Calcium influx through \( I_f \) channels in rat ventricular myocytes

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Yu X, Chen X-W, Zhou P, Yao L, Liu T, Zhang B, Li Y, Zheng H, Zheng L-H, Zhang CX, Bruce I, Ge J-B, Wang S-Q, Hu Z-A, Yu H-G, Zhou Z. Calcium influx through \( I_f \) channels in rat ventricular myocytes. Am J Physiol Cell Physiol 292: C1147–C1155, 2007. The hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels, or cardiac \( I_f \)/neuronal \( I_h \) time- and voltage-dependent inward cation current channels, are conventionally considered as monovalent-selective channels. Recently we discovered that calcium ions can permeate through HCN4 and \( I_h \) channels in neurons. This raises the possibility of \( Ca^{2+} \) permeation in \( I_h \), the \( I_h \) counterpart in cardiac myocytes, because of their structural homology. We performed simultaneous measurement of fura-2 \( Ca^{2+} \) signals and whole cell currents produced by HCN2 and HCN4 channels (the 2 cardiac isoforms present in ventricles) expressed in HEK293 cells and by \( I_h \) in rat ventricular myocytes. We observed \( Ca^{2+} \) influx when HCN4 channels were activated. \( Ca^{2+} \) influx was increased with stronger hyperpolarization. Cesium, an \( I_f \) channel blocker, inhibited \( I_f \) and \( Ca^{2+} \) influx at the same time. Quantitative analysis revealed that \( Ca^{2+} \) influx contributed to \( 0.5\% \) of current produced by the HCN2 channel or \( I_h \). The associated increase in \( Ca^{2+} \) influx was also observed in spontaneously hypertensive rat (SHR) myocytes in which \( I_h \) current density is higher than that of normotensive rat ventricle. In the absence of EGTA (a \( Ca^{2+} \) chelator), preactivation of \( I_f \) channels raised the possibility of \( Ca^{2+} \) influx through these channels. Influx through these channels contributes to transmitter release, axon guidance, or muscle contraction (3, 4, 30, 32).

In neurons the time- and voltage-dependent inward cation current, \( I_h \), is generated by hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels (10, 14, 23). It has been shown that activation of \( I_h \) channels in crayfish neurons facilitates secretion (2). However, only monovalent cations were expected to permeate through the \( I_h \) channels. Recently, we demonstrated (28) that the presence of a fractional \( Ca^{2+} \) current through \( I_h \) channels in dorsal root ganglion (DRG) neurons. 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, 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 \( I_f \) channels 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 study, we demonstrated that a fractional \( Ca^{2+} \) current is present in currents induced by HCN2 and HCN4 channels, which were ectopically expressed in human embryonic kidney (HEK)293 cells, and in \( I_f \) of rat ventricular myocytes, designated as \( P_f \) (29). Preliminary results toward understanding its potential in cardiac function are shown, and 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 pCMV-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 \( \mu \)g/ml streptomycin. When cells approached confluence they were seeded into 35-mm dishes and subsequently transfected with the HCN plasmids by 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 experiments after 2–6 days in culture. Single ventricular cardiac myocytes were isolated from adult Sprague-Dawley rats (2–3
mo old; weight 225–300 g; from Shanghai SLAC Laboratory Animal Company, Shanghai and Animal Center of Peking University, Beijing, China) by a previously described Langendorff method (21). Briefly, the heart was removed, placed in Tyrode solution containing (in mM) 137 NaCl, 0.5 MgCl₂, 10 glucose, 5.4 KCl, 1.8 CaCl₂, and 11.8 HEPES (pH adjusted to 7.4 with NaOH), and squeezed gently to expel the blood. Ventricular myocytes were prepared with a Langendorff perfusion apparatus. Briefly, the hearts were removed and perfused with calcium-free Tyrode containing (in mM) 130 NaCl, 1.2 MgSO₄, 5.4 KCl, 1.2 KH₂PO₄, and 6 HEPES-NaOH (pH adjusted to 7.2 with NaOH), with collagenase (Liberase Blendzyme 4, 0.1 mg/ml, Roche Molecular Biochemicals) for ~9 min. After the collagenase was washed out with calcium-free Tyrode, single cells were dissociated by mincing the ventricle and shaking the tissue in Kraftbrühe (KB) solution containing (in mM) 83 KCl, 30 K₂HPO₄, 5 MgSO₄, 5 Na-pyruvate, 5 Na-β-hydroxybutyrate, 5 creatine, 20 taurine, 10 glucose, 0.5 EGTA, 5 HEPES-KOH, and 5 ATP-Na₂ (pH adjusted to 7.2 with KOH). Cells were washed and resuspended in KB solution.

