Dissecting the Structural and Functional Roles of the S3-S4 Linker of Pacemaker (Hyperpolarization-activated Cyclic Nucleotide-modulated) Channels by Systematic Length Alterations*

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**I** or **I**h, a key player in neuronal and cardiac pacing, is encoded by the hyperpolarization-activated cyclic nucleotide-modulated (HCN) channel gene family. We have recently reported that the S3-S4 linker (i.e. residues 229EKMDSEVY237 of HCN1) prominently influences the activation phenotypes of HCN channels and that part of the linker may conform a secondary helical structure. Here we further dissected the structural and functional roles of this linker by systematic alterations of its length. In contrast to voltage-gated K**v** channels, complete deletion of the S3-S4 linker (Δ229–237) did not produce functional channels. Similarly, the deletions Δ229–234, Δ232–234, and Δ232–237 also abolished normal current activity. Interestingly, Δ229–231, Δ233–237, Δ234–237, Δ235–237, Δ229–231/Δ233–237, Δ229–231/Δ234–237, and Δ229–231/Δ235–237 all yielded robust hyperpolarization-activated inward currents, indicating that loss-of-function caused by deletion could be rescued by keeping the single functionally important residue Met232 alone. Whereas shortening the linker by deletion generally shifted steady-state activation in the depolarizing direction (e.g. ΔV1/2 of Δ229–231, Δ233–237, Δ235–237 > +10 mV relative to wild type), linker prolongation by duplicating the entire linker (Dup229–237) or by glutamine insertion (InsQ233Q, InsQ235Q and InsQQ233QQQ, or InsQQQ233QQQ produced length-dependent progressive hyperpolarizing activation shifts (−35 mV < ΔV1/2 < −4 mV). Based on these results, we conclude that only Met232 is prerequisite for channels to function, but the length and other constituents of the S3-S4 linker shape the ultimate activation phenotype. Our results also highlight several evolutionary similarities and differences between HCN and voltage-gated K**v** channels. Manipulations of the S3-S4 linker length may provide a flexible approach to customize HCN gating for engineering electrically active cells (such as stem cell-derived neuronal and cardiac pacemakers) for gene- and cell-based therapies.

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The abbreviations used are: HCN, hyperpolarization-activated cyclic nucleotide-modulated; K**v**, voltage-gated K**v**; WT, wild type; GFP, green fluorescent protein; **I**f, funny current; **I**h, hyperpolarization-activated, cation-selective current.

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Consequences of Altering S3-S4 Linker Length of HCN Channel

EXPERIMENTAL PROCEDURES

Molecular Biology and Heterologous Expression—Murine HCN1 (kindly provided by Drs. Siegelbaum and Santoro) was subcloned into the pGHE expression vector (5). Mutations were created using PCR with overlapping mutagenic primers. The desired mutations were confirmed by DNA sequencing. cRNA was transcribed from NheI-linearized DNA using T7 RNA polymerase (Promega, Madison, WI). HCN1 and other channel constructs investigated in the present study. Functional and nonfunctional constructs are labeled green and purple, respectively. Met232 is highlighted in red.

Electrophysiology—Two-electrode voltage clamp recordings were performed at room temperature using a Warner OC-725B amplifier.

RESULTS

FIG. 1. Putative transmembrane topology of HCN1. A, the six putative transmembrane segments (S1–S6) of a monomeric HCN1 subunit. The S3-S4 linker is thickened. B, S3-S4 linker sequences of WT HCN1 and other channel constructs investigated in the present study. Functional and nonfunctional constructs are labeled green and purple, respectively. Met232 is highlighted in red.

Discussion. Emergent insights from these results may even lead to a novel genetic approach for fine tuning the activity of cells such as electrically active neuronal and cardiac pacemakers where HCN channels are central to their physiological functions. A preliminary report of this work has appeared (15).

m_n = 1/(1 + exp((V_h - V_{1/2})/k))

where $V_h$ 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.

