Helical Secondary Structure of the External S3-S4 Linker of Pacermaker (HCN) Channels Revealed by Site-dependent Perturbations of Activation Phenotype*

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I\textsubscript{f}, encoded by the hyperpolarization-activated cyclic nucleotide-modulated channel family (HCN1–4), contributes significantly to neuronal and cardiac pacuing. Recently, we reported that the S3-S4 residue Glu-235 of HCN1 influences activation by acting as a surface charge. However, it is uncertain whether other residues of the external S3-S4 linker are also involved in gating. Furthermore, the secondary conformation of the linker is not known. Here we probed the structural and functional role of the HCN1 S3-S4 linker by introducing systematic mutations into the entire linker (defined as 229–237) and studying their effects. We found that the mutations K230A (−62.2 ± 3.4 mV versus −72.2 ± 1.7 mV of wild type (WT)), G231A (−64.4 ± 1.3 mV), M232A (V\textsubscript{1/2} = −63.1 ± 1.1 mV), and E235G (−65.4 ± 1.5 mV) produced depolarizing activation shifts. Although E229A and M232A decelerated gating kinetics (<2–3-fold), D233A, S234A, K230A and G231A accelerated both activation (−13- and 3-fold, respectively), K230A and G231A accelerated both activation and deactivation (<−2–3-fold). D233A, S234A, V236A, and Y237A channels exhibited WT properties (p > 0.05). Shortening the linker (EVY235–237AΔA) caused depolarizing activation shift and slowed kinetics that could not be explained by removing the charge at position 235 alone. Secondary structural predictions by the modeling algorithms Spro2 and PROF, along with refinements by our experimental data, suggest that part of the S3-S4 linker consitutes a helical structure with the functionally important residues Met-232, Glu-235, and Gly-231 (|ΔΔG| > 1 kcal/mol) clustered on one side.

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EXPERIMENTAL PROCEDURES

Molecular Biology and Heterologous Expression—mHCN1 (kindly provided by Drs. Siegelbaum and Santoro) was subcloned into the pGHE expression vector (9). Mutations were created by using PCR with overlapping mutagenic primers and confirmed by DNA sequencing. cRNA was transcribed from ψNhe-linearized DNA using T7 RNA polymerase (Promega, Madison, WI). HCN1 channel constructs were heterologously expressed and studied in Xenopus oocytes as described previously (16, 20–22). Briefly, stage IV to VI oocytes were surgically removed from anesthetized female frogs after immersion in 0.3% tricaine (3-aminobenzoic acid ethyl ester) followed by digestion with 1 mg/ml collagenase (type IA) in OR-2 solution containing (in mM) 88 NaCl, 2 KCl, 1 MgCl\textsubscript{2}, and 5 mm HEPES (pH 7.6) for 30–60 min. After defolliculation of cells by incubation in OR-2 with 10% fetal bovine serum, oocytes were thoroughly washed and transferred to an incubation medium (IM-1) containing (in mM) 100 NaCl, 5 KCl, 1 MgCl\textsubscript{2}, 10 HEPES (pH 7.6), 1.5 CaCl\textsubscript{2}, and 10 glucose and then micropipetted with a low resistance pipette into molar solution, a 100 μl volume of which was maintained in a resealable 1 ml Nunc-culture vessel. Transfection was performed by using a standard micropipet technique (23). Methods for measuring currents used were similar to those previously described (23–25).
serum for 15–30 min, isolated oocytes were injected with cRNA (50 or 100 ng per cell) and stored in ND96 solution containing (in mM) 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, and 5 HEPES (pH 7.6) supplemented with 50 μg/ml gentamicin, 5 mM pyruvate, and 0.5 mM theophylline.

**Electrophysiology, Experimental Protocols, and Data Analysis**—Two-electrode voltage clamp recordings were performed at room temperature (23–25 °C) using a Warner OC-725B amplifier (Hamden, CT) 1–2 days after cRNA injection as described (16). The regular recording bath solution contained (in mM): 97.8 KCl, 2 NaCl, 10 HEPES, and 1 MgCl₂ (pH 7.5). MgCl₂ concentration was increased in certain experiments as indicated.