The animal protocols in this study were reviewed and approved by the Animal Research Advisory Committees in the Shanghai Institutes of Biological Sciences and Peking University.

**Whole cell patch-clamp recordings.** Ionic currents were studied in the whole cell patch-clamp configuration with an EPC-9 amplifier (HEKA Elektronik). 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 (100 ms) controlled electronically among seven channels (Inbio, Wuhan, China; Ref. 29).

Experiments on chromaffin and HEK293 cells were conducted at 32–35°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.

### Table 1. Composition of solutions

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<th>HEK293 Cells</th>
<th>Rat Myocytes</th>
<th>Chromaffin Cells</th>
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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. CdCl₂ was used to prevent Ca²⁺ entry from voltage-dependent Ca²⁺ channels 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, Iᵦ, which masks the activation of cardiac time-and voltage-dependent cation current Iᵥ. 4-AP was used to inhibit the activation of the transient outward potassium current, Iₒ, which can overlap the deactivation of Iᵥ.

As in our previous work, fluorescence calibration for Pᵣ was 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 with a monochromator-based system (TILL Photonics), and the resulting fluorescence signals were measured with a cooled charge-coupled device. Relative changes in [Ca²⁺]ᵢ were calculated from the ratio of fluorescence at 340 nm (F₃₄₀) to fluorescence at 380 nm (F₃₈₀), which were sampled at 1 Hz. The image data were transferred and analyzed by Igor software (WaveMetrix) (28).

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

\[
Pᵣ = \frac{\int I_{HCΝ,Ca} \, dt}{\int I_{HCΝ} \, dt} \tag{1}
\]

where \(I_{HCΝ} \) is the HCN current, \(I_{HCΝ,Ca} \) is the proposed fractional HCN current carried by Ca²⁺, and \(f_{max} \) is the maximum value of \(f \) = F₃₄₀/F₃₈₀.

The change of f₀, \(\Delta F₀\), is the “modified Ca²⁺-sensitive fura-2 signal” immediately before (F₀) and after (Fₙ) a voltage-pulse-induced Ca²⁺ influx (30). Under the condition that all entering calcium ions are bound by fura-2, F₀ is a measure of Ca²⁺ influx (30). Fₙ is determined by the difference of fluorescence signals at 340 and 380 nm.

\[
F₀ = F₃₄₀ - F₃₈₀ \tag{2}
\]

\[
\Delta F₀ = F₉₀ - F₄₉₀ \tag{3}
\]

\(f_{max}\) is determined by measuring Ca²⁺ influx through voltage-gated Ca²⁺ channels in chromaffin cells under the condition that intracellular fura-2 is sufficiently high (>0.4 mM; Ref. 32).

Under physiological conditions, only calcium ions contribute to the Ca²⁺ channels (30), so \(Pᵣ = 100\%\). From Eq. 1 we have

\[
f_{max} = \frac{\Delta F₀}{\int \Delta F \, dt} \tag{4}
\]

where \(I_{Ca} \) is the current through voltage-gated Ca²⁺ channels. Although the calibration of \(f_{max}\) is measured in chromaffin cells, the accuracy of \(Pᵣ(Iᵦ)\) 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 Ca²⁺/Ca⁻⁻ independent fluorescence signal F₃₆₀ (32), which can be calculated from F₃₄₀ and F₃₈₀.

\[
F₃₆₀ = F₃₄₀ + \alpha F₃₈₀ \tag{5}
\]

where \(\alpha\) is the “isocoefficient”. According to Eq. 5, \(\alpha\) can be determined by any experimental recording that shows rapid changes in Ca²⁺ concentration. In our setup, \(\alpha = 0.35\). Since F₃₆₀ is Ca²⁺ independent, it can be used as an indicator of the intracellular fura-2 concentration ([fura]₀). After the whole cell recording configuration was established, fura-2 was dialedized into the cell. Dialysis was accompanied by a proportional F₃₆₀ increase. Once F₃₆₀ reached a steady-state level, we assumed that [fura]ᵦ, was equal to the fura-2 concentration in the pipette (see Fig. 2 and Ref. 32).

