Voltage-dependent Gating of Hyperpolarization-activated, Cyclic Nucleotide-gated Pacemaker Channels

MOLECULAR COUPLING BETWEEN THE S4–S5 AND C-LINKERS*

Niels Decher, Jun Chen‡, and Michael C. Sanguinetti§

From the Department of Physiology and Nora Eccles Harrison Cardiovascular Research and Training Institute, University of Utah, Salt Lake City, Utah 84112-5000

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Hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels have a transmembrane topology that is highly similar to voltage-gated K⁺ channels, yet HCN channels open in response to membrane hyperpolarization instead of depolarization. The structural basis for the “inverted” voltage dependence of HCN gating and how voltage sensing by the S1–S4 domains is coupled to the opening of the intracellular gate formed by the S6 domain are unknown. Coupling could arise from interaction between specific residues or entire transmembrane domains. We previously reported that the mutation of specific residues in the S4–S5 linker of HCN2 (i.e. Tyr-331 and Arg-339) prevented normal channel closure presumably by disruption of a crucial interaction with the activation gate. Here we hypothesized that the C-linker, a carboxyl terminus segment that connects S6 to the cyclic nucleotide binding domain, interacts with specific residues of the S4–S5 linker to mediate coupling. The recently solved structure of the C-linker of HCN2 indicates that an α-helix (the A'-helix) is located near the end of each S6 domain, the presumed location of the activation gate. Ala-scanning mutagenesis of the end of S6 and the A'-helix identified five residues that were important for normal gating as mutations disrupted channel closure. However, partial deletion of the C-linker indicated that the presence of only two of these residues was required for normal coupling. Further mutation analyses suggested that a specific electrostatic interaction between Arg-339 of the S4–S5 linker and Asp-443 of the C-linker stabilizes the closed state and thus participates in the coupling of voltage sensing and activation gating in HCN channels.

Biophysical experiments and the recently described crystal structures of bacterial K⁺ channels have provided remarkable insights into the physical basis for the activation and inactivation gates, selective ion permeability, and voltage sensing mechanisms of ion channels (1–3). In contrast, the physical basis for voltage sensor-to-gate coupling remains a relative mystery (4). The coupling of voltage sensor movement to pore opening could involve interactions between specific residues or a more indirect interface interaction between transmembrane domains of the voltage sensor (S1–S4) and pore (S5–S6) regions. Equally puzzling is how the coupling mechanism results in opposite voltage dependence of activation for voltage-gated K⁺ (Kv) and hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels. HCN channels (5–7) conduct the pacemaker current of sinoatrial nodal cells in the heart (8). Opposite to Kv channels, HCN pacemaker channels are closed at potentials that are positive to ~30 mV and open when the membrane is hyperpolarized. This inverted scheme of channel activation does not have an obvious structural correlate. The general topology is conserved between HCN and Kv channels, including the presence of an S4 domain with basic amino acid residues located in every third position that contributes to the sensing of transmembrane potential and an activation gate formed by the crossing of the carboxyl-terminal end of the S6 helices (10).

How the movement of the voltage sensor is coupled to the opening and closing of the HCN or Kv channel pore is unknown, but it may involve global rearrangements of the S4, S5, and S6 transmembrane domains without the need for specific amino acid interactions (11). However, three recent studies (12–14) suggest that physical coupling may involve specific interactions. First, voltage-dependent gating of KcsA-Shaker chimera channels required two complementary domains, the S4–S5 linker and the carboxyl-terminal end of S6 (12). Second, channel closing is disrupted by a charge-reversing mutation of a single acidic residue (D540K) in the S4–S5 linker of HERG (13). D540K HERG channels open in response to either membrane depolarization or hyperpolarization, resulting in an inverted bell-shaped voltage dependence of activation; channels are nearly fully closed at a potential of ~90 mV. Hyperpolarization-activated opening of D540K HERG is specifically abolished by a second mutation of a single residue (Arg-665) located in the carboxyl-terminal end of S6 (14), suggesting that an electrostatic repulsion between Lys-540 and Arg-665 mediates the unusual gating phenotype. These findings indicate that only a minor change in channel architecture is required to introduce HCN-like channel gating into HERG. This may be more than mere coincidence because HCN channels are more closely related to HERG than to other Kv channels. Third, the S4–S5 linker also appears to be a critical component of the coupling mechanism that mediates the hyperpolarization-dependent opening of HCN channels. Ala-scanning mutagenesis of the S4–S5 linker in HCN2 revealed that three amino acids were especially critical for normal gating. Mutation of Tyr-331