The voltage dependence of HCN channel activation was assessed by plotting tail currents measured immediately after pulsing to −140 mV as a function of the preceding 3-s test pulse voltage normalized to the maximum tail current recorded. Data were fit to the Boltzmann functions using the Marquardt-Levenberg algorithm in a non-linear least squares procedure as shown in Equation 1,

\[
m_a = \frac{1}{1 + \exp((V_t - V_{1/2}/k))}
\]

(Eq. 1)

where \(V_t\) is the test potential, \(V_{1/2}\) is the half-point of the relationship, \(k = RT/zF\) is the slope factor, and \(R, T, z,\) and \(F\) have their usual meanings.

Changes in free energy (\(\Delta G\)) associated with steady-state activation shifts caused by amino acid substitution were calculated using Equation 2,

\[
\Delta G = RT\left(\frac{V_{1/2,\text{Mutant}}}{k_{\text{Mutant}}} - \frac{V_{1/2,\text{WT}}}{k_{\text{WT}}}ight)
\]

(Eq. 2)

A cutoff of 1 kcal/mol in the absolute magnitude of \(\Delta G\) was used to indicate significance in this study.

For simplicity, the time constants for activation (\(\tau_{act}\)) and deactivation (\(\tau_{deact}\)) were estimated by fitting macroscopic and tail currents, respectively, with a mono-exponential function. However, it should be noted that sigmoidicity was observed during the onset of some HCN1 currents. Similarly, tail currents at certain voltages also exhibited an initial delay. The mechanism underlying such complex kinetic behavior of HCN channels is not understood; further analysis using multiple exponential components is beyond the scope of the present article.

All data reported are mean ± S.E. Statistical significance was deter-

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**FIG. 1.** Primary amino acid sequence of the S3-S4 linker of HCN1 channels and its corresponding secondary structure predictions by various modeling algorithms as indicated. The numbers below the PROF assignments refer to the confidence limits for this modeling algorithm (on a scale of 10). The sequences of HCN2–4 are also shown for comparison.

**FIG. 2.** Effects of E229A and K230A mutations on HCN1 steady-state activation. A, representative traces of hyperpolarization-activated currents through WT, E229A, and K230A HCN1 channels. The electrophysiological protocol used to elicit currents is in Fig. 3A, inset. B, steady-state activation relationships of the same channels from A. Despite their opposite charge changes, both E229A and K230A channels were similarly shifted in the depolarizing direction.
mined for all individual data points and fitting parameters using one-
way analysis of variance and the Tukey HSD post-hoc test at the 5%
level.

**Modeling Algorithms**—The secondary structure of the S3-S4 linker of
HCN1 channel was evaluated separately by the algorithms PROF
(cubic.bioc.columbia.edu/predictprotein), SSpro2 (promoter.ics.uci.edu/
BRNN-PRED), and APSSP2. PROF is an improved version of PHDhtm,
which uses evolutionary information as input to predict the locations of
transmembrane helices in integral membrane proteins by dynamic
programming (25, 26). SSpro2 is an algorithm for secondary structure
predictions based on bidirectional recurrent neural networks and PSI-
BLAST-derived profiles (27). The advanced protein secondary structure
prediction (APSSP2) server employs PSI-BLAST as well as the modified
example based learning technique.

**RESULTS**

**Modeling Algorithms Predict a Helical Secondary Conforma-
tion for the HCN1 S3-S4 Linker**—Recently, we reported (22)
that the S3-S4 residue Glu-235 of HCN1 influences activation
gating by acting as an external surface charge of the channel.
In stark contrast to Glu-235 mutations, charge neutralization
or reversal of the nearby anionic residue Asp-233, however, did
not alter wild-type (WT) HCN1 gating properties. Further-
more, D233C (in the background of C318S to eliminate the
intrinsic sensitivity of WT channels to sulfhydryl reactive com-
pounds (20)) was also not reactive to the externally applied
sulfhydryl modifier methanethiosulfonate ethyl ammonium

**TABLE I**

| Channel | $V_1$ (mV) | $h$ | $n$ |
|---------|------------|----|----|
| WT      | -72.2 ± 1.7 | 15.4 ± 0.8 | 15 |
| E229A   | -70.2 ± 1.2 | 9.3 ± 0.9* | 13 |
| K230A   | -62.2 ± 3.4* | 12.7 ± 1.1 | 7  |
| G231A   | -64.4 ± 1.3* | 9.4 ± 1.0* | 6  |
| M232A   | -98.1 ± 2.0* | 15.9 ± 1.3 | 6  |
| D233A   | -71.0 ± 5.2 | 10.5 ± 2.8* | 4  |
| S234A   | -72.6 ± 2.1 | 9.6 ± 0.6* | 7  |
| E235G   | -65.4 ± 1.5* | 13.9 ± 0.7 | 7  |
| V236A   | -70.3 ± 2.1 | 13.6 ± 1.2 | 6  |
| Y237A   | -76.3 ± 2.1 | 11.4 ± 1.2* | 7  |
| EVY235-237ΔΔΔ | -54.5 ± 1.7* | 15.4 ± 1.5 | 8  |