For simplicity, the time constants for activation ($\tau_{act}$) and deactivation ($\tau_{deact}$) were estimated by fitting macroscopic tail currents with monoexponential function although sigmoidicity with an initial delay was observed before the onset of HCN1 currents at some voltages. The mechanism underlying such complex kinetic behavior of HCN channels is not understood. Further analysis using multiple exponential components is beyond the scope of this work.

The data are presented as the means ± S.E. Statistical significance was determined for individual data points and fitting parameters using the Marquardt-Levenberg algorithm in a nonlinear least squares procedure.
did not lead to the expression of functional currents even after hyperpolarization to $-140$ mV. Similar to $\Delta 229-237$, the deletions $\Delta 229-234$, $\Delta 232-234$, and $\Delta 232-237$ also completely abolished normal current activity. To investigate whether this loss of channel function caused by linker deletion was resulted from folding, trafficking, or gating defects, we created the fusion constructs HCN1-GFP and HCN1$\Delta 232-234$-GFP, which contained the green fluorescent protein (GFP) fused to the C termini of WT and $\Delta 232-234$ HCN1 channels, respectively. Fig. 3A shows that GFP fluorescence signals of both HCN1-GFP and HCN1$\Delta 232-234$-GFP channels were localized to the membrane surface, in a manner similar to that of WT channel, suggesting the deletion did not cause folding or trafficking defects. B, steady-state $I-V$ relationships of S3-S4 deletion constructs. The steady-state $I-V$ relationship was determined by plotting the HCN1 currents measured at the end of the 3-s pulse. Inset, electrophysiological protocol used to elicit currents. The data shown are the means $\pm$ S.E.
length-dependent progressive shifts in the linker residues present (see Fig. 8, ground also appeared to positively correlate to the number of – Similarly, restoring the segment containing 229–237, producing a depolarizing shift. Although the steady-state activation curves for WT, Δ229–231/233–237, Δ229–231/234–237, and Δ229–231 normalized to the maximum current recorded. Trace a, –120 mV; trace b, –100 mV; trace c, –80 mV. B, steady-state activation curves for WT, Δ229–231/233–237, Δ229–231/234–237, Δ229–231/235–237, and Δ229–231. Increasing the S3-S4 linker length as observed from these constructs caused sequential depolarizing activation shifts (see text and Fig. 8).

Effects of Shortening the S3-S4 Linker Length on HCN1 Activation—The different activation thresholds of our deletion S3-S4 constructs that were functional, as evident from their steady-state current-voltage (I-V) relationships (Fig. 3), hint that their activation properties might have been altered. To explore in detail the functional effects of linker shortening, we next examined steady-state activation properties (Fig. 4). A, Δ229–231/Δ233–237 (i.e. only Met232 remained) significantly shifted steady-state activation in the hyperpolarizing direction by ~30 mV (versus deletion of the entire linker rendered channels nonfunctional). Interestingly, gradual extension of the linker C-terminal to Met232 caused progressive depolarizing activation shifts (i.e. Δ229–231/Δ233–237 < Δ229–231/Δ234–237 < Δ229–231/Δ235–237 < Δ229–231; p < 0.05). These length-dependent progressive shifts in the Δ229–231 background also appeared to positively correlate to the number of linker residues present (see Fig. 8, solid squares; r = 0.99). Similarly, restoring the segment containing 229–231 N-terminal to Met232 (i.e. Δ233–237) followed the same trend by producing a depolarizing shift. Although the steady-state activation curves of Δ234–237 and Δ235–237 were also positively shifted relative to WT (which contained the complete string of 231–237 residues), the depolarizing effect appeared to have saturated and started to decline when the linker was further prolonged (Fig. 8; also see later). The steady-state gating parameters of our deletion constructs are summarized in Table I.

