PACEMAKER CHANNELS PRODUCE AN INSTANTANEOUS CURRENT

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Running title: Pacemaker Channels Produce an Instantaneous Current
SUMMARY

Spontaneous rhythmic activity in mammalian heart and brain depends on pacemaker currents (I_h) which are produced by hyperpolarization-activated, cyclic nucleotide gated (HCN) channels. Here, we report that the mouse HCN2 pacemaker channel isoform also produced a large instantaneous current (I_{inst(HCN2)}) in addition to the well characterized, slowly activating I_h. I_{inst(HCN2)} was specific to expression of HCN2 on the plasma membrane and its amplitude was correlated with that of I_h. The two currents had similar reversal potentials, and both were modulated by changes in intracellular Cl^- and cyclic AMP (cAMP). A mutation in the S4 domain of HCN2 (S306Q) decreased I_h but did not alter I_{inst(HCN2)}, and instantaneous currents in cells expressing either wildtype HCN2 or mutant S306Q channels were insensitive to block by Cs^+. Co-expression of HCN2 with the accessory subunit, MiRP1, decreased I_h and increased I_{inst(HCN2)}, suggesting a mechanism for modulation of both currents in vivo. These data suggest that expression of HCN channels may be accompanied by a background conductance in native tissues and are consistent with the existence of at least two open states of HCN channels. I_{inst(HCN2)} is produced by a Cs^+- and voltage-insensitive open state; hyperpolarization produces an additional Cs^+-sensitive open state, which results in I_h.
INTRODUCTION

Hyperpolarization-activated "pacemaker" currents in mammalian heart (I_h) and brain (I_h or I_q) contribute to generation and modulation of spontaneous rhythmic activity (1, 2). These currents are carried by Na^+ and K^+ and have reversal potentials of about -20 mV in physiological solutions (3). Pacemaker currents — collectively referred to here as I_h — activate slowly upon hyperpolarization negative to a threshold of ~ -45 mV (depending on the tissue). Cyclic AMP (cAMP) shifts the midpoint of I_h activation to more positive potentials through a direct, phosphorylation-independent mechanism that has no effect on the maximal conductance (4). Extracellular Cs^+ is a voltage-dependent blocker of I_h (5). I_h is not carried by Cl^-, but extracellular Cl^- is required for production of I_h (6) and variations in intracellular Cl^- have been shown to modify the conductance of I_h (7).

The hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels that produce I_h have been recently cloned (8-11). Their amino acid sequence predicts a structure similar to that of voltage-gated potassium (K_v) channels and cyclic nucleotide-gated (CNG) channels. Thus, HCN subunits are thought to assemble as tetramers, with each subunit containing six transmembrane domains. Although HCN channels are activated by hyperpolarization, a positively charged S4 domain appears to act as a voltage sensor (12, 13) as it does in depolarization-activated channels (14, 15). Some mutations in the S4 domain of HCN channels reduce I_h but produce large instantaneous currents (12, 16). HCN channels also have a C-terminal cyclic nucleotide binding domain (CNBD) that is homologous to those of CNG channels and other cyclic nucleotide binding
proteins (17). The CNBD is thought to inhibit activation of the channels by hyperpolarization; direct binding of cAMP is thought to shift the voltage dependence of the channels by relieving this inhibition (18).

As for other voltage gated channels, HCN channels probably exist as tetramers of different HCN α subunits (19, 20) complexed with other proteins. For instance, it has recently been shown that association of HCN2 with MinK-related peptide 1 (MiRP1) increases expression levels and activation rate (21). MiRP1 is a member of the MinK family of single transmembrane-spanning proteins which have been shown to associate with an ever-growing list of voltage-gated potassium channels, including KCNQ1 (22, 23), HERG (24), and Kv4.2 and Kv4.3 (25). In several cases, association with a MiRP converts channels from a voltage-gated to a "leak" phenotype (26, 27, 28). Leak currents are a critical, but often overlooked, determinant of excitability. A relevant example is found in the sino-atrial (SA) node of the heart where a sodium-sensitive background leak current (I_h, (29)) has been suggested to contribute to the diastolic depolarization phase of the cardiac action potential (30, 31). The molecular identity of this background current has not been determined.