* $p < 0.05.$

**FIG. 3. Effects of E229A and K230A on gating kinetics.** Effects of E229A and K230A mutations on activation (i.e., $\tau_{act}$; A and B, respectively) and deactivation ($\tau_{deact}$; C) kinetics. The electrophysiological protocols used for inducing activation and deactivation current tracings of WT, E229A, and K230A channels are shown in the corresponding left panels.
Fig. 4. Effects of G231A, M232A, and E235G on HCN1 steady-state activation. A, representative currents through G231A, M232A, and E235G channels; B, the corresponding steady-state activation curves.

Fig. 5. Effects of G231A, M232A, and E235G on gating kinetics. A and C, normalized activation and deactivation current tracings of WT and M232A channels at −100 mV. Gating kinetics of M232A channels were drastically slowed. Summary of the effects of G231A, M232A, and E235G mutations on $\tau_{\text{act}}$ (B) and $\tau_{\text{deact}}$ (D). The same electrophysiological protocols as described in Fig. 3 were used for inducing activation and deactivation.
Fig. 6. Effects of D233A, S234A, V236A, and Y237A mutations on HCN1 gating. A, representative HCN1 currents through D233A, S234A, V236G, and Y237A channels. B, steady-state activation curves; C, gating kinetics ($\tau_{\text{act}}$, open symbols; $\tau_{\text{deact}}$, filled symbols) of the same channels shown in A. All of D233A, S234A, V236G, and Y237A mutants studied displayed gating properties not different from that of WT HCN1 channels. Data of D233A channels from (22) are shown for comparison.

Fig. 7. Effects of shortening the S3-S4 linker on HCN1 activation gating. A, representative currents through EVY235-237AAA channels. B, steady-state activation curves; C, gating kinetics of the same channel constructs from A. EVY235-237AAA produced significant depolarizing activation shifts compared with WT (dotted line), suggesting that the S3-S4 linker length influences activation gating.
HCN1 S3-S4 Linker Is a Helical Structure

Effects of Neutralizing the S3-S4 Residues Glu-229 and Lys-230 on Activation Gating—To determine experimentally the structural and functional role of the HCN1 S3-S4 linker, we first studied the effects of neutralizing Glu-229 and Lys-230 with alanine substitution (i.e. E229A and K230A correspond to a net charge change of +1 and −1 at these channel sites, respectively). Figs. 2 and 3 compare the gating properties of E229A, and K230A mutants with those of WT channel E229A produced a somewhat positive yet statistically insignificant shift (p > 0.05) in the steady-state activation relationship of HCN1 channels. Despite its opposite charge change (relative to E229A), K230A significantly shifted steady-state activation in the same depolarizing direction (p < 0.05). Although the slope factor of K230A was unchanged, the slope factor of E229A was smaller than WT (p < 0.05). These steady-state gating parameters are summarized in Table I.

To obtain kinetic insights into the gating changes associated with E229A and K230A channels, we next examined their activation and deactivation properties. Interestingly, E229A channels displayed drastically decelerated gating kinetics (Fig. 3, A and C). In contrast, activation and deactivation kinetics of K230A channels were significantly accelerated (Figs. 3, B and C). These kinetic changes were accompanied by a depolarizing shift of the peak of their voltage dependence concomitant with the corresponding rightward shift of the steady-state activation midpoint (cf. Fig. 2B). Collectively, these observations are consistent with the notion that the S3-S4 linker is functionally important for HCN1 gating.