TABLE I

| Channel | $V_{1/2}$ | $k$ | $N$ |
|---------|----------|-----|-----|
| WT      | –72.4 ± 1.4 | 9.8 ± 0.7 | 15  |
| Δ229–231| –61.2 ± 1.8a | 9.5 ± 0.7 | 5   |
| Δ229–234| NEa       | 5   |     |
| Δ229–237| NE         | 5   |     |
| Δ229–231/Δ233–237 | –98.8 ± 1.6a | 14.5 ± 0.5a | 5  |
| Δ229–231/Δ234–237 | –88.9 ± 0.4a | 12.4 ± 0.5 | 10  |
| Δ229–231/Δ235–237 | –66.9 ± 3.6 | 9.3 ± 0.5 | 10  |
| Δ232–234| NE         | 5   |     |
| Δ232–237| NE         | 5   |     |
| Δ233–237| –51.1 ± 1.3a | 11.6 ± 0.7 | 7   |
| Δ234–237| –65.4 ± 2.2 | 14.1 ± 0.9a | 15 |
| Δ235–237| –53.3 ± 2.3a | 8.1 ± 0.5 | 5   |
| InsQQ233Q | –76.2 ± 1.7 | 12.4 ± 0.7 | 9   |
| InsQQ233QQQ | –81.9 ± 1.5a | 10.8 ± 0.6 | 10  |
| InsQQ233QQQ | –92.2 ± 0.7a | 12.7 ± 0.9 | 5   |
| 237InsQQQ | –82.6 ± 0.95a | 13.4 ± 0.9 | 6   |
| Dup229–232| –83.8 ± 1.6a | 11.6 ± 0.7 | 6   |
| Dup229–237| –104.6 ± 0.5a | 10.8 ± 0.2 | 9   |

*a p < 0.05 versus WT values; one-way analysis of variance followed by Tukey HSD post-hoc test.

b NE, not expressed.
length of the S3-S4 linker, in addition to its composition, critically influences HCN1 gating.

**Extensive Prolongation of the S3-S4 Linker Produced Opposite Hyperpolarizing Activation Shifts**—To complement the above linker deletion experiments, we also studied the effect of prolonging the S3-S4 linker on HCN1 activation gating. Initially, we inserted glutamines to flank Asp233 to create the insertion constructs InsQ233Q, InsQQ233QQ, and InsQQQ233QQQ. Asp233 was chosen because it faces the opposite side of the critical GME231–235 cluster, and its substitutions do not affect functions (14). Furthermore, for instance, InsQQQ233QQQ would be expected to displace the original glutamate at position 235 with glutamine without altering the overall linker charge. The effect of Ins237QQQ was also tested. Fig. 5 shows that as the linker prolonged, HCN1 activation progressively shifted in the hyperpolarizing direction \( (p < 0.05) \).

To further test whether the hyperpolarizing shifts resulted from linker prolongation or simply the insertion of glutamines, we duplicated the linker segment containing residues 229–232 as well as the entire linker to create Dup229–232 and Dup229–237, respectively. As shown in Fig. 6, the steady-state activation curves of both duplication constructs were also shifted in the negative direction. In fact, the extent of the shifts was also well correlated to the linker length (see Fig. 8, open circles; \( r = 0.98 \) after saturation of the depolarizing effect). Table I summarizes the steady-state gating parameters of these glutamine and duplication constructs.

**S3-S4 Linker Length Alterations Generally Led to Decelerated Gating Kinetics**—To obtain kinetic insights into the gating changes associated with our deletion and insertion constructs, we studied their activation and deactivation properties. Fig. 7 shows that InsQ233Q, InsQQ233QQ, and InsQQQ233QQQ displayed drastically decelerated activation kinetics (up to 15-fold of InsQQQ233QQQ at \(-70 \text{ mV}\)). Similarly, deactivation kinetics of the same channels were also significantly slowed. Interestingly, linker shortening \( \Delta229–231, \Delta229–231/\Delta234–237, \Delta233–237, \text{ and } \Delta235–237 \) also produced decelerating effects on activation and deactivation of HCN1 channels, except for \( \Delta234–237 \), whose kinetic properties closely resemble those of WT. Unlike steady-state activation properties, however, there was no obvious correlation between the linker length and the decelerating effects. Therefore, changing the linker length
slowed both the activation and deactivation kinetics of the HCN1 channels (Fig. 7).

