Structural Elements of Instantaneous and Slow Gating in Hyperpolarization-activated Cyclic Nucleotide-gated Channels*

Received for publication, January 16, 2004
Published, JBC Papers in Press, January 29, 2004, DOI 10.1074/jbc.M400518200

Vincenzo Macri and Eric A. Accili‡

From the Ion Channel Laboratory, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada

Hyperpolarization-activated cyclic nucleotide-gated (HCN) subunits produce a slowly activating current in response to hyperpolarization (Iᵩ) and an instantaneous voltage-independent current (Iᵩᵢ) when expressed in Chinese hamster ovary (CHO) cells. Here we found that a mutation in the S4-S5 linker of HCN2 (Y331D) produced an additional mixed cation instantaneous current. However, this current was inhibited by external Cs⁺ like Iᵩ and unlike Iᵩᵢ. Together with a concomitant reduction in Iᵩ, the data suggest that the Y331D mutation disrupted channel closing placing the channel in a "Iᵩ-like," and not an "Iᵩᵢ-like," state. The "Iᵩ-like" instantaneous current represented ~70% of total Iᵩ over voltages ranging from +20 to −150 mV in high K⁺ solutions. Iᵩ activated at more depolarized potentials and the activation curve was less steep, whereas deactivation was significantly slowed, consistent with the idea that the mutation inhibited channel closing. The data suggest that the mutation produced allosteric effects on the activation gate (S6 segment) and/or on voltage-sensing elements. We also found that decreases in the ratio of external K⁺/Na⁺ further disrupted channel closing in the mutant channel. Finally, our data suggest that the structures involved in producing Iᵩᵢ are similar between the HCN1 and HCN2 isoforms and that excess HCN protein on the plasma membrane of CHO cells relative to native cells is not responsible for Iᵩᵢ. The data are consistent with Iᵩᵢ flowing through a "leaky" closed state but do not rule out flow through a second configuration of recombinant HCN channels or up-regulated endogenous channels/subunits.

Hyperpolarization-activated cyclic nucleotide-gated (HCN) subunits produce a slowly activating current in response to hyperpolarization known as Iᵩ, Iᵩᵢ, or Iᵩ (1) and are thus involved in regulating membrane potential and spontaneous activity in a variety of excitable cells (2–4). In addition to Iᵩ instantaneous currents were noted or are apparent in experiments describing the expression of wild-type HCN subunits in mammalian cells (5–9) and Xenopus oocytes (10). We recently described a mixed cation instantaneous current, which we refer to as "Iᵩᵢ" that appeared in addition to Iᵩ when the HCN2 isoform was expressed in Chinese hamster ovary (CHO) cells (11). Mutations that reduced or eliminated the trafficking of HCN2 to the plasma membrane also reduced or eliminated Iᵩᵢ associated with HCN2 expression (12, 13). Furthermore, the overexpression of a protein, with a single transmembrane segment and found on the plasma membrane, did not produce Iᵩᵢ. Finally, we found that the amplitudes of Iᵩᵢ and Iᵩ were directly correlated. Thus, Iᵩᵢ was not simply the result of overexpression of protein but was correlated specifically with the amount of HCN protein expressed on the plasma membrane.

We subsequently examined the role of the selectivity filter and the positively charged S4 region in producing Iᵩᵢ. This current was not affected by a mutation in the selectivity filter (HCN2 G404S), extracellular perfusion of Cs⁺, or a mutation in the S4 segment (S306Q) unlike Iᵩ, which was reduced or eliminated by these mutations as well as by Cs⁺ (14). Finally, the amplitude of Iᵩᵢ, but not Iᵩ, could be predicted on the basis of independent flow of Na⁺ and K⁺. Thus, Iᵩ and Iᵩᵢ were both mixed cation conductances, but these could be structurally and functionally separated. These results leave open the question of whether the Cs⁺-insensitive Iᵩᵢ flows through the same pore as Iᵩ (for example through a "leaky" closed state) or whether it flows through a second pore that is found within the same channel, formed by a second configuration of HCN channel subunits or associated with HCN channels in the form of up-regulated endogenous channels or subunits.

In the present study, we continued to examine regions of HCN channels potentially involved in regulating Iᵩᵢ in order to understand how this current is generated. A recent study has shown that mutations of residue Tyr³³³ of the S4-S5 linker in HCN2 produced large instantaneous currents and reduced Iᵩ, suggesting a disruption of channel closure (10). However, the nature of the instantaneous currents was not determined in those studies, and thus it was not clear whether the up-regulated current had properties which were similar to Iᵩᵢ or Iᵩ. Here, we found that Y331D up-regulated an instantaneous current when expressed in CHO cells, but its properties were more like Iᵩ than like Iᵩᵢ. We also found that Iᵩ amplitude was reduced, the activation curve of the remaining Iᵩ was shifted to more positive voltages and was less steep, and Iᵩ deactivation was slowed in the mutant channel. Together, the data support the idea that the Y331D mutation disrupted channel closing through local allosteric effects on the activation gate (S6 segment) and/or on the voltage-sensing elements. Interestingly, our data also suggest that decreasing the ratio of external K⁺ versus Na⁺ further disrupted the closing of the mutant channel possibly through a separate “foot in the door” mechanism. Finally, we found that the HCN1 isoform, when expressed on...
the plasma membrane of CHO cells in amounts similar to native HCN channels, produced $I_{\text{inst}}$, which was proportional in size to $I_{\text{f}}$. These data suggest that the structural elements involved in producing $I_{\text{inst}}$ are similar between HCN1 and HCN2 and that excess expression of HCN subunits on the plasma membrane of CHO cells, relative to native cells, is not responsible for producing $I_{\text{inst}}$. The data are consistent with $I_{\text{inst}}$ flowing through a leaky closed state but do not rule out flow through a second configuration of recombinant HCN channels or up-regulated endogenous channels/subunits.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Expression**—The Y331D mutant was constructed by overlapping PCR mutagenesis from a mouse HCN2 template as previously described (14). The amplified mutagenetic product and wild-type mHCN2, in the mammalian expression vector pcDNA3 (8), were subsequently digested using NheI and BlpI. The fragment digested out of wild-type mHCN2 was replaced by the complementary fragment carrying the mutation. The mutation was confirmed by restriction analysis and automated sequencing (The Centre for Molecular Medicine and Therapeutics, DNA Sequencing Core Facility, BC Children’s and Women’s Hospital, University of British Columbia, Vancouver, Canada).

**Cell Culture—**CHO-K1 cells were obtained from ATCC (Manassas, VA), maintained in Ham’s F-12 medium supplemented with antibiotics and 10% fetal bovine serum (Invitrogen) and incubated at 37 °C with 5% CO2. Cells were plated onto glass coverslips and 1 day after splitting were transiently co-transfected with mammalian expression vectors encoding wild-type and/or mutant mHCN2 channels (2 µg/5-mm dish) along with a green fluorescent protein (GFP) reporter plasmid (0.3 µg/dish) using the FuGENE 6 transfection reagent (Roche Applied Science).

**Electrophysiology and Analysis**—Cells expressing GFP were chosen for whole-cell recordings 24–48 h after transfection or co-transfection with mHCN2 and mHCN1 constructs. The pipette solution contained 130 mM potassium aspartate, 10 mM NaCl, 0.5 mM MgCl2, 1 mM EGTA, 5 mM HEPES, pH adjusted to 7.4 with KOH. The extracellular solution contained varying concentrations of NaCl, KCl, and NMG (see figure legends for each experimental condition), 1.8 mM CaCl2, 0.5 mM MgCl2, 5 mM HEPES, pH adjusted to 7.4 with NaOH. For solution changes, a 200-µl bath was completely exchanged and perfused (0.5–1 µl/min) for at least 1 min prior to collecting data. Whole-cell patch clamp currents were recorded using an Axopatch 200B amplifier and Clampex software (Axon Instruments, Union City, CA) at room temperature (20–22 °C). Currents were not leak-subtracted, and capacitance compensation was not used. Patch clamp pipettes were pulled from borosilicate glass and were fire-polished before use. The pipette resistance was 2.5–4.9 MΩ. Data were filtered at 2 kHz and were analyzed using Clampfit (Axon Instruments), Origin (Microlab, Northampton, MA) and Excel (Microsoft, Seattle, WA) software. Current densities were determined by dividing measured currents by the capacitance, which was estimated by the ClampEx software from the time constant of the current elicited by a 2-mV test pulse at the beginning of whole-cell recording.