Mutations of Gly-231, Met-232, and Glu-235 but Not Residues 233–234 and 236–237 Altered Gating—Because these results were encouraging, we proceeded to study the effects of replacing all the remaining S3-S4 residues individually by alanine (except glycine for residue Glu-235 because we had previously studied E225A (22); see Table I). The mutation M232A led to a significant depolarizing activation shift (Fig. 4) with significantly slowed gating kinetics (up to 3- and 1.3-fold increase in $\tau_{act}$ and $\tau_{deact}$, respectively) whose peaks of voltage dependence were also displaced in the same positive direction (Fig. 5). As anticipated from our recently published results (22) on various charge-altering Glu-235 mutations (such as Asp, Ala, Pro, His, Lys, and Arg), the charge-neutralized glycine substitution E235G (charge change = +1) also shifted the steady-state activation curve in the depolarizing direction without altering the slope factor (Fig. 4). The kinetic changes associated with this mutation were largely insignificant except for the range of −70 to −40 mV where the peaks of voltage dependence of activation and deactivation were positively shifted (Fig. 5). The gating phenotypes of G231A channels were similar to E235G (Fig. 4) except their deactivation kinetics were somewhat hastened over the entire voltage range examined (Fig. 5). Unlike the above-mentioned S3-S4 sites (i.e. 229, 230, 231, 232, and 235), mutating Asp-233, Ser-234, Val-236, and Tyr-237 to alanine led to gating properties indistinguishable from those of WT (p > 0.05; Fig. 6). These observations indicate that the functional changes observed with E229A, K230A, G231A, M232A, and E235G substitutions were site-specific. Our results are further summarized in Fig. 8A by plotting the absolute energetic changes ($\Delta G$) associated with the activation shifts resulting from various single S3-S4 mutations relative to WT HCN1.

S3-S4 Deletion Mutants Decelerated Gating Kinetics—To explore further the role of the S3-S4 linker, we next investigated the effects of shortening the linker. We created the deletion (MTSEA). One possibility that potentially explains these observations is that the HCN1 S3-S4 linker (defined as residues 229–237 here) has a helical secondary structure such that residues 233 and 235 face different sides of the helix thereby exerting distinct functional effects when mutated (22). To test this hypothesis, we first evaluated the secondary conformation of the S3-S4 linker using the modeling algorithms PROF and SSpro2 (see “Experimental Procedures”). Fig. 1 summarizes the results generated from PROF and SSpro2 for this channel region. According to PROF, the S3-S4 residues 234–237 are likely to conform a helical secondary structure (confidence levels >7 on a scale of 10 for these positions) with residues 229–233 being a random coil (note that the confidence levels for residues 232 and 233, however, were comparatively low) that connects the S3-S4 linker to S3. This prediction by PROF is in complete accordance with the notion that the S3-S4 linker, or part of it, is helical. In fact, similar predictions were also generated by the algorithms SSpro2 and APSSP2 (Fig. 1). Motivated by these modeling results, we next sought to experimentally test, and refine where necessary, the above structural predictions. Indeed, we had employed previously a similar approach to successfully identify backbone helices in the Na+ channel pore (28).

Fig. 8. The HCN1 S3-S4 linker as a helical structure. A, energetic changes ($\Delta G$) of steady-state activation shifts associated with various S3-S4 substitutions relative to WT were plotted against their positions. The cutoff was set to be 1 kcal/mol (see “Experimental Procedures”). B, helical wheel representation of HCN1 S3-S4 residues. In this model, Met-232, Gly-235, and Gly-231 are on the same side of the helix, opposite that of the functionally non-consequential Asp-233, Ser-234, Val-236, and Tyr-237 to alanine led to gating properties indistinguishable from those of WT ($p < 0.05$). These steady-state gating parameters are summarized in Table I.

To obtain kinetic insights into the gating changes associated with E229A and K230A channels, we next examined their activation and deactivation properties. Interestingly, E229A channels displayed drastically decelerated gating kinetics (Fig. 3, A and C). In contrast, activation and deactivation kinetics of K230A channels were significantly accelerated (Figs. 3, B and C). These kinetic changes were accompanied by a depolarizing shift of the peak of their voltage dependence concomitant with the corresponding rightward shift of the steady-state activation midpoint (cf. Fig. 2B). Collectively, these observations are consistent with the notion that the S3-S4 linker is functionally important for HCN1 gating.

