Mechanistic Role of $I_f$ Revealed by Induction of Ventricular Automaticity by Somatic Gene Transfer of Gating-Engineered Pacemaker (HCN) Channels

Tian Xue, PhD*; Chung-Wah Siu, MBBS*; Deborah K. Lieu, PhD; Chu-Pak Lau, MD; Hung-Fat Tse, MD; Ronald A. Li, PhD

Background—Although $I_f$, encoded by the hyperpolarization-activated cyclic-nucleotide-modulated (HCN) channel gene family, is known to be functionally important in pacing, its mechanistic action is largely inferential and indeed somewhat controversial. To dissect in detail the role of $I_f$, we investigated the functional consequences of overexpressing in adult guinea pig left ventricular cardiomyocytes (LVCMs) various HCN1 constructs that have been engineered to exhibit different gating properties.

Methods and Results—We created the recombinant adenoviruses Ad-CMV-GFP-IRES (CGI), Ad-CGI-HCN1, Ad-CGI-HCN1-ΔΔΔ, and Ad-CGI-HCN1-Ins, which mediate ectopic expression of GFP alone, WT, EVY235-7ΔΔΔ, and Ins HCN channels, respectively; EVY235-7ΔΔΔ and Ins encode channels in which the S3–S4 linkers have been shortened and lengthened to favor and inhibit opening, respectively. Ad-CGI-HCN1, Ad-CGI-HCN1-ΔΔΔ, and Ad-CGI-HCN1-Ins, but not control Ad-CGI, transduction of LVCMs led to robust expression of $I_f$ with comparable densities when fully open ($\sim 22$ pA/pF at $-140$ mV; $P>0.05$) but distinctive activation profiles ($V_{1/2} = -70.8 \pm 0.6, -60.4 \pm 0.7$, and $-87.7 \pm 0.7$ mV; $P<0.01$, respectively). Whereas control (nontransduced or Ad-CGI–transduced) LVCMs were electrically quiescent, automaticity (206±16 bpm) was observed exclusively in 61% of Ad-HCN1-ΔΔΔ–transduced cells that displayed depolarized maximum diastolic potential ($-60.6 \pm 0.5$ versus $-70.6 \pm 0.6$ mV of resting membrane potential) of control cells; $P<0.01$) and gradual phase 4 depolarization (306±32 mV/s) that were typical of genuine nodal cells. Furthermore, spontaneously firing Ad-HCN1-ΔΔΔ–transduced LVCMs responded positively to adrenergic stimulation ($P<0.05$) but exhibited neither overdrive excitation nor suppression. In contrast, the remaining 39% of Ad-HCN1-ΔΔΔ–transduced cells exhibited no spontaneous action potentials; however, a single ventricular action potential associated with a depolarized resting membrane potential and a unique, incomplete “phase 4–like” depolarization that did not lead to subsequent firing could be elicited on simulation. Such an intermediate phenotype, similarly observed in 100% of Ad-CGI-HCN– and Ad-CGI-HCN1-Ins–transduced LVCMs, could be readily reversed by ZD7288, hinting at a direct role of $I_f$. Correlation analysis revealed the specific biophysical parameters required for $I_f$ to function as an active membrane potential oscillator.

Conclusions—Our results not only contribute to a better understanding of cardiac pacing but also may advance current efforts that focus primarily on automaticity induction to the next level by enabling bioengineering of central and peripheral cells that make up the native sinoatrial node. (Circulation. 2007;115:1839-1850.)

Key Words: genes ■ ion channels ■ pacemakers ■ sinoatrial node ■ tissue engineering

$I_f$, a depolarizing, mixed Na$^+$/K$^+$ inward cardiac membrane current encoded by the hyperpolarization-activated cyclic-nucleotide-modulated (HCN1–4) channel gene family,¹ is known to functionally modulate pacing. For instance, human HCN mutations have been linked to sinus node dysfunction.²,³ Although $I_f$ has been presumed to exert its effect on pacing by driving diastolic depolarization to the action potential (AP) threshold (or takeoff potential [TOP]) after each excitation cycle, the precise mechanistic role remains largely inferential and somewhat controversial.¹⁴–¹² Indeed, the physiological relevance of $I_f$ has even been questioned because of its intrinsically slow kinetics and negative activa-

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tion relative to the time scale and voltage range of cardiac pacing.\textsuperscript{13} For instance, HCN1, the fastest isoform, activates at $\approx -80$ mV, with opening time constants in the range of seconds (versus typical maximum diastolic potential [MDP] of $\approx -62$ mV and cardiac cycle length of $\approx 800$ ms in humans). Although overexpression of wild-type (WT) HCN2 or HCN4 alone in spontaneously contracting, $I_f$-expressing cultured neonatal left ventricular cardiomyocytes (LVMCs) hastens their firing rate,\textsuperscript{14,15} neither suffices to cause automaticity in normally quiescent adult LVMCs that lack $I_f$. Along with the finding that genetic suppression of the inward rectifier (Kir), a stabilizer of the resting membrane potential (RMP; $\approx -80$ mV), alone suffices to induce pacing in ventricular myocytes (albeit the induced firing is $\approx$3-fold slower than normal), it has been postulated that $I_f$ merely plays a secondary role in the generation of cardiac rhythms.\textsuperscript{16,17}