In order to determine the voltage dependence of block by Ca2+ of instantaneous current in cells expressing HCN2 Y331D (15), the following equation was used,

$$\frac{I_{\text{inst}}}{I_{\text{f}}} = \frac{(k_{i}/[\text{Ca}])\exp(\Delta V/RT)}{1 + (k_{i}/[\text{Ca}])\exp(\Delta V/RT)}$$

(Eq. 1)

where $I_{\text{inst}}$ and $I_{\text{f}}$ represent the HCN2 Y331D instantaneous current in control conditions and after Ca2+, $[\text{Ca}]$ is Ca2+ concentration (2 mM), $k_{i}$ is the dissociation constant of Ca2+ binding to the block site at zero voltage, and $\Delta V$ is the “electrical” distance of the block site from the external membrane surface. Equation 1 was used to fit the data in Fig. 6, A and C, and values for $k_{i}$ and $[\text{Ca}]$ were obtained (see legend to Fig. 6). $\Delta V$ values were also obtained, indicating how well the points were fit. This analysis was also used to examine Ca2+ block of $I_{\text{inst}}$ in CHO cells expressing wild-type HCN2.

The voltage dependence of activation for HCN2 and HCN2 Y331D were determined by tail currents generated at −30 mV (see Fig. 7 for voltage protocol) with the tail current amplitudes normalized and plotted as a function of test potential. The values were fit with a Boltzmann function,

$$f(V) = I_{\text{inst}}/V_{1/2} + 1 + e^{(V_{1/2}-V)/\varepsilon}$$

(Eq. 2)
to determine the midpoint of activation ($V_{1/2}$) and the slope factor ($\varepsilon$).

To assess rates of $I_{\text{f}}$ activation and deactivation, single exponential fitting procedures were used. A delay occurred prior to both activation and deactivation, which was not well described by a single exponential function and therefore was not used in our fits. Student’s paired or unpaired $t$ test was used to determine significance when comparisons were made between groups of cells ($p < 0.05$).

**Immunocytochemistry and Confocal Microscopy**—At 24–48 h post-transfection, cells on coverslips were washed with PBS and fixed in 2% paraformaldehyde in PBS for 5 min. 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 (Affinity Bioreagents, Jersey, Jersey, Isreal) 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 cyanine 3 (Jackson Laboratories, West Grove, PA) at a dilution of 1:200 in PBS with 1% NGS for 1 h at room temperature in the dark. The antibody was removed, cells were washed in PBS, and the coverslips with cells were mounted on slides using Permount (Fisher). Using an inverted Zeiss TurboPascal confocal microscope, serial sections were taken in 0.8–1.0 µm steps using a ×63 oil immersion objective lens and an excitation wavelength of 543 nm.

**RESULTS**

A Mutation in the S4-S5 Linker of HCN2 Y331D Up-regulates a Mixed Cationic “Instantaneous” Current and Reduces $I_{\text{f}}$—In order to examine whether the S4-S5 linker was involved in producing the $I_{\text{inst}}$ associated with wild-type HCN2, we mutated the tyrosine at position 331 to aspartic acid (Y331D) in mouse HCN2 and expressed both the mutant and wild-type channels in CHO cells. We chose this residue because a previous study suggested that Xenopus oocytes expressing HCN2 with mutations of this residue had larger instantaneous currents and a smaller $I_{\text{f}}$, compared with currents in oocytes expressing wild-type HCN2 (10). The largest effects were produced by a mutation of this tyrosine to aspartic acid (Y331D). However, whether the up-regulated instantaneous current was carried by Na+ and K+ and whether the current was sensitive to Cs+ or other $I_{\text{f}}$ blockers were not determined in those experiments.

We anticipated three possible scenarios upon transfection of HCN2 Y331D in CHO cells. 1) If the instantaneous current were significantly larger than $I_{\text{inst}}$ measured in cells expressing the wild-type channel and sensitive to Cs+, then this would suggest that the mutation favored an “I-like” open state. 2) If the instantaneous current were significantly larger than $I_{\text{inst}}$ measured in cells expressing the wild-type channel but insensitive to Cs+, then this would suggest that the mutation favored an “Iinst-like” state. 3) If the instantaneous current were not significantly larger than $I_{\text{inst}}$ measured in cells expressing the wild-type channel and were insensitive to Cs+, then this would suggest that the mutation did not affect $I_{\text{inst}}$. We expected that the instantaneous currents would be carried by Na+ and K+ in any of the above scenarios. Based on the possibility that $I_{\text{inst}}$ results from a leaky closed state, we hypothesized that the Y331D mutation would increase $I_{\text{inst}}$ and thus that this current would be significantly up-regulated by the mutation, insensitive to Cs+, approximately linear with respect to changes in voltage and carried by Na+ and K+ (11, 14). A comparison of the S3 segment, the S4 segment, and the S4-S5 linker among mouse HCN1 and HCN2, sea urchin (Strongylocentrotus purpuratus) HCN, and the recently crystallized KvAP (16) shows the position of the HCN2 Y331D mutation (Fig. 1). Using the whole-cell patch clamp approach, we determined the densities of $I_{\text{f}}$ and instantaneous currents recorded from cells expressing wild-type HCN2 and HCN2 Y331D in order to control for variability in cell surface area. Cells expressing HCN2 Y331D produced significantly less $I_{\text{f}}$. 

Downloaded from http://www.jbc.org/
and significantly more instantaneous current than did cells expressing wild-type HCN2, as shown in representative current traces (Figs. 2A and 3A). The increase in instantaneous current in cells expressing HCN2 Y331D is apparent in I-V curves generated at each of three varying concentrations of extracellular K⁺ or Na⁺ (Figs. 2, B–D, and 3, B–D). The increase in instantaneous current amplitude due only to the Y331D mutation is shown in I-V curves that represent the difference in instantaneous currents between cells expressing HCN2 and HCN2 Y331D (Figs. 2E and 3E).

The reversal potentials for instantaneous currents at each concentration and the movement of the reversal potentials to more positive potentials at increasing concentrations of external K⁺ or Na⁺, are consistent with the mixed cationic nature of instantaneous current in cells expressing HCN2 that we described previously (I_{inst}) as well as with the up-regulated instantaneous current in cells expressing HCN2 Y331D.

Interestingly, the I-V curves for I_{inst} in cells expressing wild-type HCN2 were linear, whereas the I-V curves for the instantaneous current in cells expressing HCN2 Y331D rectified in the inward direction especially at the elevated extracellular K⁺ and Na⁺ concentrations. Inward rectification of instantaneous currents was also observed in experiments in *Xenopus* oocytes expressing HCN2 Y331D (10). The rectification suggested that the mixed cation instantaneous currents in cells expressing HCN2 Y331D and HCN2 were different. Previous studies using HER cells expressing mouse HCN2 also showed that the fully activated Iₚ had greater inward rectification at 30 mm K⁺ as compared with 5.4 mm K⁺ (8). Overall, the results suggested that the up-regulated instantaneous currents we observed in cells expressing HCN2 Y331D had similarities to both Iₚ and I_{inst}.