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‡ Present address: Abbott Laboratories, Abbott Park, IL 60064.
§ To whom correspondence should be addressed: Nora Eccles Harrison Cardiovascular Research and Training Institute, University of Utah, 95 S 2000 E, Salt Lake City, UT 84112-5000, Tel.: 801-581-3058; Fax: 801-581-3128; E-mail: michael.sanguinetti@hmbg.utah.edu.

The abbreviations used are: Kv, voltage-gated K⁺; HCN, hyperpolarization-activated, cyclic nucleotide-gated; CNBD, cyclic nucleotide binding domain; WT, wild-type; ntHCN2, N-terminal truncated HCN2.
or Arg-339 (and to a lesser extent, Glu-324) disrupted channel closure (15).

Based on our findings with HERG, and KcA-Shaker chimeras by Lu et al. (12), we hypothesized that interactions between the S4–S5 linker and a domain near the intracellular activation gate contribute to channel opening and closing of HCN2 channels. We focused on the C-linker (Fig. 1) of HCN2 in our search for residues that might participate in coupling. The structure of the C-linker was recently solved by x-ray crystallography by Zagotta et al. (16) and is defined as the portion of the carboxyl terminus that connects the end of S6 with the cyclic nucleotide binding domain (CNBD).

Here we used Ala-scanning mutagenesis and voltage clamp of Xenopus laevis oocytes expressing mutant channels to identify specific residues within or near the C-linker of HCN2 that are important for normal channel closure. Our findings suggest that a salt bridge between Asp-443 of the C-linker and Arg-339 (and to a lesser extent, Glu-324) disrupted channel closure (15). The HCN2 channel cDNA was cloned from Marathon-Ready (Clontech) mouse brain cDNA into the pSP64T oocyte expression vector (17). To facilitate subcloning, the very GC-rich N-terminal portion of the HCN2 channel (encoding amino acids 2–130) was deleted. The wild-type (WT) N-terminal-truncated channel is referred to as ntHCN2. As described previously (17), the biophysical properties of ntHCN2 channels are similar to full-length HCN2 channels. All mutations were introduced into ntHCN2 cDNA as described (18). Restriction mapping and DNA sequencing of the PCR-amplified segment were used to confirm the presence of the desired mutation and the lack of extra mutations. cRNA for injection into oocytes was prepared with SP6 Capscribe (Roche Applied Science) after linearization with EcoRI. RNA quality and quantity were determined by gel electrophoresis and UV spectroscopy.

Stage IV and V X. laevis oocytes were isolated and injected (19, 20) with 5–25 ng of cRNA encoding WT or mutant HCN2 channels. Oocytes were cultured in Barth’s solution supplemented with 50 μg/ml gentamycin and 1 mM pyruvate at 18 °C for 1–3 days before use. Barth’s solution contained 88 mM NaCl, 1 mM KCl, 0.4 mM CaCl2, 0.33 mM Ca(NO3)2, 1 mM MgSO4, 2.4 mM NaHCO3, and 10 mM HEPES, pH 7.4. For voltage clamp experiments, oocytes were bathed in a modified ND96 solution containing 96 mM NaCl, 4 mM KCl, 1 mM MgCl2, 1 mM CaCl2, and 5 mM HEPES, pH 7.6. Currents were recorded at room temperature (23–25 °C) with standard two-microelectrode voltage clamp techniques (21). HCN2 channel currents were induced by hyperpolarizing pulses of 1–3 s, from a holding potential of −30 mV, a potential at which WT HCN2 channels were nearly completely closed. Instantaneous tail currents measured at −130 mV were corrected for leakage by subtracting the average value of the leak (less than −150 nA at −130 mV) recorded from a matched set of uninjected oocytes for each experiment. A normalized tail current amplitude (I) was plotted versus the test potential and fitted with a Boltzmann function, \( I = (1 - \min_P/[1 + \exp((V - V_{1/2})/k)] + \min_P, \) to obtain the voltage required for half-maximal activation (\( V_{1/2} \)), slope factor (k), and the minimum open probability (\( \min_P \)), defined as the minimum value of relative tail current (15). Data are expressed as mean ± S.E. (n = number of oocytes).