In the course of examining the properties of HCN2 channels expressed in Chinese hamster ovary (CHO) cells, we have observed a large instantaneous current (I_{inst(HCN2)}) in addition to I_h.\(^2\) We show here that I_{inst(HCN2)} was specifically associated with expression of HCN2, and the amplitude of I_{inst(HCN2)} was correlated with that of I_h. I_{inst(HCN2)} had many properties in common with I_h — both were modulated by cAMP, were modified, but not carried, by intracellular Cl\(^-\), and had similar reversal potentials. A mutation in the S4 domain (S306Q) that reduced I_h had little effect on the
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instantaneous current ($I_{\text{inst}(S306Q)}$). Furthermore, neither $I_{\text{inst}(HCN2)}$ nor $I_{\text{inst}(S306Q)}$ was blocked by Cs$^+$. These data suggest that $I_{\text{inst}(HCN2)}$ flows through a "leaky," Cs$^+$-insensitive state of the HCN2 channel. Co-expression of HCN2 with MiRP1 similarly reduced $I_h$ but increased $I_{\text{inst}(HCN2)}$, suggesting a possible mechanism for modulation of the two currents in vivo.
**EXPERIMENTAL PROCEDURES**

*Mutagenesis and expression.*

The mutant HCN2 channels ∆CNBD and S306Q were generated from a mouse HCN2 template (10) using overlapping PCR mutagenesis. Amplified inserts bearing the mutations were confirmed by automated sequencing (Biotechnology Laboratory, University of British Columbia).

CHO-K1 cells were obtained from American Type Culture Collection (Manassas, VA), maintained in Hams F-12 media supplemented with antibiotics and 10% fetal bovine serum, and incubated at 37 °C with 5% CO₂. Cells were plated onto glass coverslips in 35 mm dishes. One day after plating, mammalian expression vectors encoding wildtype or mutant HCN2 channels, the pHOOK cell surface marker (Invitrogen Canada, Burlington, Ontario), and/or MiRP1 were transiently co-transfected (2 µg per dish) into the cells along with a green fluorescent protein (GFP) reporter plasmid (0.3 µg per dish) using the FuGene6 transfection reagent (Roche Biochemical, Indianapolis, IN). Cells expressing the transfected proteins were identified by green fluorescence. Cells expressing pHOOK on their membranes were additionally identified by binding of Capture-Tec antigen-coated beads (Invitrogen Canada).

*Electrophysiology and analysis*

One to two days following transfection, a shard of coverslip plated with cells was transferred to a recording chamber (~ 200 µl volume) and continually perfused (0.5 - 1.0 ml/min) with a low K⁺ extracellular solution. The composition of all solutions used for electrophysiology is described in Table I. Following rupture of the patch membrane, the solution was changed to a recording solution as specified in the text. The patch pipettes were filled with either a low Cl⁻ or high Cl⁻ solution, which was supplemented with 1 mM cAMP in some experiments as noted. Whole-cell currents were measured using the patch clamp method with borosilicate glass electrodes, which had
resistances of 2.0 - 4.0 MΩ when filled with the intracellular solution. Cells were held at −35 mV. Currents were recorded using an Axopatch 200B amplifier and Clampex software (Axon Instruments, Union City, Ca). Data were filtered at 2 kHz and were analyzed using Clampfit (Axon Instruments) and Origin (Microcal, Northampton, MA) software. All experiments were conducted at room temperature (20–22 °C). Series resistance was not compensated. Instantaneous currents were taken as the peak current measured after the capacitive transient and, for cells expressing HCN2, were recorded only for cells in which a time-dependent current was also detected (~95% of the green fluorescent cells). The voltage-dependence of activation was determined from tail currents at -35 mV following 4 s test pulses; interpulse intervals were 15 s to ensure complete deactivation of HCN2 channels. Normalized tail current amplitudes were plotted as a function of test potential and values were fit with a Boltzmann function,

\[ f(V) = \frac{I_{\text{max}}}{1 + e^{\left(\frac{V - V_{1/2}}{k}\right)}} + C \]

to determine the midpoint of activation (\(V_{1/2}\)) and slope factor (k). The single test pulses used in some experiments were followed by a 200-500 ms pulse to +5 mV to rapidly close the channels, and we always waited for the resting current to return to its baseline value before giving another pulse. Statistical comparisons were performed using an unpaired Student's t-test (except in Figures 3D and 5D where paired t-tests were used), or an ANOVA with either a Tukey-Kramer or Dunn's test for multiple comparisons (depending on whether the standard deviations varied significantly). Significance was assumed if the \(p\) value was < 0.05. Data are reported as mean ± SEM, and \(n\) values represent the number of cells measured, which were from a minimum of three separate transfections for each comparison reported.
Immunocytochemistry and Confocal Microscopy

Two to three days after transfection, cells on coverslips were washed with PBS and fixed in 2% paraformaldehyde in PBS for 5 minutes. The cells were then washed with PBS, permeabilized using 0.2% Triton X-100, and blocked with 10% normal goat serum (NGS). After one wash with PBS containing 1% NGS, the cells were incubated with a rabbit polyclonal antibody to HCN2 (Alomone Labs, Jerusalem, Israel) at a dilution of 1:400 in PBS with 1% NGS for 48 h at 4°C. The antibody was removed and cells were again washed with PBS. Cells were then incubated with a donkey anti-rabbit antibody tagged with cy3 (Jackson Laboratories, West Grove, Pennsylvania), at a dilution of 1:200 in PBS with 1% NGS, for one hour at room temperature in the dark. This antibody was removed, cells were washed in PBS, and the coverslips with cells were mounted on slides using Permount (Fisher Scientific, Fair Lawn, New Jersey).