Using a mathematical approach, we have previously provided a conceptual framework of how $I_f$ may actively contribute to pacing.\textsuperscript{13} With this computational model as a guide, we subsequently demonstrated that in vivo gene transfer of an engineered HCN1 construct to mediate $I_f$ expression in a large animal porcine model of sick sinus syndrome substantially reduces the dependence on electronic device for supportive pacing.\textsuperscript{18} Although these proof-of-concept experiments indicate that our gene-based approach of reconstructing a bioartificial sinoatrial (SA) node is functionally feasible, the fundamental operating mechanism of $I_f$ requires more thorough investigation and detailed analysis. Furthermore, the biological properties of pacemaker-like cells converted from cardiac muscle cells need to be further characterized. For instance, do they exhibit override suppression and/or excitation? Given that a healthy SA node, unlike the Purkinje fibers, is resistant to override suppression (or undesirable long pauses will result in the event of an ectopic rhythm), these are important properties to consider if HCN-based gene therapy is to be further developed for treating defects of cardiac impulse generation. Clearly, a better understanding is crucial for fine-tuning $I_f$-induced pacing.

In the present study, we focused on mechanistically dissecting the intricate interrelationships between various biophysical parameters of $I_n$, $I_{K1}$, and cardiac automaticity. Using HCN1 constructs that have been engineered to exhibit different gating properties, we investigated the functional consequences of their overexpression in adult guinea pig LVMCs and explored the underlying correlations. Our results not only contribute to a better understanding of cardiac pacing but also may advance current efforts that focus primarily on automaticity induction to the next level by enabling bioengineering of central and peripheral cells that make up the native SA node (via a direct gene transfer\textsuperscript{18} or an ex vivo stem cell approach\textsuperscript{19–21}).

Methods

Molecular Biology, Adenoviral Transduction

Polymerase chain reaction–based mutagenesis of mouse HCN1 (generously provided by Dr Steve Segalbaum, Columbia) was performed with overlapping oligos as described previously.\textsuperscript{22,23} Although HCN4 is the predominant isofrom expressed in the SA node (at least in rabbit),\textsuperscript{24} HCN1 was chosen because its structure-function properties were investigated more extensively in our previous studies\textsuperscript{13,22,23,25–30} and the library of constructs available. However, because HCN channels were engineered to exhibit particular gating profiles for our experiments, the ultimate biophysical properties of a given recombinant channel override the specific species and isoform used. The bicistronic adenosine shuttle vectors pAdCMV-GFP-IRES (pAdCGI) have been described elsewhere.\textsuperscript{31} Internal ribosomal entry site (IRES) allows the simultaneous translation of 2 transgenes with a single transcript and, in our experiments, GFP and an HCN1 construct. WT HCN1, HCN1-Ins, or HCN1-$\Delta$$\Delta$ was cloned into the second position of pAdCGI at EcoRI and XmnI to generate pAdCGI-HCN1, pAdCGI-HCN1-Ins, or pAdCGI-HCN1-$\Delta$$\Delta$, respectively. Adenosines were generated by Cre-lox recombination of purified φ5 viral DNA and shuttle vector DNA using Cre4 cells as previously described.\textsuperscript{32} The recombinant products were plaque purified, amplified, and purified again by CsCl gradients, yielding concentrations on the order of $10^9$ plaque-forming units per 1 mL.

Adenosine-Mediated Gene Transfer and Isolation of LVMCs

Adult guinea pigs ($\approx$250 g) were euthanized by injection of pentobarbital (80 mg/kg IP). The hearts were quickly excised, followed by perfusion with enzymatic solutions using a customized Langendorff apparatus as previously described.\textsuperscript{33} LVMCs were cultured on laminin-coated glass coverslips in 24-well dishes ($\approx 5 \times 10^4$ per well) in 5% CO$_2$, 37°C water-jacket incubator initially with medium containing 5 mmol/L carnitine, 5 mmol/L creatine, 5 mmol/L taurine, 100 µg/mL penicillin-streptomycin, and 10% fetal bovine serum in medium 199 (Sigma-Aldrich Corp, St Louis, Mo) for 2 hours. For transduction, the medium was replaced by a serum-free culture medium containing adenosiral particles at a concentration of $\approx 2 \times 10^9$ plaque-forming units and the same supplements described above. The adenosine-containing medium was replaced again by fresh serum-free medium after 24 hours. A transduction efficiency of $\approx 70$% to 80% typically could be achieved with this protocol. In some initial experiments, LVMCs were freshly isolated from hearts of animals that underwent in vivo intracardiac injection of adenosinoviruses as described recently\textsuperscript{34} and recorded within 24 hours (see Figure I in the online Data Supplement for typical images of LVMCs isolated from injected hearts). Because identical data trends were obtained (see Electrophysiology for specific data presented and our recently published article for details\textsuperscript{35}), we switched to the in vitro transduction system by which a single batch of isolated LVMCs could be used to study >1 adenoviral construct to increase the amount of data that could be collected and to minimize the need of euthanizing animals. Such an in vitro system of adult guinea pig LVMCs has been previously used by us\textsuperscript{34} and others.\textsuperscript{35}