We applied Eqs. 1–4 to determine the \(Pᵣ\) of HCN2 and Iᵥ channels by measuring the fura-2 signals.
Data were analyzed with IGOR Pro3.12 software (Wavemetrics, Lake Oswego, OR). Unless otherwise stated, data are presented as means ± 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 HEK293 cells. In a nontransfected HEK293 cell held at −70 mV, a hyperpolarizing pulse to −120 mV induced neither a time-dependent inward I_{HCN} nor Ca^{2+} flux (Fig. 1B, left). On the other hand, in response to the same hyperpolarizing pulse a HCN2-transfected cell exhibited a typical I_{HCN2} and Ca^{2+} flux at the same time (Fig. 1B, right), 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 (arrows in Fig. 1B, top) so that Ca^{2+} flux could be measured by the fura-2 signals (ΔF/Δt; see Ref. 30). Similar results were observed in five nontransfected cells and five 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, Fig. 1A). However, the Ca^{2+} flux appeared in the

![Diagram A: HCN-transfected HEK293 cell](image1)

![Diagram B: non-transfected cell vs HCN-transfected cell](image2)

Fig. 1. Hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels induced Ca^{2+} influx in HEK293 cells expressing HCN4. In response to a hyperpolarizing pulse to −120 mV for 5 s (arrows 1 and 3) from the holding potential of −70 mV, Ca^{2+} influx (arrow 1) was abolished when Ca^{2+} was removed from the bath (arrow 2) and reappeared after Ca^{2+} was added back to the bath (arrow 3). Similar results were observed in 10 cells. F_d, modified Ca^{2+}-sensitive fura-2 signal; AU, arbitrary unit. B: requirement of HCN channels for hyperpolarization-induced Ca^{2+} influx. Left: there were no time-dependent inward HCN current (bottom) and Ca^{2+} influx (top) signals in response to a 50-s hyperpolarization pulse (inset shows pulse protocol) in a nontransfected HEK293 cell. Right: in a HEK293 cell transfected with GFP-HCN2, a 10-s hyperpolarization pulse induced time-dependent inward HCN2 current (bottom inset) and Ca^{2+} influx (top) simultaneously.
presence of 2 mM Ca\(^{2+}\) (arrows 1 and 3, Fig. 1A). 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 calcium ions 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, \(I_{\text{HCN2}}\) was elicited by a step to \(-120\) mV for 3, 10, and 20 s (1–3, respectively, in protocol shown in Fig. 2B, inset) from a holding potential of \(-40\) mV. Measurement of Ca\(^{2+}\) fluorescence (Fig. 2, A and C) showed a rise in [Ca\(^{2+}\)], (arrow 1 in Fig. 2, A and C), and this rise was increased with longer pulse durations of 10 (arrow 2) and 20 (arrow 3) s. 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 s did not activate HCN4 channels (Fig. 3B, middle) and induced no Ca\(^{2+}\) signal (Fig. 3A; Fig. 3B, top), whereas a step to \(-120\) mV for 3 s activated the channels (Fig. 3B, middle) and simultaneously induced Ca\(^{2+}\) influx (Fig. 3A; Fig. 3B, top). The pulse protocol is shown in Fig. 3B, bottom. In Fig. 3A, the peaks between \(-120\) mV-3 s and \(-70\) mV-3 s marks correspond to hyperpolarizing steps to \(-120, -110, -100, -90\), and \(-80\) mV. Decreasing amplitudes of these peaks at various potentials suggest a voltage-dependent change in Ca\(^{2+}\) influx, which simultaneously accompanies the voltage-dependent activation of the channels. Figure 3C elaborates the relationship between normalized \(\Delta F_d\) and total ion inflow for HCN4 channels at tested pulses. The superimposed traces indicate a correlation between the HCN4 currents and the Ca\(^{2+}\) influx through HCN4 channels.