Cells were examined using wide field (Olympus BX 40) and confocal microscopy (Zeiss LSCM-510). For confocal microscopy, serial sections were taken in 0.8 - 1.0 micron steps using a 63x oil immersion objective lens and an excitation wavelength of 488 nm.
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RESULTS

A large instantaneous current accompanies expression of HCN2

Figure 1A shows representative current traces from Chinese hamster ovary (CHO) cells expressing either the green fluorescent protein (GFP) transfection marker, or GFP along with the mouse HCN2 pacemaker channel isoform (HCN2). Cells were perfused with the high K⁺ (135 mM) extracellular solution to maximize current amplitude, and pipettes were filled with the high Cl⁻ intracellular solution (the composition of all solutions used for electrophysiology is given in Table I). Surprisingly, cells expressing HCN2 produced not only the expected Iₘ-like current but also a large instantaneous current (I<sub>inst(HCN2)</sub>). In contrast, only a very small instantaneous current was seen in the GFP control cells (I<sub>inst(GFP)</sub>). Values for I<sub>inst(GFP)</sub> and I<sub>inst(HCN2)</sub> were normalized to cell capacitance and are plotted as a function of test voltage in Figure 1B. Both I<sub>inst(GFP)</sub> and I<sub>inst(HCN2)</sub> had a roughly linear voltage-dependence with a slight degree of inward rectification negative to about -60 mV. However, the slope conductance, which was calculated from the linear portion of the curve, was significantly (p < 0.05) greater for I<sub>inst(HCN2)</sub> than for I<sub>inst(GFP)</sub> (0.60 ± 0.28 nS/pF, n = 28, and 0.33 ± 0.04 nS/pF, n = 18, respectively). The reversal potentials of the instantaneous currents also differed significantly indicating a different ionic selectivity for I<sub>inst(HCN2)</sub> compared to I<sub>inst(GFP)</sub> (-5.9 ± 0.6 mV and -10.7 ± 2.0 mV, n = 29, respectively).

To determine whether Iₘ and I<sub>inst(HCN2)</sub> were related processes, we looked for a correlation in their amplitudes. I<sub>inst(HCN2)</sub> density in response to voltage steps to -150 mV was plotted as a function of Iₘ density in the same cells (Figure 1C, n = 138 cells). A Pearson correlation revealed a significant positive covariation between these amplitudes (slope = 0.16, p = 0.003, R = 0.25). These observations suggested that the same process governs production of Iₘ and I<sub>inst(HCN2)</sub>, and perhaps that both currents flow through the HCN2 channel.
We wanted to ensure that \( I_{\text{inst}(\text{HCN2})} \) was not a Cl\(^-\) current because endogenous Cl\(^-\) currents in CHO cells (e.g., (32)) could have been upregulated by HCN2 expression. Thus, we measured slope conductances and reversal potentials for \( I_{\text{inst}(\text{HCN2})} \) and \( I_{\text{inst}(\text{GFP})} \) in intracellular solutions containing a low (11 mM) or high (131 mM) concentration of Cl\(^-\) (aspartate was substituted for Cl\(^-\) in the low Cl\(^-\) intracellular solution; see Table I for details). The high and low Cl\(^-\) solutions yielded theoretical reversal potentials for Cl\(^-\) of 0 mV and -67 mV, respectively (values which were independent of the extracellular solution used). We found that the slope conductance of \( I_{\text{inst}(\text{HCN2})} \) remained significantly larger than that of \( I_{\text{inst}(\text{GFP})} \) when measured using the low Cl\(^-\) intracellular solution and high K\(^+\) extracellular solution (0.40 ± 0.10 and 0.13 ± 0.04 nS/pF, respectively), as it had been in the high Cl\(^-\) solution.