**DISCUSSION**

Previous studies of K channels have shown that the length of their S3-S4 linker is a determinant of gating kinetics (10–12). Complete deletion of S3-S4 linker in the Kv channel still yielded robust K current (11), suggesting that the linker probably does not participate in large conformational changes during channel activation (11, 12). This in turn implies that during activation of Kv channels, either the S4 voltage sensor moves only a short distance or the S3-S4 linker can move along with S4 (cf. paddle model in Ref. 16; see below). Despite these studies of Kv channels, the role of the S3-S4 linker length in HCN channel functions has not been fully elucidated. Our recent report has suggested that shortening the linker in deletion mutant Δ235–237 caused depolarizing activation shift and slowed kinetics that could not be explained by removing the charge at position 235 alone (13, 14). Given HCN and Kv channels are structurally analogous, this finding motivated us to investigate in detail the role of the S3-S4 linker length in HCN functions. Indeed, we found in the present study that both the length and composition of the S3-S4 linker prominently influence HCN gating.

The Length and Composition of the S3-S4 Linker: Structural and Mechanistic Insights into the Process of HCN Activation—S3-S4 linker, directly tethered to the S4 voltage sensor, may influence the energy barriers that separate the channel transitions required for channel openings by undergoing a series of conformational changes during the gating process. Our present study shows that shortening and prolonging the S3-S4 linker generally shifted the steady-state activation curves (Fig. 6). Consistent with the glutamine insertion constructs, the steady-state activation curves of the duplication constructs were also negative shifted. Activation curve of Dup229–237 was more hyperpolarized than that of Dup229–232.
changes in gating kinetic while having relatively modest alterations in their steady-state activation properties. Perhaps changing the linker length alters the transitions among different channel conformations that occur during the process of activation. Indeed, the linker may either affect the rate at which S4 voltage sensor responds to the change in membrane potential or affect conformational changes that occur after S4 has responded to the changes in membrane potential. Disregarding the sequence, these results hint at a possible role of the S3-S4 linker in establishing the time scale of HCN channel activation, as previously proposed for L-type Ca\(^{2+}\) channels (17).

Interestingly, Δ234–237 nearly restores normal HCN1 gating properties with \(V_{\text{1/2}}\) activation and deactivation kinetics indistinguishable from those of WT. Based on our previously proposed model (i.e. residues 231–237 conform a helical arrangement with Glu\(^{229}\) and Lys\(^{230}\) forming a coil structure that connects the S3-S4 linker to S3) (14), Δ234–237 is predicted to contain one helical turn. Coincidentally, a S3-S4 linker consisting of only 3 amino acids (i.e. one helical turn) is also sufficient to restore WT activation phenotype for Shaker K\(^+\) channels (12). Although \(V_{\text{1/2}}\) of Δ234–237 was statistically indistinguishable from that of WT (\(p > 0.05\)), activation was modestly shifted in the depolarizing direction, presumably as a result of the deletion of Glu\(^{235}\), which is known to influence activation gating by acting as a surface charge (13).

Another feature of the HCN1 S3-S4 linker is that Met\(^{232}\) but not other linker residues is prerequisite for channels to function. We have previously demonstrated that alanine substitution of residue 232 significantly altered gating (14). When