Electrophysiology

Electrical recordings were performed with the whole-cell patch-clamp technique with an Axopatch 200B amplifier and the pClamp 9.2 software (Axon Instruments Inc, Foster City, Calif). A xenon arc lamp was used to view green fluorescent protein fluorescence at 488/530 nm (excitation/emission). Successfully transduced cells were recognized by their green epifluorescence. Patch pipettes were prepared from 1.5-mm thin-walled borosilicate glass tubes using a Sutter micropipette puller P-97 and had typical resistances of 3 to 5 MΩ when filled with an internal solution containing (mmol/L) 110 K\textsuperscript{+} aspartate, 20 KCl, 1 MgCl\textsubscript{2}, 0.1 Na-GTP, 5 Mg-ATP, 5 Na\textsubscript{2}-phosphocreatine, 1 EGTA, and 10 HEPES, pH adjusted to 7.3 with KOH. The external Tyrode’s bath solution was composed of (mmol/L) 140 NaCl, 5 KCl, 1 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, 10 glucose, and 10 HEPES, pH adjusted to 7.4 with NaOH. Voltage- and current-clamp recordings were performed at body temperatures ($\approx 37°C$) within 24 to 36 hours after culturing. To avoid contaminations of automaticity resulting from time-dependent $I_n$ reduction\textsuperscript{19} in culture, control cells were recorded during the above time window; $I_n$ density at $\approx 100$
mV of cultured LVCMs (−16.4±1.2 pA/pF; n=9) was modestly reduced but not significantly different (P>0.05) compared with those recorded from freshly isolated LVCMs (−20.4±1.9 pA/pF; n=9). At the same time, there was an insignificant change in cell capacitance from the freshly isolated LVCMs (100.5±6.9 pF) to their cultured counterparts (122.2±10.4 pF; P>0.05). Despite such a modest reduction in I\textsubscript{K} after culturing for 24–36 hours, AP parameters were not altered (eg, RMP, −71.7±0.5 and −70.6±0.6 mV; APD\textsubscript{50}, 289±32 and 281±28 ms; n=9 and 8, respectively; P>0.05). Absolutely no automaticity was detected from cultured control LVCMs recorded over the same period.

**Electrophysiological Protocols and Data Analysis**

To elicit inward currents, cells were held at −30 mV and pulsed from 0 to −140 mV at 10-mV increments for 2 seconds, followed by a 1-second −100-mV pulse. I\textsubscript{f} was defined as 10 μmol/L ZD7288-sensitive, 1 mmol/L Ba\textsuperscript{2+}-insensitive, time-dependent currents. For recording APs, cells were held at 0 pA without (for electrically active cells) or with stimulation of 0.1 to 1 mA for 5 ms, just enough to elicit a response.

The steady-state current-voltage (I-V) relationship was determined by plotting the currents measured at the end of the 2-second test pulses of the above-mentioned protocol against the test potentials. The voltage dependence of HCN channel activation was assessed by plotting time-dependent tail currents at −100 mV measured immediately after a 2-second test pulse (0 to −140 mV) as a function of the test pulse voltage. Currents were normalized to the maximum tail current recorded. These recordings were made in the presence of 1 mmol/L BaCl\textsubscript{2} to block I\textsubscript{K1}. Data were fit to the Boltzmann functions using the Marquardt-Levenberg algorithm in a nonlinear least-squares procedure:

\[ \text{m} = \frac{1}{1 + \exp \left( \frac{V - V_{1/2}}{k} \right)} \]

where V\textsubscript{t} is the test potential, V\textsubscript{1/2} is the half-point of the relationship, and k=RT/zF is the slope factor.

The relationships of I\textsubscript{f} with the voltage range of phase 4–like depolarization (ΔV\textsubscript{phase4}) were fitted with single exponential decay functions in a nonlinear least-squares procedure:

\[ \Delta V_{\text{phase4}} = V_{\text{max}} \times \left[ 1 - \exp \left( \frac{-t}{I} \right) \right] \]

where I is the magnitude of I\textsubscript{f} at −60 mV and V\textsubscript{max}, I\textsubscript{0}, and I\textsubscript{1} are parameters determined by fitting functions.