RESULTS

Identification of Residues in the C-linker Is Important for Normal Voltage-dependent Channel Gating—Ala-scanning mutagenesis of residues Ile-439 to Leu-442 of the S6 domain and age sensing and gating are mediated at least in part by specific interactions between residues in the S4–S5 linker and residues located near the activation gate.

EXPERIMENTAL PROCEDURES

The HCN2 channel cDNA was cloned from Marathon-Ready (Clontech) mouse brain cDNA into the pSP64T oocyte expression vector (17). To facilitate subcloning, the very GC-rich N-terminal portion of the HCN2 channel (encoding amino acids 2–130) was deleted. The wild-type (WT) N-terminal-truncated channel is referred to as ntHCN2. As described previously (17), the biophysical properties of ntHCN2 channels are similar to full-length HCN2 channels. All mutations were introduced into ntHCN2 cDNA as described (18). Restriction mapping and DNA sequencing of the PCR-amplified segment were used to confirm the presence of the desired mutation and the lack of extra mutations. cRNA for injection into oocytes was prepared with SP6 Capscribe (Roche Applied Science) after linearization with EcoRI. RNA quality and quantity were determined by gel electrophoresis and UV spectroscopy.

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RESULTS

Identification of Residues in the C-linker Is Important for Normal Voltage-dependent Channel Gating—Ala-scanning mutagenesis of residues Ile-439 to Leu-442 of the S6 domain and
Asp-443 to Glu-457 of the A’-helix of the C-linker (Fig. 1) was performed to determine the potential role of this region in HCN2 channel gating. The voltage dependence of activation for WT ntHCN2 and each Ala mutant was determined from currents elicited by 1-3-s hyperpolarizing pulses to test potentials ranging from −40 to −140 mV, applied in 10-mV increments. Currents evoked by a hyperpolarizing test pulse was characterized by an instantaneous component and a time-dependent component that activated with a time constant of ~350 ms at −120 mV. The rate of current activation was voltage-dependent, becoming faster at more hyperpolarized potentials (Fig. 2a).

Immediately after the test pulse, a second pulse to −130 mV was applied. The initial current elicited by the pulse to −130 mV was used to define min-Po, a measure of the extent of channel closure (15). Channels capable of complete closure have a min-Po of 0, whereas channels that are always open would have a min-Po of 1.0. For WT ntHCN2 channels, the average min-Po was 0.09 ± 0.01 (n = 12). Five mutant channels failed to close normally (S441A, D443A, R447A, Y449A, and Y453A (Fig. 2A)), whereas all the others had normal gating. The min-Po for all the mutant channels examined are plotted in Fig. 2b. The min-Po for the five channels with altered gating were 0.51 ± 0.02 for S441A (n = 15), 0.79 ± 0.01 for D443A (n = 9), 0.48 ± 0.01 for R447A (n = 10), 0.87 ± 0.01 for Y449A (n = 10), and 0.62 ± 0.02 for Y453A (n = 7). The voltage dependence of channel activation was assessed by plotting the instantaneous current measured at −130 mV immediately after each test pulse. The half-point (V1/2) and slope factor of WT ntHCN2 were −80.9 ± 0.5 mV and 8.1 ± 0.2 mV (n = 12), respectively. The voltage dependence for activation was altered by many but not all of the Ala mutations. A large shift in V1/2 (−15 mV) was induced by I439A, but this mutation caused only a minor increase in min-Po. The normalized voltage dependences for activation of the five mutant channels with the largest increase in min-Po are plotted in Fig. 2c. There was no apparent correlation between an increase in min-Po and the shift in V1/2 of the activation curve. The V1/2 values varied from −64 mV for Y453A to −96 mV for I439A (Table 1). The locations of four of the five residues identified in the Ala scan are indicated in the recently solved structure of the HCN2 C-linker (A’-F helices in Fig. 2d). In summary, Ala-scanning mutagenesis of the end of the S6 domain and the C-linker A’-helix identified five residues that have an important but unspecified role in stabilizing the closed state of HCN2 channels.