**HCN2 Y331D Is Expressed on the Cell Surface of CHO Cells in Significant Amounts**—The large decrease in Iₚ in cells expressing Y331D may have reflected a decrease in the total amount of protein expressed in the cell and/or localized to the plasma membrane. Therefore, we examined the localization of mutant channels labeled with an anti-HCN2 primary antibody and a cyanine 3-tagged secondary antibody using confocal microscopy, which would unmask any large reductions in protein expression. There was a very strong pattern of fluorescence along the periphery of the cells consistent with the presence of HCN2 protein on the plasma membrane (Fig. 4). Fluorescent regions were also observed in the interior of the cells, probably representing channel protein present in intracellular compartments such as the endoplasmic reticulum, Golgi apparatus, vesicles mediating transport to or from the plasma membrane, or degradatory compartments. There was relatively little fluorescence in nontransfected cells or in mock-transfected cells (not shown). We have shown previously that the wild-type channel demonstrated a similar pattern of localization and overall intensity of fluorescence, whereas mutants lacking the cyclic nucleotide binding domain and distal C terminus or the complete N terminus did not express Iₚ or instantaneous currents significantly larger than GFP-transfected cells and demonstrated a very different pattern of localization that did not include fluorescence on the periphery of CHO cells (12). The pattern of fluorescence observed is consistent with the idea that the large instantaneous currents recorded in these cells were due to HCN2 Y331D and that the large reduction of Iₚ in cells expressing HCN2 Y331D was not due to a low level of HCN2 protein in the cell or on the plasma membrane.

**Instantaneous Currents Associated with HCN2 Y331D Are Sensitive to External Cs⁺ in a Voltage-dependent Manner**—In cells expressing HCN2, I_{inst} is not sensitive to Cs⁺, whereas Iₚ is sensitive to Cs⁺ (11); the ability of Cs⁺ to block Iₚ increases at more negative potentials and occurs only when Iₚ is inward (15, 17, 18). In order to determine whether the instantaneous currents up-regulated by the Y331D mutation were more like Iₚ or I_{inst}, we examined the sensitivity of instantaneous currents to Cs⁺ in cells expressing HCN2 Y331D. We found that, in addition to blocking Iₚ, Cs⁺ dramatically reduced instantaneous currents in cells expressing HCN2 Y331D, whereas in cells expressing HCN2, Iₚ was blocked but I_{inst} was not affected (Fig. 5, A and B). The effects of Cs⁺ occurred within 4–5 s (Fig. 5C) supporting a direct action on the channels. As can be seen in the current traces in Fig. 5B, there was no effect of Cs⁺ on outward instantaneous currents recorded from cells expressing HCN2 Y331D. This can be seen more clearly in Fig. 5D, which shows the inhibitory effect of Cs⁺ on instantaneous currents as a function of test voltage. As shown previously, Cs⁺ had no effect on I_{inst} recorded from cells expressing HCN2 at any voltage tested. However, Cs⁺ did block inward, but not outward, instantaneous currents recorded from cells expressing HCN2 Y331D, and the block was greater at more negative voltages. Thus, the key characteristics of Cs⁺ block of Iₚ were manifested by the Cs⁺ block of a proportion of instantaneous currents recorded from cells expressing HCN2 Y331D. This is consistent with the idea that the up-regulated instantaneous currents observed in cells expressing HCN2 Y331D were due to an “Iₚ-like” instantaneous current and not an “I_{inst}-like” instantaneous current.

In Figs. 2E and 3E, the amplitude of instantaneous currents measured in cells expressing HCN2 was subtracted from the amplitude of instantaneous currents measured in cells expressing HCN2 Y331D in order to remove the contribution of instantaneous current due to HCN2 (Cs⁺-insensitive I_{inst}). This calculation is reasonable because the amplitude of Cs⁺-insensitive instantaneous current was not significantly different between cells expressing HCN2 and HCN2 Y331D at −150 mV (see instantaneous currents in Fig. 5D, at −150 mV, replotted in Fig. 5F). At −150 mV, Cs⁺ blocks Iₚ almost completely in cells expressing either HCN2 or HCN2 Y331D (Fig. 5F), and therefore we assumed that the block of any Iₚ-like instantaneous current would also be complete at this voltage.

We also found that the total amount of Cs⁺-sensitive current

---

**Fig. 1.** A multiple sequence alignment of part of the S3 segment, S3-S4 linker, the S4 segment, and the S4-S5 linker from mouse HCN2, mouse HCN1, sea urchin HCN, and the recently crystallized KvAP channel from *Aeropyrum pernix* (16). Amino acids highlighted in black represent identical residues, and those highlighted in gray represent conserved residues, among all of the isoforms shown. There is conservation of residues throughout this region, especially of charged residues in the S4 segment. The alignment was generated using ClustalW 1.8, available at The Baylor College of Medicine Search Launcher site on the World Wide Web (searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). Shading was carried out using Boxshade 3.21 on the Swiss EMBnet node Web site (www.ch.embnet.org/software/BOX_form.html). The asterisk shows the mHCN2 residue mutated in this study (Y331D).
FIG. 2. Instantaneous currents in CHO cells expressing HCN2 or HCN2 Y331D are permeable to K⁺ but differ in amplitude. A, representative current traces of HCN2 and HCN2 Y331D at three different concentrations of external potassium are shown: 30 mM potassium, 110 mM sodium, 0.4 mM NMG (30K); 15 mM potassium, 110 mM sodium, 15 mM NMG (15K); 5.4 mM potassium, 110 mM sodium, 25 mM NMG (5.4K). The voltage protocol consisted of 500-ms pulses ranging from +60 to −150 mV at 30-mV steps; the holding potential was −35 mV. Note the parallel reduction in HCN2 I_{inst}, HCN2 Y331D instantaneous currents, and If as the current carrying ion K⁺ is lowered from 30K to 15K to 5.4K in both HCN2 and HCN2 Y331D. The dotted line in the current traces recorded in 30K represents the zero current level. The heavy and thin arrows outline the instantaneous currents and If at a test voltage of −150 mV, respectively, for HCN2 and HCN2 Y331D. B–D, comparison of I-V relations between HCN2 (circles) and HCN2 Y331D (squares) at 30K (B), 15K (C), and 5.4K (D). Reversal potentials of HCN2 I_{inst} at 5.4K, 15K, and 30K were −16.1 ± 3.3, −14.7 ± 3.5, and −11.7 ± 3.2 mV (n = 6). Reversal potentials of HCN2 Y331D instantaneous currents at 5.4K, 15K, and 30K were −21.7 ± 1.8, −13.7 ± 1.2, and −5.3 ± 1.5 mV (n = 6). E, I-V relations for instantaneous current representing the difference between HCN2 Y331D instantaneous current and HCN2 I_{inst} for 30K (squares, reversal potential = −5 mV), 15K (circles, reversal potential = −14 mV), and 5.4K (triangles, reversal potential = −30 mV) from the same cells plotted in B–D.
Fig. 3. Instantaneous currents in CHO cells expressing HCN2 or HCN2 Y331D are permeable to Na⁺ but differ in amplitude. A, representative current traces of HCN2 and HCN2 Y331D at three different concentrations of external sodium: 30 mM potassium, 110 mM sodium, 0.4 mM NMG (110Na); 30 mM potassium, 40 mM sodium, 70.4 mM NMG (40Na); 30 mM potassium, 5.4 mM sodium, 105.4 NMG (5.4Na). The voltage protocol consisted of 500-ms pulses ranging from +60 to −150 mV at 30-mV steps; the holding potential was −35 mV. Note the parallel reduction in HCN2 I_{inst}, HCN2 Y331D instantaneous currents, and I_f as the current carrying ion Na⁺ is lowered from 110Na to 40Na to 5.4Na in both HCN2 and HCN2 Y331D. B–D, comparison of I-V relations between HCN2 (circles) and HCN2 Y331D (squares) at 110Na (B), 40K (C), and 5.4Na (D). Reversal potentials of HCN2 I_{inst} at 5.4Na, 40Na, and 110Na were −28.4 ± 0.8, −21 ± 1.2, and −8.7 ± 2.2 mV (n = 8). Reversal potentials of HCN2 Y331D instantaneous currents at 5.4K, 15K, and 30K were −21.9 ± 0.6, −15.5 ± 0.4, and −7.8 ± 0.4 (n = 8). E, I-V relations for instantaneous current representing the difference between HCN2 Y331D instantaneous current and HCN2 I_{inst} for 110Na (squares, reversal potential = −6 mV), 40Na (circles, reversal potential = 13 mV), and 5.4Na (triangles, reversal potential = −19 mV) from the same cells plotted in B–D.
was larger in cells expressing HCN2 Y331D ($I_f + \text{Cs}^+$-sensitive instantaneous current) than in cells expressing HCN2 ($I_f$ only) (Fig. 5G). The larger Cs$^+$-sensitive current in cells expressing HCN2 Y331D suggests some effect of the mutation on maximal conductance, since the channels are fully activated at −150 mV (see Fig. 7F). One possibility is that the number of functional mutant channels was larger than the number of functional wild-type channels. If this were true, we would expect Cs$^+$-insensitive $I_{\text{inst}}$ to also be larger, because it is proportional to the amount of HCN protein on the plasma membrane (see Ref. 11 for HCN2 and Fig. 10A for HCN1). We found that $I_{\text{inst}}$ associated with HCN2 Y331D was not significantly larger than $I_{\text{inst}}$ associated with HCN2, which implies a difference in single channel conductance or open probability. However, single channel measurements and assays of HCN protein on the plasma membrane will be required to accurately and directly determine whether the mutation affects expression of the channel on the plasma membrane.