The reversal potentials for \( I_{\text{inst}(\text{GFP})} \) and \( I_{\text{inst}(\text{HCN2})} \) were measured from the linear portions of instantaneous IV curves, such as those illustrated in Figure 1B. The reversal potential for \( I_h \) was determined from the average fully-activated IV relation, in which the maximal \( I_h \) current was measured at various test potentials as the difference in current following prepulses to +20 mV (where HCN channels are completely closed) and to −150 mV (where the channels are maximally open). These differences are plotted as a function of test potential in Figure 2A. The reversal potentials for \( I_{\text{inst}(\text{HCN2})} \) and \( I_h \) did not differ significantly, as illustrated in Figure 2B in the 30 mM K\(^+\) extracellular solution using either a low Cl\(^-\) or high Cl\(^-\) intracellular solution. Similar results were obtained when we used the low (5.4 mM) K\(^+\) extracellular solution (see Table I), in which average reversal potentials measured with low Cl\(^-\) in the pipette were -16.3 ± 6.7 mV, \( n = 5 \), and -15.0 ± 6.4 mV, \( n = 3 \), for \( I_{\text{inst}(\text{HCN2})} \) and \( I_h \), respectively; see also Figure 2C). These values are similar to those previously published for \( I_h \) (3). These experiments demonstrate that \( I_{\text{inst}(\text{HCN2})} \) and \( I_h \) have similar ionic selectivities, consistent with the idea that they travel through the same channel.
Surprisingly, the reversal potentials for $I_{\text{inst(HCN2)}}$ and $I_h$ were both significantly more positive when intracellular Cl$^-$ was low than when intracellular Cl$^-$ was high, whereas the reversal potential for $I_{\text{inst(GFP)}}$ did not differ significantly between the two pipette solutions (Figure 2B). This confirmed that neither $I_h$ nor $I_{\text{inst(HCN2)}}$ was carried by Cl$^-$ because the reversal potential for a Cl$^-$ current would have been more negative when intracellular Cl$^-$ was low. Furthermore, since no significant difference was seen in the reversal potential of $I_{\text{inst(GFP)}}$ in the same solutions, a change in junction potential cannot account for the more positive reversal potentials of $I_h$ and $I_{\text{inst(HCN2)}}$ in the low Cl$^-$ solution. To determine whether $I_{\text{inst(HCN2)}}$ was specific to expression of HCN2 on the plasma membrane (and not a non-specific result of over-expression of any membrane protein), we expressed the pHOOK cell surface marker, which consists of the sFv antibody fused to the transmembrane domain from the platelet-derived growth factor receptor. Cells expressing pHOOK on their surface were identified by binding of antigen-coated beads in addition to green fluorescence. Instantaneous current density in response to test pulses to -150 mV did not differ in cells expressing pHOOK compared to those expressing GFP, and in both cases the instantaneous current was significantly smaller than $I_{\text{inst(HCN2)}}$ (Figure 2C). Taken together, the similar reversal potentials of, and the peculiar effect of intracellular Cl$^-$ on, $I_{\text{inst(HCN2)}}$ and $I_h$ are consistent with the hypothesis that both currents flow through the HCN2 channel pore. They further suggest that the pore is altered by intracellular Cl$^-$. 

$I_{\text{inst(HCN2)}}$ is modulated by cAMP

Sensitivity to cAMP is a hallmark of HCN channels. To further investigate whether $I_{\text{inst(HCN2)}}$ was produced by the HCN2 channel, we asked whether it, like $I_h$, was modulated by cAMP. First, we measured $I_h$ and $I_{\text{inst(HCN2)}}$ with or without 1 mM cAMP in the pipette solution. We determined the steady state voltage-dependence of $I_h$ activation from tail currents at $-35$ mV.
following 4 second test pulses and found that it was significantly more positive when 1 mM cAMP was included in the pipette solution (-90.2 ± 2.1, n = 7, versus -101.0 ± 3.1 mV, n = 7; Figure 3A). In the same cells, we also found a reduction in the average slope conductance of $I_{\text{inst(HCN2)}}$ in the presence of cAMP, although this difference did not reach statistical significance (Figure 3B). Since leak currents not associated with expression of HCN2 (i.e., $I_{\text{inst(GFP)}}$) made up approximately 50% of the total $I_{\text{inst(HCN2)}}$ (see Figure 1B) and thus could mask a significant effect of cAMP, we next used a more sensitive approach to confirm a reduction of $I_{\text{inst(HCN2)}}$ by cAMP in individual cells. We gave 200 ms test pulses to -150 mV every five seconds in cells expressing either HCN2 or GFP and perfused with the high K$^+$ extracellular solution. After 15 seconds, the perfusing solution was changed to the same solution containing IBMX and forskolin (100 µM each), which increase cAMP in CHO cells (e.g., (33)). These concentrations of IBMX and forskolin produced a rapid and reversible decrease in $I_{\text{inst(HCN2)}}$ but not $I_{\text{inst(GFP)}}$, as illustrated for representative cells in Figure 3C. After one minute in IBMX/forskolin, the average value of $I_{\text{inst(HCN2)}}$ in 11 cells was significantly reduced compared to its initial value before cAMP elevation, whereas no difference was observed in the average value of $I_{\text{inst(GFP)}}$ (Figure 3D). The effect of cAMP on $I_{\text{inst(HCN2)}}$ was rapidly reversed when the perfusing solution was changed back to the high K$^+$ extracellular solution (Figure 3C, D). A similar reduction in $I_{\text{inst(HCN2)}}$ was observed when the perfusing solution was changed to one containing 1 µM 8-Br-cAMP (data not shown), showing that the effects of IBMX and forskolin were most likely due to increased intracellular cAMP. These results demonstrate that $I_{\text{inst(HCN2)}}$ is modulated by cAMP and strengthen the hypothesis that HCN2 produces both $I_h$ and $I_{\text{inst(HCN2)}}$.