To quantify the Ca\(^{2+}\) influx through HCN channels, we utilized a widely used calibrating approach, quantitating Ca\(^{2+}\) flux through the voltage-dependent Ca\(^{2+}\) channel expressed in

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**Figure 2. Ca\(^{2+}\) influx through HCN2 channels in HEK293 cells.**

A: Ca\(^{2+}\) signals in response to 3-s (arrow 1), 10-s (arrow 2), and 20-s (arrow 3) hyperpolarizing pulses (see protocol in B). Fluorescence signals during fura-2 loading (0.1 mM in the pipette) at 360 nm (top trace: \(F_{360}\), Ca\(^{2+}\)-insensitive fluorescence, indicating the process of fura-2 entry into the cell), 380 nm (2nd trace: \(F_{380}\), Ca\(^{2+}\)-sensitive fluorescence, indicating Ca\(^{2+}\) influx), and \(F_d = F_{380} - F_{360}\) (3rd trace: \(F_d\), modified signal indicating Ca\(^{2+}\) influx) are shown together with intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]; bottom trace). 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 3-s pulse, arrow 2 the second 10-s pulse, and arrow 3 the 20-s pulse. Similar results were observed in 14 cells. B: HCN2 currents \(I_{\text{HCN2}}\) at \(-120\) mV for 3, 10, and 20 s. The voltage protocol is shown at bottom. C: enlarged \(F_d\) signals corresponding to arrows 1–3 in A.
rat adrenal chromaffin cells, which passes 100% Ca\(^{2+}\) (Ref. 28, also see Eq. 4 for details) as calibration for fura-2 signals. A depolarizing step to 0 mV for 500 ms from a holding potential of -70 mV (Fig. 4A, bottom) activated a voltage-dependent Ca\(^{2+}\) current (i\(_{Ca}\)) and simultaneously induced an increase in Fd (DF\(_{a2}\), Fig. 4B, top). The total ion influx charge was calculated from the time integral of i\(_{Ca}\) trace (Fig. 4B, middle, shaded region). In Fig. 4A we show that in a HEK293 cell expressing HCN2 channels a hyperpolarizing step to -120 mV for 10 s (Fig. 4A, inset) activated the HCN2 current (Fig. 4A, middle) and simultaneously induced an increase in Fd (DF\(_{a1}\), Fig. 4A, top). The time integral of ion flux through HCN2 channels was calculated from the current trace (Fig. 4A, middle, shaded area). The relationship between total ion influx and the corresponding increase in Fd (DF\(_{a}\)) obtained with different durations of stimulation (Fig. 4C) was best fitted by a linear equation, indicating a correlation of the increased DF\(_{a}\) with the increased ion flux through voltage-dependent calcium channels (Fig. 4B) and HCN2 channels (Fig. 4A). Using Eq. 1, we determined P\(_f\) 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 to -150 mV (Fig. 5A, inset) the I\(_f\) current traces are 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. 5, B and D) concomitant with activation of I\(_f\) (Fig. 5C, trace I). When I\(_f\) was blocked by 2 mM cesium (trace 2 in Fig. 5C), the increased Ca\(^{2+}\) signal was blocked as well (arrow 2 in Fig. 5, B and D). 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 P\(_f\) 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 spontaneously hypertensive rat 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 could 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). Figure 6 shows a typical example in that in response to a 10-s pulse to -150 mV from the holding potential of -70 mV, both I\(_{f}\) (Fig. 6A, top) and Ca\(^{2+}\) influx (Fig. 6A, bottom) through I\(_f\) channels are significantly larger in SHR myocytes than in normal rat myocytes. The averaged I\(_f\) current density at -150 mV is increased by 55% in SHR compared with 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.

On membrane depolarization, L-type Ca\(^{2+}\) 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 (3). It is well documented that Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels causes...
channel inactivation (11, 12). On the other hand, we wondered whether if calcium ions can enter the cell through $I_f$ channels on 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 preactivating $I_f$ on action potential duration, we compared the action potential duration measured at 15% of amplitude with and without preactivating $I_f$ (Fig. 7A). A hyperpolarizing pulse to $-120$ mV for 5 s was applied to open $I_f$ channels before initiation of an action potential (Fig. 7B). Compared to the control (without a preceding hyperpolarizing pulse that opens $I_f$ channels), the action potential duration was shortened by $32 \pm 7\%$ ($n = 5$, $P < 0.05$) and returned to control when the hyperpolarizing pulse was removed (Fig. 7, A and C). In addition, when intracellular Ca$^{2+}$ was buffered by addition of 10 mM EGTA to the pipette solution, the effect of preactivation of $I_f$ on the shortening of action potential duration was eliminated ($99 \pm 3\%$, $n = 8$; 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 $-40$ 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 ($27 \pm 4\%$, $n = 4$, $P < 0.05$; Fig. 7C). In the second experiment, ZD-7288 (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 ($91 \pm 5\%$, $n = 3$; 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 allows 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 this evidence points to the direction that the fractional Ca$^{2+}$ current may present in $I_f$ in cardiac myocytes.