The voltage dependence of Cs$^+$ block of human HCN2 expressed in HEK cells has been interpreted by assuming that the ions block the channels according to a Woodhull ion block model (15). To examine the voltage dependence of block of instantaneous current by Cs$^+$ in cells expressing Y331D according to this model, we plotted the percentage of current blocked (calculated from Fig. 5D) versus voltage (Fig. 6A). The block reaches a maximum at voltages near −150 mV. We then subtracted Cs$^+$-insensitive $I_{\text{inst}}$ from the instantaneous currents in Fig. 5D (Fig. 6B), recalculated the percentage of current blocked, and plotted these new values versus test voltage (Fig. 6C). In Fig. 6, A and C, we fit both the unsubtracted and subtracted values of fractional block with Equation 1 (see “Experimental Procedures”) as carried out previously for human HCN2 (15). The fit of the subtracted current amplitudes was better than the fit of the unsubtracted amplitudes, as can be seen from the figures and as indicated by a 10 times smaller $\chi^2$ value for the former (see legend to Fig. 6). Together, the data strongly suggest that the block of instantaneous current by 2 mM Cs$^+$ was complete at the more negative voltages and that the unblocked instantaneous current at −150 mV reflected $I_{\text{inst}}$ in cells expressing HCN2 Y331D. According to the fitting, our data suggest that Cs$^+$ ions cross 0.87 of the membrane electrical thickness ($\delta$) before reaching the binding site in HCN2 Y331D channels. The dissociation constant of Cs$^+$ binding ($k_d$) was 4.04 mM. We carried out a similar analysis of Cs$^+$ block of $I_f$ in CHO cells expressing HCN2 and determined values of 0.82 and 4.02 mM for $\delta$ and $k_d$, respectively (see legend to Fig. 6). The $k_d$ and $\delta$ for the mutant and wild-type channel were approximately the same, indicating that the Y331D mutation did not affect the affinity or location of the Cs$^+$ binding site in the pore.

The Y331D Mutation Disrupts Channel Closing and Destabilizes the Closed State.—The above experiments support the suggestion that a fraction of $I_f$ is already activated by the Y331D mutation, thus producing an instantaneous current in addition to $I_{\text{inst}}$ and $I_f$. We call this current the $I_f$-like instantaneous current to differentiate it from $I_{\text{inst}}$. In order to estimate the fraction of $I_f$ activated by the mutation, we determined the ratio of $I_f$-like instantaneous current to the totally fully activated $I_f$. We used a standard leak-subtracting protocol (Fig. 7A) to determine the fully activated $I_f$ in cells expressing the wild-type channel (Fig. 7B) and Y331D (Fig. 7C). The $I_f$-like instantaneous current was determined from the difference in instantaneous currents between cells expressing HCN2 Y331D and HCN2 (Fig. 7C). The fully activated $I_f$ and the $I_f$-like instantaneous current were added to yield the totally fully activated $I_f$ for HCN2 Y331D (Fig. 7C), which crosses the x axis at 9.5 mV. This is very close to the reversal potential of fully activated $I_f$ determined from cells expressing wild-type HCN2 (10.1 ± 3.4 mV, n = 5 cells) recorded under identical conditions and suggests that Y331D did not affect selectivity of Na$^+$ and K$^+$ (both relations are plotted in Fig. 7B). Using the values in Fig. 7C, we then determined the ratio of the $I_f$-like instantaneous current to the totally fully activated $I_f$ as a function of voltage for HCN2 Y331D (Fig. 7D). The fraction of $I_f$ activated by the mutation is −0.68 on average over voltages ranging from +30 to −150 mV.

We next generated activation curves for $I_f$ using 1-s test pulses and obtaining tail currents at −30 mV (arrows in Fig. 7E) and plotting normalized tail currents versus test voltage (Fig. 7F). We found that the activation curves determined from cells expressing HCN2 Y331D were shifted to more positive voltages and were less steep than those determined from cells expressing wild-type HCN2. $I_f$ began to activate in the range of −40 mV in cells expressing HCN2 Y331D unlike $I_f$ in cells expressing the wild-type HCN2, which began to activate at much more negative voltages using the same protocol. We also found that the rates of $I_f$ activation/deactivation were shifted to more positive voltages, as would be expected from a positive shift in the activation curve, but we also found that the rates of deactivation were significantly slowed at more positive voltages by the Y331D mutation (Fig. 7H). The slower rates of $I_f$ deactivation can also be observed by comparing the tail currents at −30 mV in Fig. 7F (arrows). On the other hand, the rates of $I_f$ activation were not different between HCN2 Y331D and HCN2 at voltages in the fully activated range (−120 and −150 mV). Together, the data are consistent with a disruption of channel closing and destabilization of the closed state by the Y331D mutation.