Mutations of Gly-231, Met-232, and Glu-235 but Not Residues 233–234 and 236–237 Altered Gating—Because these results were encouraging, we proceeded to study the effects of replacing all the remaining S3-S4 residues individually by alanine (except glycine for residue Glu-235 because we had previously studied E225A (22); see Table I). The mutation M232A led to a significant depolarizing activation shift (Fig. 4) with significantly slowed gating kinetics (up to 3- and 1.3-fold increase in $\tau_{act}$ and $\tau_{deact}$, respectively) whose peaks of voltage dependence were also displaced in the same positive direction (Fig. 5). As anticipated from our recently published results (22) on various charge-altering Glu-235 mutations (such as Asp, Ala, Pro, His, Lys, and Arg), the charge-neutralized glycine substitution E235G (charge change = +1) also shifted the steady-state activation curve in the depolarizing direction without altering the slope factor (Fig. 4). The kinetic changes associated with this mutation were largely insignificant except for the range of −70 to −40 mV where the peaks of voltage dependence of activation and deactivation were positively shifted (Fig. 5). The gating phenotypes of G231A channels were similar to E235G (Fig. 4) except their deactivation kinetics were somewhat hastened over the entire voltage range examined (Fig. 5). Unlike the above-mentioned S3-S4 sites (i.e. 229, 230, 231, 232, and 235), mutating Asp-233, Ser-234, Val-236, and Tyr-237 to alanine led to gating properties indistinguishable from those of WT (p > 0.05; Fig. 6). These observations indicate that the functional changes observed with E229A, K230A, G231A, M232A, and E235G substitutions were site-specific. Our results are further summarized in Fig. 8A by plotting the absolute energetic changes ($\Delta G$) associated with the activation shifts resulting from various single S3-S4 mutations relative to WT HCN1.
constructs EVY235–237ΔΔ, whose S3-S4 residues Glu-235, Val-236, and Tyr-237 had been simultaneously removed. Fig. 7 shows that the activation midpoint of EVY235–237ΔΔΔ channels was significantly displaced in the depolarizing direction by −22 mV. Both activation and deactivation kinetics were decelerated with their voltage dependence shifted in the concomitant depolarizing direction. Given that the single mutants V236A and Y237A did not alter gating and that neutralizing Glu-235 consistently produced only about +7 mV of activation shift (cf. Fig. 4) (22), these changes in gating properties observed with EVY235–237ΔΔΔ channels could not be sufficiently explained by the elimination of the anionic residue at position 235. The results suggest that both the length and composition of the S3-S4 linker influence HCN1 gating.

DISCUSSION

S3-S4 Linker Is a Gating Determinant—Recently, we reported (22) that the anionic S3-S4 residue Glu-235 of HCN1 channels influences activation by acting as a surface charge. However, the role of other S3-S4 linker residues in gating had not been evaluated. In the present study, we identified four novel determinants within the S3-S4 linker, namely Glu-229, Lys-230, Gly-231, and Met-232, that when substituted significantly altered the gating phenotypes of HCN1 channels. These data support the notion that the S3-S4 linker of HCN1 channels is a gating determinant. Indeed, previous studies (29–33) have shown that the extracellular S3-S4 linker is also a determinant of activation in various depolarization-activated K+ and Ca2+ channels (see below for detailed comparison).

A Refined Helical Model of the HCN1 S3-S4 Linker—Whereas Glu-229, Lys-230, Gly-231, Met-232, and Glu-235 when mutated prominently influence activation gating, none of the Asp-233, Ser-234, Val-236, and Tyr-237 mutations studied altered HCN gating properties (Fig. 8A). These observations argue that perturbations of gating by S3-S4 mutations were site-specific. For instance, residues flanking Glu-235 (i.e. Ser-234 and Val-236) when substituted by alanine did not affect activation properties. These results suggest that the native side chain orientation of Glu-235 and thus its functional role are not disrupted when these nearby residues are replaced. Instead, residues Gly-231 and Met-232, which are farther away from Glu-235 in the primary sequence, altered gating when mutated. Interestingly, these observations were most consistent with the prediction by the modeling algorithms SSpro2 and PROF that part of the HCN1 S3-S4 linker is a helical structure. Fig. 8B shows a refined model of such prediction based on our experimental observations. The model is essentially the same as that generated by SSpro2 and PROF except that residues 231–233, whose predicted confidence levels were comparatively low, are now included as part of the S3-S4 helix. Such helical arrangement of residues 231–237 places the functionally important Gly-231, Met-232, and Glu-235 clustered on one side of the proposed helix but the non-consequential residues (Asp-233, Ser-234, Val-236, and Tyr-237) facing the other. Glu-229 and Lys-230, as modeled by SSPRO and PROF, plausibly form a coil that connects the S3-S4 linker to S3. When the physical properties (such as charge and flexibility) of this “connector” are altered, changes in the orientation of the S3-S4 helix and subsequently changes in gating properties result. Indeed, the hydrophilic properties of the side chains of residues 229 and 230 further raise the intriguing possibility that they are exposed to the extracellular milieu. Further experiments are needed to assess their side chain accessibility as well as to investigate whether these charged residues, like Glu-235, have direct electrostatic influences on HCN gating.