Rectification ratio\textsuperscript{37} (RR) is defined as the following:

\[ \text{RR} = \frac{I_{100mV}}{I_{-60mV}} \]

where I\textsubscript{100mV} and I\textsubscript{-60mV} for the absolute current amplitudes at −100 and −60 mV, respectively. These data are not corrected for the junction potential. All data reported were mean±SEM. Comparisons of variables between grouped were made with the unpaired Student t test; comparisons of variables of the same group in paired experimental conditions were made with the paired-sample t test when appropriate. Results were considered statistically significant at a value of P<0.05.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Adenovirus-Mediated Overexpression of I\textsubscript{f} in LVCMs**

As a first step, we created the recombinant adenoviruses Ad-CGI, Ad-CGI-HCN1, Ad-CGI-HCN1–ΔΔΔ, and Ad-CGI-HCN1-Ins, which mediate ectopic expression of GFP alone, WT, EVY235-7ΔΔΔ, and Ins HCN1 channels, respectively. EVY235-7ΔΔΔ and Ins encode HCN1 channels in which the S3–S4 linkers have been shortened by deleting residues 235 to 237 and lengthened by inserting 2 glutamines to flank each of the C- and N-terminal sides of residues 235 to 237 (ie, QQ235 to 237QQ) to favor and inhibit opening, respectively, as recently described.\textsuperscript{22,23,25}

Figure 1 shows that I\textsubscript{K1} (Figure 1A and 1E), which could be completely blocked by 1 mmol/L Ba\textsuperscript{2+} (Figure 1B and 1F), was robustly expressed in control (nontransduced or Ad-CGI-transduced) adult guinea pig LVCMs (n=20 from 4 animals). For Ad-CGI-HCN1–transduced cells, a similar Ba\textsuperscript{2+}-sensitive I\textsubscript{K1} with properties not different from those of control cells also was expressed (Figure 1C and 1E; P>0.05). In contrast, a time-dependent current component, reminiscent of nodal I\textsubscript{f} with peak current densities of −22±3 pA/pF at −140 mV also could be recorded after 1 mmol/L Ba\textsuperscript{2+} subtraction (n=12; Figure 1D). Ad-CGI-HCN1–induced I\textsubscript{f} was sensitive to the known HCN blocker Cs\textsuperscript{+} or ZD7288 (not shown), increased in magnitude, and became faster with progressive hyperpolarization (Figure 1F). The midpoint (V\textsubscript{1/2}) and slope factor (k) derived from the steady-state activation curve were −70.8±0.6 mV and 8.1±0.8, respectively (Figure 2E, solid squares).
Similarly, Ba$_2^+$-sensitive $I_{K1}$ (Figure 1E) and ZD7288-sensitive, Ba$_2^+$-insensitive, time-dependent $I_{f}$ (Figures 1F and 2C) could be recorded from Ad-CGI-HCN1-$\Delta\Delta\Delta$- and Ad-CGI-HCN1-Ins–transduced LVCMs ($P>0.05$); examination of steady-state activation revealed that Ad-CGI-HCN1-$\Delta\Delta\Delta$- and Ad-CGI-HCN1-Ins–induced $I_{f}$ had positively ($V_{1/2}=-60.4\pm0.7$ mV; $k=8.7\pm0.6$; $n=7$) and negatively ($V_{1/2}=-87.7\pm0.7$ mV; $k=11.4\pm0.4$; $n=17$) shifted activation midpoints, respectively (Figure 2E), consistent with our previous heterologous expression experiments.22,25 The differences in activation threshold

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**Figure 2.** Effects of WT or engineered HCN1 transduction on pacing ability of LVCMs. A, AP waveforms of a representative control LVCM with (solid line) and without (broken line) stimulation. The arrow indicates time of stimulation. B, The same cell generates APs in the presence of 1 mmol/L Ba$_2^+$ without stimulation. C, Representative current tracings of Ba$_2^+$-subtracted $I_{f}$ recorded from HCN1-WT–, HCN1-$\Delta\Delta\Delta$–, and HCN1-Ins–transduced LVCMs. Currents recorded at $-50$ mV are shown in red. Note that the kinetics observed here in LVCMs were faster than the same channels that we reported previously.23 Nonetheless, the difference in activation midpoints relative to WT persisted (see E). D, APs without stimulation from the same cells. Automaticity exists in $61\%$ of HCN1-$\Delta\Delta\Delta$–transduced LVCMs at an average AP firing rate of $216\pm23$ bpm but none in control, HCN1-WT–, or HCN1-Ins–transduced cells. E, The steady-state activation curves of HCN1-WT–, HCN1-$\Delta\Delta\Delta$–, and HCN1-Ins–induced $I_{f}$ currents.
Spontaneous AP Firing

Ad-CGI-HCN1-∆∆∆ but Not Ad-CGI-HCN1 or Ad-CGI-HCN1-Ins Transduction Induced Spontaneous AP Firing

Shown in Figure 2A is a typical control ventricular cell that normally was electrically quiescent with no spontaneous activity. On injection of a stimulating current (800 pA for 2 to 5 ms), the same cell generated a single AP, indicating normal ventricular excitability. Addition of 1 mmol/L BaCl2 to block I
t, destabilized the normal RMP and subsequently resulted in spontaneous firing that was similar to that induced by I
t, genetic suppression16,17 but ≈2.5-fold slower than that of genuine guinea pig nodal cells (Figure 2B). Collectively, these observations indicate that although I
t suppression unleashes latent pacemaker activity of ventricular cardiomyocytes, it did not lead to the normal frequency of endogenous nodal pacing. All AP parameters are summarized in Table 1.

