \(\Delta F_{d1}\) for HCN2 is plotted against the corresponding ion influx, \(Q_1\). In D, Pf for HCN2 is determined by Pf = \(\frac{\Delta F_{d1}}{\int I_{HCN2} dt + f_{max} k_2} = \frac{k_1}{k_2}\). with \(F_{d2}\) and \(Q_2\) corresponding to \(\Delta F_{d2}\) and ion influx of voltage-gated Ca\(^{2+}\) channels, respectively (\(n = 7\)).

The fractional Ca\(^{2+}\) current in Ca\(^{2+}\) channels is assumed to be 100% (refs. 26,30,32).
Figure 5.

I_f in rat ventricular myocytes.

(A). I_f was recorded in steps ranging from −70 mV to −150 mV with 10 mV increments (protocol in lower panel) in a rat ventricular myocyte.

(B). Ca^{2+} signals in response to a step to −120 mV in the absence (arrow #1) and presence of 2 mM CsCl (arrow #2).

(C). 2 mM Cs^+ (trace #2, corresponding to arrow #2 in panel A) blocked I_f at −120 mV (trace #1, corresponding to arrow #1 in panel A).

(D). Enlargement of fluorescent signals marked by arrows #1 and #2 in panel B. The fractional Ca^{2+} current of I_f was 0.6 ± 0.1% (n = 3). Dashed lines in A and C represent zero currents.
Figure 6. 
Ca²⁺ influx through Iᵢ channels in SHR ventricular myocytes. 
(A). Iᵢ currents (middle traces) and Ca²⁺ signals (lower traces, shown as F/F₀) in ventricular myocytes from control rat and SHR rat upon a 10-s hyperpolarization pulse to −150 mV from a holding potential of −70 mV (protocol in upper panel). 
(B). Comparison of Iᵢ current density in control (2.34 ± 0.49 pA/pF, n = 8) and in SHR (3.63 ± 0.63 pA/pF, n = 12) at −150 mV, (p < 0.05).
(C). Comparison of the rates of Ca²⁺ signal changes in control (1.27 ± 0.15 AU/s, n = 7) and in SHR (2.15 ± 0.14 AU/s, n = 5) in response to a pulse to −150 mV (p < 0.001).
Figure 7.
I_f-induced shortening in action potential duration.
(A). Action potentials (APs, upper traces) in a ventricular myocyte before (thin line, control) and after (thick line) a 5 s hyperpolarizing pulse to −120 mV. Action potentials were induced by 800 pA depolarizing current for 5 ms (lower trace). Action potential duration, APD_{15}, starts at the peak of the AP, or 100% of AP-amplitude (APA_{100}), and ends at the time when the APA has decayed to 15% of APA_{100}. A pre-hyperpolarization pulse for 5 s shortened APD_{15}. This effect was reversible (dashed line).
(B). Hyperpolarization-induced current in the same ventricular myocyte of panel A. Immediately before the AP recording under current clamp (CC), the cell was stimulated by a 5 s hyperpolarizing pulse (inset) under voltage-clamp (VC). Lower panel shows the pulse protocol.
(C). Statistic analysis. Compared with control, APD_{15} was shortened by 32 ± 7% (n = 5) by pre-activation of I_f at −120 mV for 5 s without EGTA in the pipette solution. Pre-activation of Ca^{2+} channels for 50 ms also shortened APD_{15} by 27 ± 4% (n = 4). With 10 mM EGTA in the

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pipette, pre-activation of I_f for 5 s had no effect on APD (99 ± 3%; n = 8). ZD7288 blocked
the effect (91 ± 5%; n = 3).
Table 1
Composition of solutions: internal solutions* (mM)

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* For fluorescence calibration experiments, 1 mM fura-2 potassium salt was added to internal solutions. For other Ca²⁺ imaging experiments, 0.1 mM fura-2 salt was added to internal solutions.

⁶CdCl₂ was used to prevent Ca²⁺ entry from VDCCs that can be activated by steps back from hyperpolarization to the holding potential in ventricular myocytes. BaCl₂ was used to block the background potassium current, iK₁, which masks the activation of If. 4-AP was used to inhibit the activation of the transient outward potassium current, iₒ, which can overlap the deactivation of If.