The Fraction of $I_f$ Activated by Y331D Is Modified by the Relative Amounts of External K$^+$ and Na$^+$ but Permeation Is Unaffected.—When examining the selectivity of the $I_f$-like instantaneous current for Na$^+$, we found that the amplitude of $I_f$ (measured at −150 mV) in cells expressing HCN2 Y331D increased when perfused with solutions containing decreasing amounts of external Na$^+$ while external K$^+$ was kept constant (Fig. 8A). This was unusual and was not like cells expressing wild-type HCN2 in which the amplitude of $I_f$ (also measured at −150 mV) decreased when perfused with the same solutions. However, the total $I_f$ ($I_f$ and $I_f$-like instantaneous current) determined from cells expressing HCN2 Y331D did decrease with decreasing amounts of Na$^+$ (Fig. 8, A and B). The changes in the total amount of $I_f$ flowing through the wild-type and mutated channels due to changes in external Na$^+$ were almost identical for the wild-type and mutant channels. When going from 110 to 5.4 mM Na$^+$, the amplitude of fully activated $I_f$ in
Fig. 5. The instantaneous current in cells expressing HCN2 Y331D is sensitive to Cs⁺. A and B, representative current traces of from cells expressing HCN2 or HCN2 Y331D, respectively. The voltage protocol was the same as in Fig. 2. The external solution contained 135 mM K⁺ and 5.4 mM Na⁺. The top and bottom traces were recorded before and after perfusion with 2 mM Cs⁺ in the external solution, respectively. In cells expressing HCN2, $I_f$ is blocked by Cs⁺, but $I_{\text{inst}}$ is not (A). Both $I_f$ and a large amount of inward instantaneous current are blocked by Cs⁺ in cells expressing HCN2 Y331D (B). C, current traces from cells expressing HCN2 or HCN2 Y331D elicited by 15-s test pulses at voltages of −120 and −110 mV, respectively. After 6 s, cells were perfused with an external solution containing 2 mM Cs⁺ as indicated by the arrow. The reduction of current by Cs⁺ occurred within ~4–5 s in cells expressing HCN2 or HCN2 Y331D. In addition to $I_f$, a large amount of the instantaneous current was blocked by Cs⁺ in cells expressing HCN2 Y331D, but only $I_f$ was blocked by Cs⁺ in cells expressing HCN2. D, I-V relationships of instantaneous currents recorded from cells expressing HCN2 constructs before and after block by 2 mM Cs⁺ (HCN2 (triangles), n = 13; HCN2 Y331D (squares), n = 14; HCN2 Y331D + Cs⁺ (circles), n = 14). The I-V relation for cells expressing HCN2 and perfused with 2 mM Cs⁺ is identical to the I-V curve obtained before perfusion with Cs⁺ and is not included for clarity. E, bar graph of instantaneous currents measured in response to one test pulse to −150 mV (pA/pF) in cells expressing HCN2 constructs. The instantaneous currents measured for HCN2, HCN2 + Cs⁺, and HCN2 Y331D + Cs⁺ were not significantly different (single asterisk, p < 0.05). F, bar graph of $I_f$ measured in response to one test pulse to −150 mV (pA/pF) in cells expressing HCN2 constructs. The amplitudes of $I_f$ in cells expressing HCN2 or HCN2 Y331D were significantly reduced by Cs⁺ (single asterisks, p < 0.05). G, bar graph of total Cs⁺-sensitive current measured in cells expressing HCN2 and HCN2 Y331D. The total amount of Cs⁺-sensitive current in cells expressing HCN2 Y331D is significantly larger than in cells expressing HCN2 (asterisk, p < 0.05). The values in E-G are given as means ± S.E., and the numbers in parentheses represent the number of cells for each condition.
cells expressing wild-type HCN2 decreased from −248.1 to −160.3 pA/pF (to 0.65 times the initial value), whereas the total \( I_f \) in cells expressing HCN2 Y331D decreased from −592.6 to −357.8 pA/pF (to 0.60 times the initial value). These data confirm that the permeation of Na\(^+\) was not affected by the Y331D mutation.

We also carried out identical protocols in experiments where only the concentration of external K\(^+\) was varied. When perfused with solutions containing decreasing amounts of K\(^+\) while Na\(^+\) was kept constant, we found that both the amplitude of \( I_f \) and the total \( I_f \) (\( I_f \) and \( I_f \)-like instantaneous current) decreased in cells expressing HCN2 Y331D (Fig. 8: A, C and D). As expected, the amplitude of \( I_f \) also decreased in cells expressing wild-type HCN2 (Fig. 8C). The changes in the total amount of \( I_f \) flowing through the wild-type and mutated channels due to changes in external K\(^+\) were almost identical for the wild-type and mutant channels (as they were above for changes in external Na\(^+\)). When going from 30 to 5.4 mM K\(^+\), the amplitude of fully activated \( I_f \) in cells expressing wild-type HCN2 decreased from −202.4 to −63.9 pA/pF (to 0.32 times the initial value), whereas the total \( I_f \) in cells expressing HCN2 Y331D decreased from −286.0 to −94.2 pA/pF (to 0.33 times the initial value). These data confirm that the permeation of K\(^+\) was also not affected by the Y331D mutation.

Finally, we generated \( I_f \) activation curves from tail currents in three of the five external concentrations used (the amplitudes of \( I_f \) were too small to generate accurate activation curves in the mutant channels at 5.4 and 15 mM external K\(^+\)). These indicated that \( I_f \) was fully activated at −150 mV in cells expressing the mutant channel (\( n = 2 \) cells, not shown) as it was in the high K\(^+\) solution (Fig. 7F). These data and the similarity in the changes in total \( I_f \) amplitude at different concentrations of external Na\(^+\) and K\(^+\) between cells expressing either the wild-type or mutant channels are consistent with the channels being fully activated at −150 mV.

Because the changes in total \( I_f \) were similar, the above experiments suggested that it was the relative amplitudes of \( I_f \) and the \( I_f \)-like instantaneous current produced by HCN2 Y331D that was affected and furthermore that these changes were related to changes in both external Na\(^+\) and K\(^+\). We previously showed that the rate of \( I_f \) deactivation increased as the ratio of external K\(^+\) versus Na\(^+\) concentration ([K\(^+\)]/[Na\(^+\)]) increased, and we suspected a similar relationship between the proportion of \( I_f \) and the ratio of external cations involved here. A plot of \( I_f/(I_f + I_i)-\)like instantaneous current) versus \([\text{K}^+]_o/\text{[Na}^+]_o\) showed an increase that was well fit by a single decaying exponential function. The range of greatest change was at ratios of \([\text{K}^+]_o/\text{[Na}^+]_o\) between 0 and 1 (in the “physiological range”), whereas the proportion of \( I_f \) reaches a close-to-maximum level of 0.33 at \([\text{K}^+]_o/\text{[Na}^+]_o\) value of 5.5. This exponential relationship is consistent with our findings in Fig. 7D, where the fraction of \( I_f \) was 0.32 at a \([\text{K}^+]_o/\text{[Na}^+]_o\) value of 25 (135 mM K\(^+\)/5.4 mM Na\(^+\) in the external solution).

The HCN1 Isoform Also Produces \( I_{imix} \).Because the above analysis suggested that mutations in the S4-S5 linker did not alter \( I_{inst} \) and since our previous work showed that mutations in the selectivity filter or S4 segment also did not alter \( I_{inst} \), we next took a more global approach to uncover regions involved in generating \( I_{inst} \). We looked for \( I_{inst} \) in CHO cells expressing HCN1 in order to determine whether structural elements in common between the HCN1 and HCN2 isoforms are involved. Previous results have suggested that other HCN isoforms are associated with the presence of instantaneous currents, although the nature of these currents is not always very clear (5–7, 9, 19). Fig. 9, A and B, shows representative current traces from CHO cells expressing HCN1 or GFP alone recorded in varying external K\(^+\) or Na\(^+\) solutions, respectively. The density of instantaneous currents measured at −150 mV was significantly larger in cells expressing HCN1 than in cells expressing only GFP (Fig. 9, C and D). The amplitude of instantaneous current in cells expressing HCN1 increased with increasing changes in external K\(^+\) or Na\(^+\) in a linear fashion, suggesting the current was carried by both cations (Fig. 9, E and F). Interestingly, the amplitude of instantaneous currents in cells expressing only GFP also increased with increasing concentrations of external K\(^+\) and Na\(^+\), suggesting the presence of an endogenous cation channel. This is consistent with other studies that have demonstrated endogenous mixed cation channels in CHO cells (20, 21).