We next investigated the functional consequences of Ad-CGI-HCN1−, Ad-CGI-HCN1-Ins−, and Ad-CGI-HCN1-∆∆∆−mediated I
t overexpression in the AP waveform of LVCMs (Figure 3). Interestingly, automaticity was exclusively observed in 61% of Ad-CGI-HCN1-∆∆∆−transduced cells recorded (n=19 of 31), even without I
t inhibition and pulse stimulation, but never in control LVCMs. The AP firing rate of electrically active Ad-CGI-HCN1-∆∆∆−transduced LVCMs was 206±16 bpm, comparable to the native guinea pig heart rate and much higher than that induced by BaCl2 (P<0.01). The AP duration at 50% repolarization (APD50) (239±23 ms) was not different from control LVCMs (Table 1; P>0.05). Of note, the MDP (−60.6±0.5 mV; n=19) was significantly depolarized relative to the RMP of control cells (−70.6±0.6 mV; n=8; P<0.01) and associated with a gradual phase 4 depolarization (slope=306±32 mV/s). These properties were typical of genuine nodal cells. Nonetheless, the rapid AP upstroke (V=86±8 V/s; n=13) and overshoot observed were indicative of the ventricular origin of these rhythmic “pacemaker-like” cells. These in vitro data were in complete accordance with our recently reported in vivo experiments.18

To obtain mechanistic insights, we next studied the effect of ZD7288 on Ad-CGI-HCN1-∆∆∆−transduced cells. Interestingly, both the induced I
t and AP firing could be blocked by ZD7288 (Figure 3A). Indeed, WT RMP also was restored (−69.3±0.4 mV; n=8; P>0.05) (Figure 3B, blue line). Interestingly, when given a depolarizing stimulus, silenced Ad-CGI-HCN1-∆∆∆−transduced cells could once again elicit normal single APs (Figure 3B, red line). All AP parameters before and after I
t blockade are summarized in Table 1. Collectively, these data indicated that Ad-CGI-HCN1-∆∆∆−mediated AP firing was a direct result of the induced I
t. The following experiments were designed to mechanistically dissect specific I
t properties that led to automaticity.

Intermediate Phase 4–Like Depolarization

In stark contrast to the pacemaker-like phenotype, the remaining 39% of Ad-HCN1-∆∆∆−transduced cells exhibited a depolarized RMP (−61.3±1.2 mV; n=12; P<0.01) but without spontaneous APs (as a result of a lower level of expressed I
t because of biological variation; see later); however, a single ventricular AP associated with an incomplete “phase 4–like” depolarization that did not lead to subsequent firing could be elicited on simulation (Figure 3C). Addition of ZD7288 restored RMP to the normal hyperpolarized level (−72.0±1.2 mV; n=8) (Figure 3C, blue line; Table 1). Furthermore, a normal ventricular AP also could be elicited on stimulation after I
t blockade by ZD7288 (Figure 3C, red line). Of note, the incomplete phase 4–like depolarization also disappeared. Such an intermediate phase 4–like phenotype, which could be reverted to control ventricular AP phenotype after ZD7288 blockade, was similarly observed in 100% of Ad-CGI-HCN− (Figure 3D) and 100% of Ad-CGI-HCN1-Ins–transduced LVCMs (Figure 3E), indicating that

### TABLE 1. Summary of AP Parameters

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<tr>
<th></th>
<th>RMP, mV</th>
<th>MDP, mV</th>
<th>APD50, ms</th>
<th>Pacing Rate, bpm</th>
<th>Phase 4 Slope, mV/s</th>
<th>Phase 4 Length, ms</th>
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<tr>
<td>Control (8)</td>
<td>−70.6±0.6</td>
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<td>281±28</td>
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<td>Control+ZD7288 (6)</td>
<td>−70.9±1.2</td>
<td>...</td>
<td>272±31</td>
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<td>...</td>
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<td>Intermediate phase 4–like AP</td>
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<td>HCN1-WT (15)</td>
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<td>−63.2±1.0</td>
<td>307±35</td>
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<td>HCN1-Ins (16)</td>
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<td>−65.9±0.9</td>
<td>321±29</td>
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<tr>
<td>HCN1-∆∆∆ (12)</td>
<td>−61.3±1.2*</td>
<td>−68.3±0.8</td>
<td>266±35</td>
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<td>...</td>
<td>...</td>
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<tr>
<td>HCN1-∆∆∆+ZD7288 (8)</td>
<td>−72.0±1.2</td>
<td>...</td>
<td>286±38</td>
<td>...</td>
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<td>...</td>
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<tr>
<td>Spontaneously firing cells</td>
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<td>HCN1-∆∆∆ (19)</td>
<td>−60.6±0.5†</td>
<td>...</td>
<td>239±23</td>
<td>206±16†</td>
<td>306±32†</td>
<td>85.1±8.6†</td>
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<td>Control+BaCl2 (10)</td>
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<td>296±34</td>
<td>92±15</td>
<td>46.2±7.6</td>
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*Statistically different (P<0.01) from control.  †Statistically different (P<0.01) from Ba2+ cells.
overexpression of WT or Ins HCN1 channels clearly influences the RMP and phase 4 depolarization but was insufficient to lead to AP firing, unlike HCN1-ΔΔΔ.