Alternatively, it is possible that residues 235–237 are indeed part of the S4 segment and that the actual S3-S4 linker consists of residues 229–234 only. Such arrangement also readily explains the electrostatic influences of Glu-235 (with this anionic residue as part of the voltage-sensing S4) on HCN activation (22). Perhaps, the side chain of Asp-233 faces away from S4 thereby minimizing its effects on gating. However, alterations of the physical properties of the S3-S4 linker, as discussed above, could lead to subsequent changes in gating. This second possibility is equally attractive particularly given that serine (Ser-234) has a high probability of bending helical structures of proteins (34). Disregarding the precise location of this helix, which remains to be defined, it is apparent that part of the stretch of residues spanning from Glu-229 to Tyr-237 forms a helical structure. Taken together, our present study lays the ground work required to further address this question.

Structural and Mechanistic Insights: HCN and Kv Channels Are Analogous—The helical secondary conformation of the HCN S3-S4 linker (assuming that our conclusion with HCN1 can be generalized to the other isoforms) described above is analogous to that previously proposed for the S3-S4 linker of Kv channels (35). Interestingly, our previous finding that charge neutralization or charge reversal of the anionic S3-S4 residue Glu-235 attenuated the gating responses of HCN1 channels to the charge-screening effects of external Mg2+ (22) also strongly mirrors the abolition of surface shielding effects of H+ observed with the Shaker mutant whose S3-S4 linker (including the acidic residues Glu-332 to Glu-335 and Asp-336) had been deleted (32). Furthermore, our present finding that HCN gating kinetics decelerate when the S3-S4 linker was shortened is also analogous to previous experiments with Shaker channels demonstrating that shortening the S3-S4 linker slows activation kinetics (31). Taken together, it is probable that the HCN S3-S4 linker, similar to that of Shaker (30–32), forms a protein-lined dielectric vestibule surrounding the N-terminal portion of S4 and thereby influences gating.

By using cysteine scanning mutagenesis, Larsson and colleagues (19) provided evidence that the voltage-sensing mechanisms of HCN and Kv channels are conserved. The same as Kv channels, the S4 domain of HCN channels move outward and inward during depolarization and hyperpolarization, respectively. It is possible that the S3-S4 helical face consisting of Gly-231, Met-232, and Glu-235 is in contact with the S4 voltage sensor, thereby acting as a “lubricant” for its translocation during the process of gating. Indeed, S3-S4 residues could alter the energy barriers separating the channel transitions required for channel openings, thereby providing a potential explanation for the altered gating kinetics observed with several of our S3-S4 constructs (although there is no general rule for predicting the effect of a mutation at these critical sites as was the case of many K channel studies). However, we are unable to conclude whether the S3-S4 linker undergoes major conformational changes during gating based on our present finding. Nevertheless, emerging lines of evidence appear to suggest that although HCN and Kv channels have distinctive activation profiles, their gating machineries appear to share significant structural and functional similarities (16–18, 20–22, 36–38).

Insights into Isoform-specific Gating Properties of HCN Channels—Our results and those of others (20–22, 36, 40) suggest that regions other than the S4 domain of HCN channels also prominently modulate gating. These regions include the P-loop, the extracellular S1-S2, S3-S4, S5-P linkers, as well as the cytoplasmic S4-S5 and S6-CNBD linkers. Because different HCN isoforms are known to exhibit distinct gating behaviors (8, 10, 41), it is likely that variant residues within these linkers underlie such isoform-specific gating differences. Consistent with this notion, chimeric studies of HCN1 and HCN4
channels, whose gating kinetics differ by ~40-fold, suggest that amino acid differences within the S1-S2 linker account for some but not all of the differences in activation kinetics between these isoforms (40). In addition, the slower kinetics of HCN2 can be partially attributed to the Ala to Arg pore variant at HCN1 position 352 (also an arginine in the slow HCN4) (21). Therefore, it is apparent that discrete sequence differences with small but definitive additive individual effects underlie isoform-specific gating properties. Given that the HCN1 positions 230–232 influence gating and the equivalent sites in HCN2–4 are also variant residues (e.g. Met-232 of HCN1, whose equivalent residues in HCN2 and 4 are isoleucine, is of particular interest since the mutation M232A significantly slows gating kinetics, thereby rendering HCN1 more HCN2- and HCN4-like; cf. Fig. 1), these differences are prime candidates for exploring the molecular basis of isoform-specific HCN gating.

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