To determine whether the instantaneous current was related to the amount of HCN protein on the plasma membrane, we previously showed a significant positive correlation between the amplitudes of \( I_{inst} \) and fully activated \( I_f \) measured in the same cells expressing HCN2 (11). We carried out a similar correlation of fully activated \( I_f \) and instantaneous current in CHO cells expressing HCN1. We plotted the current densities of instantaneous current versus those of \( I_f \) measured in the same cells in response to test pulses to −150 mV (Fig. 10A, \( n = \)}
2) then subtracting the HCN2 Y331D instantaneous current measured in the first current staircase from the current at each test voltage in the second staircase, which yielded the fully activated HCN2 Y331D instantaneous current component (circles plotted in C) by the total fully activated HCN2 Y331D current (triangles plotted in C). These were added to give the total fully activated I_/HCN2 Y331D (squares in B and C, reversal potential 7 9.5 mV, slope 7 5.5 nA). C, a plot of the I_/HCN2 Y331D instantaneous current component, fully activated I_/HCN2 Y331D (triangles and the total fully activated I_/HCN2 Y331D (squares) versus test voltage, D, a plot of the fractional activation of I_/HCN2 Y331D, determined by dividing the I_/HCN2 Y331D instantaneous current (circles in C) by the total fully activated I_/HCN2 Y331D (squares in C) at each test voltage, versus test voltage, E, representative I_/HCN2 and HCN2 Y331D measured in a 135 mM K\textsuperscript{+} and 5.4 mM Na\textsuperscript{+} external solution used for generation of activation curves. The voltage protocol consisted of 1-s pulses ranging from 10 to 150 mV at 20-mV steps, followed by a second pulse to #30 mV for 2 s to elicit tail currents from a holding potential of 35 mV. F, I_/ activation curves for HCN2 (circles, n 5) and HCN2 Y331D (squares, n 9) generated from tail currents as in E. I_/ activation curves were best fit with a Boltzmann function (see Equation 2), which gave V and inverse slope (k) values of 3125.8 7 2.0 and 9.8 7 0.6 mV for HCN2 and 398.5 7 2.1 and 25 7 3.0 mV for HCN2 Y331D. G, plots of I_/ activation (filled symbols) and deactivation rates (open symbols) versus test voltage for HCN2 (circles) and HCN2 Y331D (squares). Rates were determined using a single exponential function fit to activating and deactivating currents in response to test voltages elicited as shown in A. The activation rates were not significantly different for both constructs at hyperpolarized potentials of 150 and 120 mV, and the deactivation rates were significantly slowed in the mutant compared with wild-type channel at depolarized potentials of 60, 30, 0, 30 mV.

11 cells) and found a significant correlation (r 7 0.7, slope 7 0.13, p < 0.05). The linear correlation crosses the y axis at approximately 30 pA/pF. This represents the amplitude of instantaneous current in cells not expressing I_/ and is close to the experimental values of instantaneous current measured in cells expressing only GFP using identical intracellular and extracellular solutions (12, 13). This positive covariation, together with data presented above regarding the mixed cationic nature of the current, indicates that the instantaneous current associated with the expression of HCN1 in CHO cells is I_/inst.

I_/inst is not due to excess levels of HCN protein on the plasma membrane as compared with native cells—The mixed cationic nature of I_/inst and the correlation of I_/inst with I_/ do not support a nonspecific leakage phenomenon associated with the expression of HCN protein on the plasma membrane of CHO cells. Another possibility is that overexpression of HCN protein on the plasma membrane allowed for the specific entry of Na\textsuperscript{+} and K\textsuperscript{+}, which would not be observed with presumably lower levels of protein found on the plasma membrane of native cells. In order to examine this issue, we determined the densities of I_/inst and fully activated I_/ obtained by expression of HCN1 in CHO cells using "physiological" concentrations of Na\textsuperscript{+} and K\textsuperscript{+} (135 and 5.4 mM, respectively, in the extracellular solution and a high K\textsuperscript{+} solution in the pipette) and compared these with amplitudes of I_/ found in native cells using solutions with similar concentrations of Na\textsuperscript{+} and K\textsuperscript{+}. Interestingly, we have found that the electrophysiological protocol required for a direct comparison of I_/inst and "fully activated" I_/ in the same cell...
can be carried out most easily and successfully with the HCN1 isoform as compared with HCN2 and HCN4. The densities of $I_{\text{inst}}$ and fully activated $I_f$ were determined in the same cells at two concentrations of extracellular $K^+$ and $Na^+$ (as described in the legend to Fig. 10B, which shows the pulse protocol used and representative current traces) and plotted against voltage.

![Image](http://www.jbc.org/Downloadedfrom)

**FIG. 8.** Effects of $[K^+]/[Na^+]_e$ on the fraction of $I_f$ activated in CHO cells expressing HCN2 Y331D. A, HCN2 and HCN2 Y331D $I_f$ measured at $-150$ mV (same cells in Fig. 3, $n = 8$) in $110Na$ (solid bars), $40Na$ (hatched bars), and $5.4Na$ (open bars). Inset, shows representative current traces for cells expressing HCN2 (left) and HCN2 Y331D (right) $I_f$ at $-150$ mV in $5.4Na$, $40Na$, and $110Na$ recorded in the same cell. B, $I_f$-like instantaneous current and $I_f$ (added hatched regions) measured in the same cells at $5.4Na$ (diamonds), $40Na$ (triangles), and $110Na$ (squares). Values for $I_f$-like instantaneous current are the same as in Fig. 3E. C, HCN2 and HCN2 Y331D $I_f$ measured at $-150$ mV (same cells in Fig. 2, $n = 6$) in $30K$ (solid bars), $15K$ (hatched bars), and $5.4K$ (open bars). Inset, shows representative current traces for cells expressing HCN2 (left) and HCN2 Y331D (right) $I_f$ at $-150$ mV in $5.4K$, $15K$, and $30K$ recorded in the same cell. D, $I_f$-like instantaneous current and $I_f$ (hatched regions) measured in the same cells at $5.4K$ (diamonds), $15K$ (triangles), and $30K$ (squares). Values for $I_f$-like instantaneous current are the same as in Fig. 2E. E, plot of fraction of $I_f$ activated versus $[K^+]/[Na^+]_e$. The values were fit with a single decaying exponential function and yielded a $\tau$ value of 0.91.
FIG. 9. Cells expressing the HCN1 isoform have an increased instantaneous current that is similar to \( I_{\text{inst}} \). A, representative current traces from cells expressing HCN1 or GFP, recorded in 30K, 15K, and 5.4K external solutions. B, representative current traces from cells expressing HCN1 and GFP recorded in 110 mM sodium, 30 mM potassium, 0.4 mM NMG (110Na), 40 mM sodium, 30 mM potassium, 70.4 mM NMG (40Na), and 5.4 mM sodium, 30 mM potassium, 105 mM NMG (5.4Na) external solutions. The voltage protocol was the same as in Fig. 2, except that the test voltage was applied for 250 ms. C, bar graph of instantaneous current amplitudes (pA/pF) in cells expressing HCN1 (n = 6) or GFP alone (n = 4) measured at −150 mV, in external solutions containing varying amounts of K\(^+\). D, bar graph of instantaneous current amplitudes (pA/pF) in cells expressing HCN1 (n = 9) or GFP alone (n = 5) measured at −150 mV, in external solutions containing varying amounts of external Na\(^+\). The amplitude of instantaneous current in cells expressing HCN1 is significantly larger than in cells expressing GFP, at each corresponding K\(^+\) and Na\(^+\) concentration. E and F, plots of slope conductance of
as shown in Fig. 10, C and D. A shift in the reversal potential was found for both \( I_f \) and \( I_{\text{inst}} \) in the two concentrations of \( \text{Na}^+ \) and \( \text{K}^+ \), consistent with the mixed cationic nature of these currents. A comparison of \( I_f \) densities collected from studies of native cells (with relatively high levels of \( I_f \)) with the density of \( I_f \) determined from Fig. 10D is shown in Table I. The comparison is not precise, because the measurements were made using similar, but not identical, “physiological” solutions (see legend to Table I). However, the comparison indicates that the density of \( I_f \), determined in native cells may be as high or higher than those determined in our experiments. This suggests that the amount of HCN protein on the plasma membrane of CHO cells in our experiments and HCN protein found on plasma membranes in native cells do not greatly differ and thus that \( I_{\text{inst}} \) is not the result of excessive amounts of HCN protein on the plasma membrane of CHO cells relative to native cells.