To explore the basis of phase 4–like depolarization, the difference between RMPs before and after ZD7288 (ΔVphase4) was plotted against the If magnitude at 60 mV (the earliest voltage at which Ad-CGI-HCN1– and Ad-CGI-HCN1-Ins–induced If could first be recorded) of Ad-CGI-HCN1–, Ad-CGI-HCN1-ΔΔΔ–, and Ad-CGI-HCN1-Ins–transduced LVCMs (Figure 3F). The data were well fitted with a single exponential function, although statistically different plateau values were reached for the 3 groups (20.9 ± 1.2, 15.5 ± 1.8, and 12.0 ± 1.5 mV, respectively; P < 0.01). Of note, the rank order parallels the corresponding activation midpoints.

Adrenergic Stimulation of Spontaneously Firing Ad-CGI-HCN1-ΔΔΔ–Transduced LVCMs

To further characterize spontaneously-firing Ad-CGI-HCN1-ΔΔΔ–transduced LVCMs as a pacemaker, we next studied their response to adrenergic stimulation by superfusion of 1 μmol/L isoproterenol for 5 minutes. The cycle length reduced from 234 ± 5.5 to 182 ± 4.1 ms (P < 0.01; n = 4), representing a 27% increase in firing rate (Figure 4A and 4B). This acceleration could be attributed largely to the change in phase 4 slope from 295 ± 5 to 350 ± 12 mV/s, which subsequently shortened APD (P < 0.05; Figure 4C); MDP also became more depolarized (from −60.3 ± 0.4 to −55.5 ± 0.7 mV; P < 0.05; Figure 4D). However, there was no significant change in the TOP after isoproterenol application (−45.2 ± 0.4 versus −44.6 ± 0.5 mV of control; P > 0.05; Figure 4E). On inspection of the steady-state activation curves recorded from the same cells, the midpoint (V1/2) was positively shifted from −62.5 ± 1.0 to −56.9 ± 1.6 mV (P < 0.05; Figure 4F).

Effects of Acetylcholine on Spontaneous AP Firing

Furthermore, we studied the effect of acetylcholine on the pacing parameters of spontaneously firing Ad-CGI-HCN1-ΔΔΔ–transduced LVCMs. On application of acetylcholine (1 μmol/L) to the bath solution, there were no significant changes in cycle length (293 ± 17 versus 293 ± 16 ms; n = 6; P > 0.05), MDP (−59.2 ± 0.9 versus −59.0 ± 1.0 mV; P > 0.05), and phase 4 slope (236 ± 15 to 241 ± 16 mV/s) from baseline to acetylcholine (20 seconds; Figure 4G).
Effects of Tetrodotoxin on Spontaneous AP Firing

On application of the Na channel-specific blocker tetrodotoxin (TTX; 60 μmol/L) to electrically active Ad-CGI-HCN1–transduced LVCMs (n=4), there was a notable increase in cycle length from 256±11 to 328±23 ms (Figure 5A and 5B). This increase was associated with a decrease in the maximal upstroke velocity of AP from 68.8±8.3 to 5.3±0.6 V/s (P<0.01), as well as a depolarized TOP from 43.7±0.4 to 27.0±3.4 mV (P<0.05; Figure 5C and 5D). Of note, the AP overshoot also disappeared. Despite these changes, there was no statistical difference in the phase 4 slope and MDP (Figure 5E and 5F), consistent with the notion that If was unaltered in these experiments.

Effects of Overdrive Pacing

To test the response to overdrive pacing, a train of pulse stimulation of 0.1 to 1 nA for 5 ms at 6.7 Hz (ie, cycle length, 150 ms or 400 per minute) was applied to electrically active Ad-CGI-HCN1–transduced LVCMs for 30 seconds (n=6) in a manner similar to that previously used to study overdrive suppression in rabbit SA nodal cell. A representative experiment is shown in Figure 6A. Figure 6B shows on an expanded scale overlapping APs recorded before, during, and after overdrive stimulation. The cycle length significantly shortened during the stimulation, indicating that the ability of Ad-CGI-HCN1–induced biopacemaker to adapt to the rate remained intact. However, there were no detectable changes in phase 4 slope, MDP, and TOP (Figure 6C; P>0.05). These parameters are summarized in Table 2.

What Specific If Parameters Underlie the Induced Automaticity?