$I_{\text{inst(HCN2)}}$ requires HCN2 on the plasma membrane

In an attempt to determine whether the CNBD of HCN2 mediates the cAMP sensitivity of $I_{\text{inst(HCN2)}}$, we constructed a mutant channel in which the entire CNBD and downstream regions of
the carboxyl terminus were removed (ΔCNBD). Unfortunately, this mutant channel did not produce either $I_{\text{inst}}(\text{HCN2})$ or $I_h$ in CHO cells (Figure 4A). The average amplitude of the instantaneous current produced by ΔCNBD did not differ significantly from $I_{\text{inst}}(\text{GFP})$ and was significantly smaller than $I_{\text{inst}}(\text{HCN2})$ (Figure 4B). Confocal imaging using a primary antibody against HCN2 revealed a dramatic reduction in fluorescence on the periphery of cells expressing ΔCNBD compared to those expressing wildtype HCN2 (Figure 4C,D). The electrophysiological and imaging data suggest that the ΔCNBD mutation eliminates functional expression of HCN2 by reducing the amount of channel protein present on the plasma membrane. Previous work has shown that functional expression of some mutant ion channels is rescued by incubation of transfected mammalian cells at lower temperatures (e.g., (34)). This was not the case for ΔCNBD — incubation of the transfected cells at $28^\circ C$ for 48-72 hours did not rescue expression of $I_{\text{inst}}(\text{HCN2})$ or $I_h$ in cells expressing ΔCNBD ($n = 7$; data not shown). Taken together with the demonstration that $I_{\text{inst}}(\text{HCN2})$ is specific to expression of HCN2 (Figure 2C), these data suggest that the HCN2 protein must be on the plasma membrane in order for $I_{\text{inst}}(\text{HCN2})$ to be produced and further support a model in which $I_{\text{inst}}(\text{HCN2})$ travels through the HCN2 channel pore.

$I_{\text{inst}}$ produced by S306Q and wildtype channels are similar and insensitive to $Cs^+$

A previous report indicated that a mutation in the S4 segment of HCN2 (S306Q) reduced $I_h$ and produced a large instantaneous current (12). We expressed the S306Q mutant in CHO cells and compared its properties to those of HCN2. We found that $I_h$ was dramatically reduced by the S306Q mutation (Figure 5A, B), as reported previously in *Xenopus* oocytes (12). This huge reduction $I_h$ amplitude probably did not result from a decreased surface expression of the S306Q mutant channel because surface fluorescence in cells expressing S306Q was similar that seen in cells expressing the wild type HCN2 channel (Figure 5C, compare to Figure 4C). The average
amplitude of the instantaneous current in cells expressing S306Q ($I_{\text{inst(S306Q)}}$) did not differ significantly from that of $I_{\text{inst(HCN2)}}$ (Figure 5A,B), and $I_{\text{inst(S306Q)}}$ was reduced by cAMP in the same manner as $I_{\text{inst(HCN2)}}$ (data not shown). To test whether $I_{\text{inst(S306Q)}}$ was produced by an incomplete closure of the mutant channels, as has been suggested for some HCN2 S4 mutations (16), we added 2 mM Cs+, an open-channel blocker of HCN2 (10, 35), to the extracellular solution. We found that Cs+ completely blocked inward $I_h$ at -150 mV (data not shown) but had no effect on either $I_{\text{inst(S306Q)}}$ or $I_{\text{inst(HCN2)}}$; the instantaneous current densities recorded 1 minute after perfusion with a high K+ extracellular solution containing 2 mM Cs+ did not differ from those in the absence of Cs+ (Figure 5D). These data are consistent with a model in which HCN2 has at least two open states. $I_{\text{inst(HCN2)}}$ is produced by a voltage-independent, Cs+-insensitive open state, whereas $I_h$ is produced by a hyperpolarization-activated, Cs+-sensitive open state. The S306Q mutation has no effect on the instantaneous conductance but rather affects only the hyperpolarization-activated conductance.