**DISCUSSION**

**Y331D Mutation Disrupts Channel Closing**—We have shown that the Y331D mutation in the HCN2 S4-S5 linker significantly decreased \( I_f \) and significantly increased a mixed cation instantaneous current. However, the instantaneous current up-regulated by Y331D was sensitive to external Cs\(^+\), making it more similar to \( I_f \) than \( I_{\text{inst}} \), which was unchanged by the mutation. Therefore, we were able to divide the instantaneous currents measured in cells expressing HCN2 Y331D into three portions: a portion endogenous to CHO cells, a Cs\(^+\)-insensitive portion produced by expression of HCN2 channels (\( I_{\text{inst}} \)), and a Cs\(^+\)-sensitive “\( I_f \)-like” portion produced by the Y331D mutation. The “\( I_f \)-like” instantaneous current was \( \approx 70\% \) of the total \( I_f \) over a range of \(+30 \rightarrow -150 \text{ mV}\) in a high potassium external solution. In addition, the activation curve of \( I_f \) was shifted to more positive voltages and was less steep, the \( I_f \) activation/deactivation curve was shifted to more positive voltages, and deactivation was significantly slowed at depolarized potentials compared with \( I_f \) recorded from cells expressing wild-type HCN2. On the other hand, the rate of \( I_f \) activation at voltages in the fully activated range was not different between cells expressing HCN2 Y331D or wild-type HCN2. These data are consistent with the idea that the Y331D mutation shifted the closing of the channel and destabilizes the closed state and does not increase the conductance of a leaky closed state. Our findings are in line with previous studies suggesting a disruption of HCN2 channel closure by mutations of Tyr\(^{331}\) (10).

Previous studies suggested a functional and physical uncoupling of the voltage sensor from the activation gate (10, 22), but we think that the Y331D mutation does not physically sever or weaken a link between the voltage-sensing elements and the activation gate. Our reasoning is based partly on an argument made for mutations of the Shaker pore and similarities of this pore to the crystallized pores of KcsA and MthK channels by Yifrach and Mackinnon (23). These authors suggested that because mutations generally tend to destabilize protein packing, shifts toward the open state indicate pore mutations destabilized the closed conformation as opposed to stabilizing the open conformation. The packing of the inner and outer helices of closed Shaker channels were argued to be more optimal based on a comparison of the crystal structures of KcsA and MthK, closed and open channels, respectively. If Shaker channels are intrinsically more stable when they are closed, these authors argued that it follows that the voltage sensors must work to open the pore. If HCN channels have a similar pore structure, which seems plausible based on the conservation of key sequences in the pore including the GYG signature motif and a key glycine “hinge” region (24), then it follows that these channels may also be more stable when they are closed where protein packing would be optimal. Furthermore, the voltage-sensing elements would also have to work to open the pore, although this would be done by hyperpolarization and not by depolarization as in Shaker. This line of reasoning suggests that severing or weakening the link between the voltage-sensing elements and gate would favor the more stable closed state of the channel. Therefore, it seems unlikely that the Y331D mutation reduced or eliminated coupling between voltage-sensing elements and the activation gate but more likely that this mutation disrupted the closing of the channel. The slowing of \( I_f \) deactivation at depolarized potentials, without any effects on the rates of \( I_f \) activation in the fully activated range, and the shift of channel opening to more positive voltages further support a disruption of channel closing and destabilization of the closed state by Y331D rather than an uncoupling of the voltage-sensing elements and the activation gate.

We also suspect that the pore is not directly involved in the effect of Y331D for two reasons. First, permeation is unaffected by Y331D. Second, the disruption of closing by Y331D is additive with the effects of \([\text{K}^+]_e/[\text{Na}^+]_i\) on channel closing, which may be due to the effect of cations in the pore (see below). Because the two effects are additive, it seems likely that they are at least partly independent.

Based on its position, we suspect that the Y331D mutation causes a local allosteric perturbation of the activation gate (S6 segment) and/or of the voltage-sensing elements, which disrupts channel closing. An allosteric effect involving the S6 segment is supported by studies that show that HCN channels with cysteine mutations in the S6 region can be “locked” open by Cd\(^{2+}\) (25). A study using Shaker potassium channels showed that S6 mutations also constitutively activated the channel and shifted the activation curve of time-dependent current to more negative voltages, because of a perturbation of the closed to open equilibrium (26). Exactly how the Tyr\(^{331}\) residue is involved in disrupting gating will require a structural understanding of how HCN channels sense and respond to changes in voltage, and this is presently not completely clear (19, 27, 28).

\([\text{K}^+]_e/[\text{Na}^+]_i\), Ratio Further Modifies HCN2 Y331D Channel Closing—An unusual and interesting finding in the present study was a decrease in the proportion of \( I_f \)-like instantaneous current and an increase in the proportion of \( I_f \) in cells expressing HCN2 Y331D when the \([\text{K}^+]_e/[\text{Na}^+]_i\) ratio increased. The increase in the proportion of \( I_f \) could be accurately described by a decaying single exponential function. The range over which the greatest change occurred was at \([\text{K}^+]_e/[\text{Na}^+]_i\) values between 0 and 1. In CHO cells expressing wild-type HCN2, we have previously shown that a hardening of \( I_f \) deactivation and the amplitude of fully activated \( I_f \) are both related to external \( \text{K}^+ \) and \( \text{Na}^+ \) in a similar way, with the greatest change occurring...
Fig. 10. **I** correlates with the amount of **I** in CHO cells expressing HCN1, and both are increased in a high potassium external solution. **A**, graph of **I** versus **I** in cells expressing HCN1 (r = 0.7, slope = 0.13, p < 0.05). **I** and **I** were obtained from the same cells during perfusion with the high potassium solution as in **B** (n = 11). **B**, current traces recorded from the same cell in high external potassium (top trace, 135 mM K⁺, 5.4 mM Na⁺) and low external potassium (bottom trace, 5.4 mM K⁺, 135 mM Na⁺). The voltage protocol is shown above the current traces. **C** and **D**, I-V relationships generated for **I** and fully activated **I** for high potassium (squares in **C** and **D**, respectively) and low potassium (circles in **C** and **D**, respectively). **I** was obtained in the first staircase, and the fully activated **I** was generated by subtracting **I** from the current at each test voltage in the second staircase. These were plotted versus test voltage. The slope conductances for **I** in low potassium and high potassium were 0.16 ± 0.01 nS/pF (n = 6) and 0.36 ± 0.01 nS/pF (n = 11), respectively. The slope conductances for fully activated **I** in low potassium and high potassium were 0.32 ± 0.04 nS/pF (n = 6) and 1.31 ± 0.06 nS/pF (n = 11), respectively. The reversal potentials for **I** (low potassium = −15.7 ± 4.4 mV; high potassium = 0.6 ± 1.8 mV) and **I** (low potassium = −21.7 ± 3.2 mV; high potassium = 9.0 ± 1.9 mV) were both shifted to more positive potentials in high potassium conditions.
Instantaneous and Slow Gating Elements in HCN Channels

Table I

| Values for \( I_{\text{f}} \) slope conductance and density from this study | are within a range found in native cells |
|---|---|
| **Literature values** | **Present study** |
| **Slope conductance (nS/pF) (−100 mV)** | \(-1.3^a\) | 0.26 |
| | 0.26 ± 0.07\(^b\) | 0.32 ± 0.04 |
| | 0.24 ± 0.02\(^c\) | 0.23 ± 0.04 |
| | 0.16 ± 0.01\(^d\) | 0.18 ± 0.02 |
| **Current density (pA/pF)** | \(-130\) mV | 27.6 ± 5.0\(^d\) | 34 |
| | +20 mV | 14.9 ± 1.3\(^d\) | 14 |

\( a \) See Ref. 54 (also personal communication); cochlear octopus cells; pipette solution: 108 mM potassium gluconate; extracellular solution 138 mM NaCl, 3 mM KCl.