To mechanistically dissect the role of If in spontaneous AP firing, we next performed correlation analysis to examine in details the relationships between various AP parameters and If properties. Figure 7 shows that for electrically active Ad-CGI-HCN1–induced LVCMs, the induced pacing rate (A), MDP (B), and phase 4 slope (C) were all positively correlated to the If density at −50 mV (r=0.94, 0.70, and 0.81, respectively). Whereas net outward whole-cell currents of LVCMs (from 40 to 70 mV) tend to hyperpolarize the cells, Ad-CGI-HCN1–transduced cells had zero or inward currents over the same voltage range (Figure 8A), which tend to depolarize the cells. All these in turn translated into a significantly smaller rectification ratio (Figure 8B). When the net total currents at −50 mV (IIf−IK1) of control and Ad-CGI-HCN1–transduced cells were plot-
ted against the corresponding RMP or MDP (Figure 8C), an interesting pattern emerged: RMP or MDP became more depolarized with increasingly less positive \( I_{f} + I_{K1} \), consistent with that presented in Figure 7B; furthermore, net negative \( I_{f} + I_{K1} \) occurred only in spontaneously firing cells (ie, 61% of Ad-CGI-HCN1–transduced cells; squares in Figure 8), never in the quiescent ones (including control cells, 39% of Ad-CGI-HCN1–transduced cells, and electrically active Ad-CGI-HCN1–transduced LVCMs that had been silenced by ZD7288, as represented by inverted triangles, circles, and triangles, respectively).

**Discussion**

Although \( I_{f} \) is most abundant in the SA node, the region that normally paces the entire heart, it is also found at various levels in cardiac tissues such as the atria and ventricles. Unlike rhythmic nodal pacemaker cells, however, adult atrial and ventricular cells are normally electrically quiescent unless they are stimulated by pacemaker activity arising elsewhere. This is due to the intense expression in the atria and ventricles of the cardiac inward-rectifier K⁺ current or \( I_{K1} \), encoded by the Kir2 gene family, that stabilizes a negative resting membrane potential (\( \sim 80 \) mV) and thus suppresses any latent spontaneous electrical activity while maintaining the cells fully excitable. In contrast, \( I_{f} \), an “opponent” of \( I_{o} \) is absent in nodal pacemaker cells, rendering the latter more prone to oscillations. Miake and colleagues\(^{16,17} \) have demonstrated that latent pacemaker activity of normally silent ventricular myocytes can be unleashed to produce spontaneous firing activity by genetic inhibition of Kir2-encoded \( I_{K1} \). However, \( I_{K1} \) suppression–induced pacemaking activity is \( \sim 3 \) times slower than normal, indicating that other genuine, active players are involved in pacemaking nodal cells. In the present study, we focused on dissecting the mechanistic role of \( I_{f} \).

The experimental results presented in this study fully support the notion that \( I_{f} \) is a crucial, intrinsic oscillator, rather than just a secondary modulator, of the membrane potential. However, its contribution is heavily dependent on the relative expression level of \( I_{K1} \). Mechanistically, the RMP is \( \sim 71 \) mV when \( I_{K1} \) dominates and the net current is outward with an amplitude of at least 1.6 pA/pF (ie, \( |I_{f} + I_{K1}| < 50 \text{mV} \)). However, when the net outward current is \( \sim 1.6 \) pA/pF (ie, 0 pA < \( |I_{f} + I_{K1}| < 1.6 \) pA/pF) because of increasing levels of \( I_{f} \) expression, \( I_{f} \) partially counterbalances \( I_{K1} \) and thereby depolarizes the RMP (up to \( \sim 62 \) mV) or even drive membrane depolarization, leading to the incomplete, phase 4–like slope observed in our intermediate AP phenotypes (cf Figure 3C through 3E).

Within the range of 0 to 1.6 pA/pF, \( I_{f} \) amplitude is proportional to the rising speed of phase 4–like depolarization (Figure 7C) but is still insufficient to cause electrical oscillations. When \( I_{f} \) is sufficiently expressed to dominate \( I_{K1} \), so that the net current becomes inward (ie, \( |I_{f} + I_{K1}| < 0 \) and net current becomes inward (ie, \( |I_{f} + I_{K1}| < 0 \))
pA/pF), spontaneous firing can be induced. Once induced, both the firing rate (Figure 7A) and MDP (Figure 7B) are directly proportional to $I_f$ (up to at least $\frac{6}{pA/pF}$).

In recent years, various gene- and cell-based approaches have been tested to generate a functional biological pacemaker as a potential replacement for or supplement to conventional electronic pacemakers that are commonly used to treat impulse generation defects (e.g., bradycardias). Understanding the mechanistic action of $I_f$ in cardiac automaticity is invaluable for achieving this goal. Based on the substantial groundwork previously done by others, our recent in silico analysis, in vivo experiments, and present mechanistic study clearly indicate that genetic induction of pacing and, more important, its fine-tuning, can be achieved by tuning the relative activity of $I_f$ and $I_K1$. This can perhaps be accomplished best by custom tailoring the activation gating properties of $I_f$ via engineering of such HCN channel functional motifs as the S3–S4 linker or the S4 segment. Of note, although $I_f$ and $I_K1$ counterbalance each other within a certain voltage range, $I_K1$ also hyperpolarizes the RMP, which in turn can result in a higher probability of opening for the so-called hyperpolarization-activated $I_f$ channels. This is particularly true under the dynamic conditions of pacing as a result of hysteretic properties of HCN channels. Therefore, the strategies of $I_K1$ inhibition and $I_f$ overexpression to induce pacing in nonnodal cardiomyocytes, which carry distinct compositions of other ion channel types, are not necessarily synergetic. Clearly, a fine balance between the 2 is needed for the most desirable outcome. Further experiments to fully explore the intricate interrelationships between $I_f$ and $I_K1$ in cardiac pacing are warranted.