To strengthen the link between the Ca$^{2+}$ flux and $I_f$ channel activation at very negative potentials, we need cardiac cells.
that natively express either higher or no $I_f$ channels. We chose SHR ventricular myocytes, in which $I_f$ channel expression is significantly higher compared with 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 Ca$^{2+}$ indeed permeates $I_f$ channels, although at $-150 \text{ mV}$ we cannot exclude the possible contribution of other ionic mechanisms such as Na$^+$/Ca$^{2+}$ exchanger current and Ca$^{2+}$ release from sarcoplasmic reticulum. The higher percent increase in Ca$^{2+}$ influx (69%) than in $I_f$ current (55%) may also reflect the possibility of involving another ionic mechanism. Nonetheless, these data point to a potential role of the fractional Ca$^{2+}$ influx through $I_f$ channels during diastole in pathophysiological ventricles where $I_f$ 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 Ca$^{2+}$ current, generated by Ca$^{2+}$ influx through L-type channels on membrane depolarization. The contribution of this Ca$^{2+}$ inward current to the action potential duration is limited by the inactivation of L-type Ca$^{2+}$ channels partially caused by Ca$^{2+}$ influx (3). Within every heartbeat, the amount of calcium that enters the cell during depolarization will have to get out of the cell when the membrane repolarizes to the resting potential via the Na$^+$/Ca$^{2+}$ exchanger and calcium pump (3). That means that, 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 the next action potential. The Ca$^{2+}$ influx through $I_f$ 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 nAChRs (2.5%; Ref. 32), glutamate channels (10% for NMDA; Ref. 17), AMPA/kainate receptors (0.5–5%; Refs. 4, 24), CNG channels (10–80%; Ref. 8), and voltage-dependent calcium channels (100%; Ref. 30), the P of HCN/I\(f\) channels is relatively small (0.47% in HCN2 and 0.6% in I\(f\) channels). However, given the nature of local calcium signaling, this small Ca\(^{2+}\) flux through I\(f\) channels may be sufficient

**Fig. 6.** Ca\(^{2+}\) influx through I\(f\) channels in spontaneously hypertensive rat (SHR) ventricular myocytes. A: I\(f\) currents (middle traces) and Ca\(^{2+}\) signals (bottom traces, shown as ratio of fluorescence to fluorescence at time 0 (F/F\(_0\)) in ventricular myocytes from control and SHR rats on a 10-s hyperpolarization pulse to \(-150\) mV from a holding potential of \(-70\) mV (protocol at top). B: comparison of I\(f\) 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 Ca\(^{2+}\) 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 (\(P < 0.001\)).

**Fig. 7.** I\(f\)-induced shortening in action potential duration. A: action potentials (APs, top 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 (bottom 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 APA has decayed to 15% of APA\(_{100}\). A prehyperpolarization pulse for 5 s shortened APD\(_{15}\). This effect was reversible (dashed line). B: hyperpolarization-induced current in the same ventricular myocyte as in 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). Bottom: pulse protocol. C: statistical analysis. Compared with control, APD\(_{15}\) was shortened by 32 ± 7% (\(n = 5\)) by preactivation of I\(f\) at \(-120\) mV for 5 s without EGTA in the pipette solution. Preactivation of Ca\(^{2+}\) channels for 50 ms also shortened APD\(_{15}\) by 27 ± 4% (\(n = 4\)). With 10 mM EGTA in the pipette, preactivation of I\(f\) for 5 s had no effect on APD (99 ± 3%; \(n = 8\)). ZD-7288 blocked the effect (91 ± 5%; \(n = 3\)).
to increase the local calcium concentration near the intracellular side of L-type Ca\(^{2+}\) channels, effectively accelerating \(I_{\text{Ca}}\) 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 50 mV in cardiac pacemaker sinoatrial (25) and atrial (18) myocytes and −70 mV in 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 could be tested. This can finally establish the physiological relevance of fractional Ca\(^{2+}\) current through HCN.

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