**MiRP1 co-expression reduces $I_h$ and increases $I_{\text{inst(HCN2)}}$**

The MinK (KCNE) family of single transmembrane-spanning proteins has been shown to increase instantaneous currents and decrease time-dependent currents when co-expressed with voltage-dependent K+ channels of the KvLQT (KCNQ) family (26). One member of this family, MiRP1 (KCNE2), has been recently shown to associate with, and increase the expression of, HCN2 (21) when expressed in *Xenopus* oocytes. We co-expressed MiRP1 with HCN2 to determine whether their interaction affected $I_{\text{inst(HCN2)}}$ and $I_h$ in CHO cells. We found that MiRP1 decreased $I_h$ and increased $I_{\text{inst(HCN2)}}$ (Figure 6A, B, C). Expression of MiRP1 alone did not significantly alter the instantaneous current compared to expression of GFP (Figure 6A, B), and no time-dependent current was produced by either MiRP1 or GFP (Figure 6A, C). These findings suggest a possible physiological mechanism for modulation of $I_{\text{inst(HCN2)}}$ and $I_h$ by MiRP1.
DISCUSSION

In this paper, we have shown that expression of the HCN2 pacemaker channel isoform in CHO cells produced a large instantaneous current ($I_{\text{inst(HCN2)}}$) in addition to the well-characterized time-dependent current ($I_h$). $I_{\text{inst(HCN2)}}$ was shown to be specific to expression of HCN2 because its amplitude was strongly correlated with that of $I_h$ and because overexpression of the pHOOK cell surface marker did not produce any non-specific increase in instantaneous current. Furthermore, expression of the HCN2 protein on the plasma membrane was shown to be required for $I_{\text{inst(HCN2)}}$ because a truncated HCN2 channel, which did not express on the plasma membrane did not produce either $I_h$ or $I_{\text{inst(HCN2)}}$.

The upregulation of an endogenous channel is a possible explanation for $I_{\text{inst(HCN2)}}$. However, it seems more likely that the two currents are produced by the same channel because they have so many common properties: both currents have similar ionic selectivity, and both are modified by changes in intracellular Cl⁻ and by elevation of cAMP. It will be interesting in future work to compare the relative ionic permeabilities of $I_h$ and $I_{\text{inst(HCN2)}}$ under various conditions, although the contamination of $I_{\text{inst(HCN2)}}$ by variable amounts of non-specific leak in different cells will make this challenging in the absence of a specific blocker for $I_{\text{inst(HCN2)}}$ (or $I_h$). The S306Q mutation provides further support for the idea that $I_{\text{inst(HCN2)}}$ travels through the HCN2 channel pore. Previous work has established that S4 mutations, such as S306Q, produce large instantaneous currents (12, 16). We have shown here that $I_{\text{inst(S306Q)}}$ does not differ from $I_{\text{inst(HCN2)}}$, indicating that both flow through the HCN2 channel pore.
Overall, the data presented here are consistent with a model in which HCN2 channels have at least two open states,

\[ C \leftrightarrow L \leftrightarrow O \]

\( I_{\text{inst}(HCN2)} \) flows through a "leaky" open state (L) of HCN2, while \( I_h \) flows through the "normal" hyperpolarization-activated open state (O). The L state is insensitive to Cs\(^+\) and voltage. The lack of Cs\(^+\) sensitivity and reduction by cAMP of both \( I_{\text{inst}S306Q} \) and \( I_{\text{inst}(HCN2)} \) indicates that the two open states are distinct, and suggests that they may represent different conformations of the channel. According to this model, the S306Q mutation does not affect the L state, but disrupts the ability of the channel to enter the O state. This is consistent with the conclusion of Chen et al (12) that Ser-306 is crucial for gating. However, our experiments with Cs\(^+\) and cAMP, and the fact that the amplitudes of \( I_{\text{inst}S306Q} \) and \( I_{\text{inst}(HCN2)} \) did not differ in CHO cells, lead us to conclude that the mutant channels are stabilized in the L rather than the O state — the channels are prevented from opening rather than prevented from closing.

Like the S306Q mutation, the MiRP1-HCN2 interaction also appears to disrupt the ability of HCN2 channels to enter the O state (although further studies will be required to determine whether the mechanisms of S306Q and MiRP1 are the same). The block of \( I_h \) and increase in \( I_{\text{inst}(HCN2)} \) produced when MiRP1 is co-expressed with HCN2 are similar to its effects when co-expressed with \( K_vLQT1 \), where it decreases time-dependent and increases instantaneous currents (26). MiRP1 is expressed in the heart, and a direct interaction between MiRP1 and HCN2 has been shown (21). This interaction is likely to be responsible for the increase in \( I_{\text{inst}(HCN2)} \) and the reduction in \( I_h \) we
have described in CHO cells. These effects were not observed when the two proteins were co-expressed in *Xenopus* oocytes (21), but this may be due to the difference in expression system. Indeed, it is similar to the influence of expression environment on the conversion of KCNQ1 to a permanently open channel by MiRP1, which was observed only in mammalian cells and not in oocytes (26, 36). Yu et al. (21) also used a lower ratio of MiRP1 to HCN2 than we used in the CHO cells. In preliminary experiments, we have found that lower ratios had much less effect on $I_{\text{inst}(\text{HCN2})}$.