\( b \) See Ref. 55; SA node cells; pipette solution: 55 mM KCl, 75 mM K\(_2\)SO\(_4\), extracellular solution: 130 mM NaCl, 5.4 mM KCl.

\( c \) See Ref. 56; newborn (top) and adult (bottom) SA node cells; pipette solution: 10 mM NaCl, 130 mM potassium aspartate; extracellular solution: 140 mM NaCl, 5.4 mM KCl.

\( d \) See Ref. 57: spider and spindle-shaped cells from SA node cells; pipette solution: 100 mM potassium aspartic acid, 30 mM KCl; extracellular solution: 137 mM NaCl, 4.0 mM KCl.

ring over the same range of \([K^+]_o/[Na^+]_o\) values (14). Therefore, it seems reasonable to suggest that the effects on channel closing and permeation are connected and are due to a competition for the occupancy of site(s) in the pore by Na\(^+\) and K\(^+\). In HCN2 channels, K\(^+\) is the preferred cation, whereas Na\(^+\) moves through the pore less efficiently, and the channel itself conducts only when a minimum amount of external K\(^+\) is present (6, 14, 29, 30). Based on similarities to the pore and gate of voltage-gated K\(^+\) channels (31–33), the slower passage of Na\(^+\) suggests a “sodium foot in a potassium channel door” mechanism whereby the presence of Na\(^+\) in the pore interferes with the closing of the HCN2 channel. An interesting feature of this mechanism in HCN2 channels is that the effects occur in a range of \([K^+]_o/[Na^+]_o\) found in vivo, and thus it is likely to play an important physiological role (14). The effect of \([K^+]_o/[Na^+]_o\) also explains the larger proportion of constitutively activated current found previously in *Xenopus* oocytes expressing HCN2 Y331D, where a value of close to 100% was reported (10). Using the value of \([K^+]_o/[Na^+]_o\) in those experiments (\([K^+]_o/[Na^+]_o\) = 4 mM/96 mM = 0.04), we would predict a fraction of activated \(I_f\) of greater than 95% using our exponential relationship generated in Fig. SE.

\( I_{\text{inst}} \) Is Also Associated with HCN1, Implicating Regions Conserved among HCN Channels in Generating This Current—We also found that the HCN1 isoform produced an instantaneous current identical to \( I_{\text{inst}} \) produced by HCN2 expression in CHO cells restricting the structural elements involved to those common between these isoforms. These include conserved regions in the proximal N and C termini as well as those in the transmembrane segments including the pore. The presence of instantaneous currents in the same tissues as \( I_f \) and HCN channels (34–36) and an apparent up-regulation of instantaneous currents in cells with up-regulated HCN channels or \( I_f \) (37–40) strongly suggest a direct association between them. Whether \( I_{\text{inst}} \) is intrinsic to the HCN channel (e.g. through a leaky closed state) or whether it results from a specific association of HCN channels with endogenous subunits or channels remains unknown.

We did find that \( I_{\text{inst}} \) was not likely to be an artifact of excess protein on the plasma membrane of CHO cells expressing HCN1, because fully activated \( I_f \) amplitudes were similar between our experiments and studies of \( I_f \) in native cells. It is possible that, in both heterologous expression systems and native cells, HCN proteins may cluster in certain areas of the plasma membrane (e.g. see Refs. 41–44). Molecular crowding of proteins on the cell surface may influence the function of membrane proteins (45). Thus, it is possible that the clustering of HCN protein on the plasma membrane, alone or together with other proteins, leads directly or indirectly to cation-permeable channels underlying the instantaneous current we observed.

It is also possible that \( I_{\text{inst}} \) results from the specific up-regulation of an endogenous channel, since CHO cells have a mixed cation instantaneous current. Furthermore, we have yet to find a mutation that modifies \( I_{\text{inst}} \) without affecting trafficking, suggesting that the current may not be intrinsic to the channel. On the other hand, if \( I_{\text{inst}} \) does result from a leaky closed state, it may be difficult to find a mutation that would disrupt flow through the pore of the closed channel without disrupting the activation gate. Also, we have found that increases in \( I_{\text{inst}} \) are directly proportional to the amount of \( I_f \) in the same cell and hence directly proportional to the amount of HCN protein on the plasma membrane in that cell. Therefore, the up-regulation of an endogenous channel or protein and the amplitude of \( I_{\text{inst}} \) might not increase in a linear fashion but might be expected to saturate at higher levels HCN expression. Finally, the pore represents a region that is highly conserved between HCN1 and HCN2 and therefore may be responsible for \( I_{\text{inst}} \).

Are closed channels leaky? Recent experiments have shown that closed Shaker channels have a resting conductance and nonzero open probability, although they are very low, at least 100,000-fold lower than fully activated channels (46, 47). Cyclic nucleotide-gated channels show a spontaneous \( P_o \) of about 0.002 in the absence of ligand, suggesting that these “closed” channels also conduct some current (48). If \( I_{\text{inst}} \) flows through a closed HCN channel, we estimate a closed state resting conductance of −10 times less than the open state in conditions of high external potassium (after subtraction of instantaneous currents endogenous to the CHO cell). This would make HCN channels considerably more leaky than Shaker and CNG channels but would make them similar to inwardly rectifying plant K\(^+\) channels, which are associated with relatively large instantaneous currents across a range of positive and negative voltages when expressed in COS or CHO cells (49).

HCN1 has been localized to myocytes and conducting tissue of the heart and neurons (50–52), and therefore \( I_{\text{inst}} \) may play an important role in these cells regardless of how this current is generated. As we have discussed previously for HCN2 (11), the presence of HCN1 protein in sinoatrial myocytes of the heart (51) suggests that \( I_{\text{inst}} \) may form the sodium-sensitive background current (\( I_{\text{b,Na}} \)) which is found in these cells. \( I_{\text{b,Na}} \) has similar properties to \( I_{\text{inst}} \) (34), and its amplitude relative to \( I_f \) in sinoatrial node myocytes (35) is similar to the amplitude of \( I_{\text{inst}} \) relative to \( I_f \) determined here and previously with HCN2 (11). \( I_{\text{b,Na}} \) provides depolarizing current during diastole, which probably contributes to the diastolic depolarization along with \( I_{\text{f}} \) (53). Perhaps not coincidentally, \( I_{\text{b,Na}} \) plays an important role in stabilizing pacemaker frequency together with \( I_f \) (35). The HCN channel would therefore provide a common point for tightly co-regulating the expression of \( I_{\text{b,Na}} \) and \( I_f \).

Acknowledgments—We thank Dr. Andreas Ludwig (Technische Universität München) for the mHCN2 clone and Dr. Bina Santoro (Columbia University) for the mHCN1 clone. Discussions with Profs. D. DiFrancesco and Anna Moroni (Università di Milano) regarding HCN protein clustering on the plasma membrane, Professor Donatella Ortel (University of Wisconsin) regarding the large current densities in neurons, and Dr. Cathy Proenza (Harvard University) on conductance through closed channels were greatly appreciated. The technical assistance and input from Damiano Angoli was also greatly appreciated.
Structural Elements of Instantaneous and Slow Gating in Hyperpolarization-activated Cyclic Nucleotide-gated Channels
Vincenzo Macri and Eric A. Accili

J. Biol. Chem. 2004, 279:16832-16846.
doi: 10.1074/jbc.M400518200 originally published online January 29, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400518200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 57 references, 17 of which can be accessed free at http://www.jbc.org/content/279/16/16832.full.html#ref-list-1