Fig. 7. Effects of prolonging (top panels) and shortening (bottom panels) the S3-S4 linker on activation (\(\tau_{\text{act}}\)) (A) and deactivation (\(\tau_{\text{deact}}\)) (B) kinetics. The electrophysiological protocols used for inducing activation and deactivation are displayed.
substituted by an alanine (i.e. M232A), activation is shifted in the depolarizing direction, and gating kinetics are decelerated. Our present results further suggest that the presence of Met232 may be critical for maintaining the structural integrity of the S4 voltage sensor. Consistent with the latter possibility, non-functional constructs such as Δ229–234, Δ229–237, Δ232–234, and Δ232–237 have their N-terminal S4 segments (residues 238–240) changed from the original helical conformation to a coiled structure based on the predictions generated by the modeling algorithm S3pro2 (data not shown). In contrast, constructs that express robust currents (e.g. Δ229–231, Δ234–237, and S4QQQ233QQQ) have intact helical not different from that of WT. Alternatively, it is also possible that without Met232 S4 can no longer move properly in response to voltage changes or its movements cannot lead to subsequent channel openings. Our present data do not allow us to distinguish between the two possibilities. However, our confocal experiments do not support a role of Met232 in the synthesis, maturation, or assembly of functional HCN1 channels because the expression of Δ232–234 channels appears to localize on the membrane surface.

Our observations with Δ229–231/Δ233–237, Δ229–231/Δ234–237, and Δ229–231/Δ235–237 also suggest that even very short S3-S4 linkers enable sufficient displacements of the S4 voltage sensor that are required for HCN channel opening. Several models are available for describing how the S4 transmembrane domain moves during activation of Kc channels. According to one, the S4 segment moves across the membrane as a sliding helix during activation perpendicular to the lipid bilayer with a transfer of at least 10 amino acid residues across the plasma membrane (18, 19). Such a large movement of S4 would be constrained by the S3-S4 linker length if S3 is immobile relative to S4. At first glance, our finding that the presence of Met232 alone is sufficient for channels to gate does not appear to favor this model. However, it is possible that the S3 and S4 segments move simultaneously during HCN gating, as predicted from the paddle model (16). Further studies are needed to test whether this gating model also applies to HCN channels.

Evolutionary Similarities and Differences between HCN and K Channels—As mentioned, HCN and K channels are structurally homologous, although they are functionally distinctive. Our previous studies have already identified several structural and functional similarities of these two oppositely gated channels (6, 7, 13, 14, 20). For instance, the S3-S4 linkers of both channels appear to be partly helical (12, 14), neutralization of the anionic S3-S4 residue Glu235 attenuated the gating responses of HCN channels to the charge-screening effects of external Mg2+ (13) in a manner similar to the abolition of surface shielding effects of H+ observed with the Shaker mutant whose acidic residues (i.e. Glu333/Glu334/Asp336) within the S3-S4 linker had been deleted (21), etc. Our present results further demonstrated that, like K channels, the S3-S4 linker length of HCN channels also prominently influence activation gating. Despite these similarities, noticeable differences between HCN and Kc channels also exist. For instance, complete deletion of the S3-S4 linker rendered HCN channels nonfunctional, at least over the voltages examined, whereas Kc channels remained fully functional even after removal of the entire S3-S4 linker (11).

Implications for Tissue Engineering—Recently, our laboratory has shown that lentivirus-mediated gene transfer of HCN1-encoded pacemaker channels can modify the beating rate of spontaneously contracting human embryoid bodies derived from pluripotent human embryonic stem cells (22). In a separate study, we demonstrated that pacing can be fine tuned by manipulating the biophysical properties of Iκ conductance, activation threshold, gating kinetics, or their combinations. Given that mutations introduced into S4 often drastically shift activation (by ≈25mV) (8), manipulations of the S3-S4 linker length may provide a flexible way to customize HCN gating for fine tuning the electrical activity of endogenous and engineered cells, such as cardiac and neuronal pacemakers (native or adult and embryonic stem cell-derived), where HCN channels play a pivotal role in their physiological functions for achieving various gene- and cell-based therapies.

Conclusions—In summary, we conclude that the S3-S4 linker residue Met232 is prerequisite for channels to function. However, other constituents and the length of the S3-S4 linker contribute to the ultimate activation phenotype. Our results also highlight several evolutionary similarities and differences between HCN and Kc channels. In combination with gene transfer or transgenesis techniques, the constructs described in this study may be useful for engineering rhythmic electrically active tissues.

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