The data presented here suggest that native cells that express HCN channels may have an instantaneous current in addition to $I_h$. Evidence for one such instantaneous current exists in the sino-atrial (SA) node of the heart. The "sodium sensitive background current,' ($I_b$) together with $I_h$, has been suggested to be important for the generation and modulation of pacemaker activity in the SA node (29-31). This current shares several properties with $I_{\text{inst}(\text{HCN2})}$; in addition to being instantaneous, $I_b$ reverses at about -20 mV in physiological solutions, and is insensitive to Cs$^+$ (29, 38). Interestingly, ventricular myocytes of mice overexpressing the β adrenoceptor had an increased amount of HCN4 transcript and a larger Cs$^+$-insensitive background current compared to control littermates (37). These data, and the fact that $I_b$ demonstrates many similarities with $I_{\text{inst}(\text{HCN2})}$, suggest that expression of HCN channels may be accompanied by a background current in cardiac myocytes. It will be interesting to compare further the properties of $I_b$ in cardiac myocytes and $I_{\text{inst}(\text{HCN2})}$. Curiously, some neurons that express HCN subunits have low or nonexistent levels of $I_h$ (39). For instance, in neurons of the reticular nucleus, little or no $I_h$ was observed despite the
"prominent" presence of HCN2 channel transcript (39); coincidentally, these cells appeared to have a large instantaneous current (see figure 10 in (39)). Further studies may reveal whether neurons that express HCN channels but lack I_h have a background conductance similar to I_{inst(HCN2)}. Overall, the data presented here suggest that HCN channels may produce background conductances in vivo. Modulation of I_{inst(HCN2)}, for instance by cAMP or interaction with MiRP1, may be a way in which membrane excitability could be dynamically controlled.
REFERENCES


FOOTNOTES

1 The abbreviations used are: \( I_h \), hyperpolarization-activated current; HCN2, hyperpolarization-activated cyclic nucleotide gated channel, isoform 2; \( I_{\text{inst(HCN2)}} \), instantaneous current in cells expressing HCN2; cAMP, cyclic AMP; \( I_{\text{inst(GFP)}} \), instantaneous current in cells expressing GFP; PCR, polymerase chain reaction; SA, sinoatrial; CHO, Chinese hamster ovary; \( I_h \), hyperpolarization-activated current; CNBD, cyclic nucleotide binding domain; \( K_v \), voltage-gated potassium channel; \( I_b \), sodium-dependent background current;


ACKNOWLEDGMENTS

This work was supported by grants from the Heart and Stroke Foundation of BC & Yukon (to EAA and CP) and the Canadian Institutes for Health Research (to EAA). We thank Andreas Ludwig for the mHCN2 clone and Steve Goldstein for the MiRP1 clone.
FIGURE LEGENDS

Figure 1. Instantaneous current is larger in HCN2-expressing cells than in GFP-expressing cells. (A) Current families measured from representative CHO cells expressing GFP (top) or expressing GFP and HCN2 (bottom) in response to hyperpolarizing voltage steps from a holding potential of -35 mV to test potentials from -40 to -150 mV in 10 mV increments. Dashed lines indicate zero current level. Scale bars are 500 pA and 500 ms. Cells were bathed in the high K+ extracellular solution and pipettes were filled with the high Cl- solution (see Table I). (B) In the same solutions, average voltage-dependence of instantaneous current density in cells expressing GFP (n = 18, filled squares) or HCN2 (n = 28, open circles) determined from the current level measured at the onset of 200 ms test pulses from +60 to -150 mV in 30 mV increments, as indicated by the voltage protocol and representative currents for an HCN2 cell shown in the inset. Scale bars as in (A). Cells were held at -35 mV and test pulses were followed by a 500 ms pulse to +5 mV to ensure that the channels closed completely. (C) Correlation between $I_{\text{inst(HCN2)}}$ and $I_h$ amplitudes. $I_{\text{inst(HCN2)}}$ density as a function of $I_h$ density in the same cells (n = 138) in response to voltage steps to -150 mV in the same solutions as in (A) and (B). The line is a linear fit of all the data and indicates a positive covariance between $I_{\text{inst(HCN2)}}$ and $I_h$.

Figure 2. Effects of intracellular Cl– on $I_h$ and $I_{\text{inst(HCN2)}}$. (A) Average (n = 5) fully-activated current-voltage relation for $I_h$ in low Cl– intracellular and 30 K+ extracellular solutions. The reversal potential for $I_h$ was determined from the linear portion of the curve near the $x$ intercept. The voltage protocol used to generate the IV plot and currents recorded from a representative cell are shown in the inset. (B) Reversal potentials for $I_{\text{inst(GFP)}}$, $I_{\text{inst(HCN2)}}$, and $I_h$ in high (131 mM, solid bars) versus low (11 mM, hatched bars) Cl– intracellular solutions and in an extracellular solution containing 30...
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mM K+. Numbers in parentheses indicate number of cells tested and the asterisks indicate significant differences from the corresponding high Cl⁻ reversal potentials. (C) Instantaneous current density recorded in high K⁺ extracellular and low Cl⁻ intracellular solutions in response to voltage steps to -150 mV in cells expressing GFP (n = 9), pHOOK (n = 11), or HCN2 (n = 25). The asterisk indicates a statistically significant difference from either GFP or pHOOK (p < 0.05).