Because biological pacemakers are powered by passive transports driven by the ionic gradients, they do not require battery replacement like electronic pacemakers. More important, another major advantage over the electronic counterpart is the ability to maintain the in vivo responsiveness of pacing

### TABLE 2. Effects of Overdrive Pacing on Ad-CGI-HCN1-ΔΔΔ Spontaneously Firing Cells

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>After Overdrive</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pacing rate, bpm</td>
<td>217±13</td>
<td>214±15</td>
<td>0.32</td>
</tr>
<tr>
<td>MDP, mV</td>
<td>−61.3±0.4</td>
<td>−61.6±0.7</td>
<td>0.94</td>
</tr>
<tr>
<td>Phase 4 slope, mV/s</td>
<td>157±12</td>
<td>157±13</td>
<td>0.99</td>
</tr>
<tr>
<td>TOP, mV</td>
<td>−48.1±2.4</td>
<td>−43.7±3.4</td>
<td>0.07</td>
</tr>
</tbody>
</table>

All values are mean±SEM ($n=6$ from a single animal).
to endogenous neuronal and hormonal inputs. Although individual isoforms exhibit different sensitivities to cAMP modulation (mediated via the cyclic nucleotide-binding domain), the response of If-induced biological pacemaker to sympathetic and parasympathetic agents can likewise be engineered. In fact, individual amino acid substitutions designed to alter gating and cAMP sensitivity can be combined simultaneously to achieve a particular phenotype. In other words, HCN-based biopacemaker can be “programmed,” unlike the binary nature of IK suppression.

The SA node is undoubtedly a complex tissue consisting of a heterogeneous population of pacemaker cells. For instance, there are gradual changes in such phenotypic properties as AP profile, ionic current densities, and gap junction expression from central (dominant or the leading pacemaker site) to peripheral (subsidiary) nodal cells. These differences and anatomic arrangements ensure that the leading center cells are protected from any overhyperpolarizing effects from the surrounding mass of atrial cardiomyocytes and that the depolarization wave front is propagated in the proper directions. An interesting feature of our Ad-CGI-HCN1-ΔΔΔ-induced biopacemaker is the lack of overdrive suppression, mimicking the native peripheral nodal cells. In the presence of TTX, AP firing exhibits such features of peripheral nodal cells as an increased cycle length, a depolarized TOP, and a slowed AP upstroke. Although it is well known that L-type...
Ca\(^{2+}\) current plays an obligatory role in pacemaking of both central and peripheral nodal cells,\(^{43}\) Ca\(^{2+}\) and Na\(^{+}\) loads have been demonstrated to lead to overdrive excitation and suppression, respectively.\(^{44}\) The contribution of these mechanisms to our observed phenotypes requires further studies. Nevertheless, our present results imply that efforts may now focus on other ionic currents and electrogenic pumps to focus on other ionic currents and electrogenic pumps to fine-tune \(I_f\)-induced pacemaking activity and other properties essential for the SA node to function properly.

Although conceptually attractive, a number of technical issues still need to be addressed. For instance, both the distribution of the transgene in the heart and the expression level need to be carefully targeted to produce a gradient effect, or chaotic heartbeats would result. Even if this can be achieved, it will still be necessary to demonstrate the ability of such a gene-based bioartificial SA to overcome the electrotonic sink resulting from cell-to-cell coupling and to reliably generate a sustained source of depolarizing currents for initiating self-propagating heart rhythms. In our recent in vivo study using a large animal (porcine) sick sinus syndrome model that exhibits cardiac anatomic and functional features similar to those of humans, we overcame some of the above hurdles by demonstrating that in situ focal expression of HCN1-\(\Delta\Delta\) in the atrium can replace or supplement electronic pacemaker.\(^{18}\) Long-term follow-up experiments using recombinant adeno-associated viruses for permanent transgene integration (versus transient expression with adenoviruses) are currently underway in our laboratory.

**Conclusions**

Our present experiments provide a detailed analysis of the mechanistic role of \(I_f\) in cardiac automaticity. Overall, our results not only contribute to a better understanding of cardiac pacing but also may advance current efforts that focus primarily on automaticity induction to the next level by enabling the bioengineering of central and peripheral cells that make up the native SA node.

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**Disclosures**

None.

**References**


Mechanistic Role of $I_f$ Revealed by Induction of Ventricular Automaticity by Somatic Gene Transfer of Gating-Engineered Pacemaker (HCN) Channels

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