**Figure 3.** $I_{\text{inst(HCN2)}}$ is modulated by cAMP. Average normalized tail current amplitudes (A) or average $I_{\text{inst(HCN2)}}$ density (B) in the same cells in the absence (open circles, n = 7) or presence (closed squares, n = 7) of 1 mM cAMP in the low Cl⁻ pipette solution. All cells were perfused with the high K⁺ extracellular solution. Tail currents were measured at -35 mV following 4 second test pulses from -150 to -40 mV, in 10 mV increments, as indicated by the voltage protocol and representative currents recorded from one cell in the absence of cAMP (A, inset). Interpulse intervals were 15 seconds. (C) Time course of $I_{\text{inst(HCN2)}}$ (circles) or $I_{\text{inst(GFP)}}$ (squares) in response to repeated applications of IBMX and forskolin (100 µM each, black bars). $I_{\text{inst(HCN2)}}$ or $I_{\text{inst(GFP)}}$ was measured in response to 200 ms test pulses to -150 mV, which were given every 5 seconds from a holding potential of -35 mV. (D) Average $I_{\text{inst(GFP)}}$ (n = 10) or $I_{\text{inst(HCN2)}}$ (n = 11) in the control high K⁺ extracellular solution, 1 minute after the application of IBMX and forskolin, and 1 minute after washout. The asterisk indicates a statistically significant (paired t test, p < 0.05) difference from $I_{\text{inst(HCN2)}}$ in the control solution. Time points illustrated in (D) are indicated by arrowheads in (C).

**Figure 4.** $I_{\text{inst(HCN2)}}$ requires HCN2 on the plasma membrane. Representative current traces (A) and average current density (B) elicited by pulses to −150 mV in CHO cells expressing HCN2 (n = 43), GFP (n = 16), or ΔCNBD (n = 36) in high K⁺ extracellular and high Cl⁻ intracellular solutions.
Asterisks indicate statistically significant differences from HCN2. Confocal micrographs of CHO cells transfected with HCN2 (C) or ΔCNBD (D). Arrows point to plasma membrane regions to indicate presence or absence of fluorescence.

**Figure 5.** $I_{\text{inst}}$ in wildtype and S306Q mutant channels is insensitive to Cs$^+$. Representative current produced by a cell expressing S306Q (A) and average current densities in cells expressing S306Q (n = 21) or HCN2 (n = 43) (B) in response to voltage steps to –150 mV. $I_h$ indicates the difference in current between the beginning and end of the test pulse and the asterisk indicates a significant reduction in $I_h$ current compared to cells expressing HCN2. Cells were bathed in the high K$^+$ extracellular solution and the pipettes were filled with the low Cl$^-$ intracellular solution. (C) Confocal micrograph of CHO cell transfected with S306Q. The arrow indicates a region of surface fluorescence. (D) Average instantaneous current densities in cells expressing S306Q (n = 14) or HCN2 (n = 12) in response to voltage steps to –150 mV. Control currents (black bars) were recorded when cells were perfused in a high K$^+$ extracellular solution. The Cs$^+$ values (gray bars) were measured from the same cells 1 minute after changing to a high K$^+$ extracellular solution containing 2 mM Cs$^+$. Pipettes were filled with the low Cl$^-$ solution for all measurements.

**Figure 6.** MiRP1 co-expression reduces $I_h$ and increases $I_{\text{inst(HCN2)}}$

(A) Representative current traces elicited by voltage pulses to –150 mV in cells expressing MiRP1, HCN2, or MiRP1 and HCN2 (at a 1:1 ratio). Average current density for instantaneous current (B) or time-dependent ($I_h$) current (C) measured for 1 second pulses to –150 mV in cells expressing GFP (n = 9), MiRP1 (n = 11) HCN2 (n = 25), or MiPR1 + HCN2 (n = 19). $I_h$ was taken as the difference in current between the beginning and end of the test pulse. Average current densities and error bars for GFP or MiRP1 are hidden by the axis. Asterisks indicate a significant difference
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between HCN2 and MiRP1 + HCN2 (t-test, \( p < 0.05 \)). Currents were measured using high K\(^+\) extracellular and low Cl\(^-\) intracellular solutions.

**Table 1. Solutions for electrophysiology.** All concentrations given in mM, and all solutions were adjusted to pH = 7.4.

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<th>forskolin</th>
<th>EGTA</th>
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Figure 1
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Figure 6
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J. Biol. Chem. published online December 7, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106974200

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