**Further Mutagenesis of C-linker Residues that Disrupt Channel Closure**—The five residues we identified with the Ala scan were subjected to further mutational analysis. One conservative and several non-conservative mutations were introduced at each site, and the effect on min-Po was determined. The substitution of Ser-441 with Thr did not substantially alter gating, but substitution with another polar amino acid lacking a −OH (Asn) or Cys disrupted channel closure and increased min-Po, similar to that observed for the Ala mutation (Fig. 3a). Mutation of Asp-443 to another acidic residue (Glu) had no appreciable effect on gating, but mutation to Asn, Cys, Tyr, or basic residues (Arg or Lys) increased min-Po to 0.7 or more (Fig. 3b). This finding suggests that Asp-443 might form a salt bridge with a nearby basic residue to affect activation gating. Substitution of Arg-447 with Tyr increased min-Po to 0.5, similar to R447A, whereas substitution with a conserved Lys resi...
Idue or Glu did not affect $P_o$ (Fig. 3c). Surprisingly, closure of R447D channels ($P_o = 0.02 \pm 0.01, n = 5$) was more complete than WT nHCN2 channels. The mutation of Tyr-449 to the hydrophobic residues Ala or Leu disrupted channel closure (Fig. 3d). Y449G and Y449D mutant channels closed properly. It is unclear why mutation to Ala but not Gly disrupted channel gating; also unusual were the gating behaviors of channels in which Tyr-449 was mutated to different aromatic residues. Y449F closed normally ($P_o = 0.17 \pm 0.006, n = 11$), but closure of Y449W channels was impaired ($P_o = 0.64 \pm 0.055, n = 16$) (Fig. 3d). Finally, Tyr-453 was subjected to further mutational analysis. Y453F, Y453W, and Y453D gated normally, whereas mutations to Gly, Ala, or Leu increased $P_o$ to approximately 0.6 (Fig. 3e). In summary, to maintain normal channel closure, a Ser or Thr amino acid was preferred at position 441, an acidic residue was required at position 443, a charged residue was preferred at position 447, and an aromatic residue was optimal at position 453; however, no consistent feature was found for position 449.

It has been proposed that HCN2 channels have two distinct open states, and occupancy of one state produces an instantaneous current that is insensitive to blockage by Cs$^+$, whereas occupancy of the other state produces the typical time- and voltage-dependent $I_n$ that is blocked by Cs$^+$ (22). Therefore, we determined the effect of Cs$^+$ on the instantaneous current induced by some of the C-linker mutations. Both the large instantaneous and smaller time-dependent currents conducted by WT, D443R, and Y449A HCN2 channels ($n = 3$ for each channel type) were completely blocked by the addition of 5 mM CsCl to the extracellular bath solution (data not shown).

**Intact C-linker Is Not Required for HCN2 Channel Gating**—Truncation of all but three residues (DSS, where D is Asp-443) of the carboxyl terminus of HCN2 channels led to the loss of functional expression (23). In contrast, the equivalent deletion in HCN1 is tolerated, and the channels retain the ability to generate hyperpolarization-activated currents (23). These findings are surprising considering the similarity in sequence of HCN1 and HCN2. Because truncation of the carboxyl terminus of HCN2 was not tolerated, we explored the effects of the mutation of residues important for the structural integrity of the C-linker. Cyclic AMP binding to the CNBD of HCN2 is likely to cause a conformational change in the C-linker that is coupled to an increased pore opening ultimately via the N-terminal end of the A‘-helix (16). Three Tyr residues (Tyr-476, -477, and -481) located in the B‘-helix of the C-linker were proposed (16) to form H-bonding interactions with specific residues in the C‘-helix of the neighboring subunit. Presumably, mutations that prevented this coupling would cause a negative shift in the voltage dependence of HCN2 activation, similar to the effect of the absence of cAMP binding to the CNBD. However, partial disruption of these interactions by mutation of single Tyr residues to Ala or the mutation of Pro-466 (to Val or Gln), located in the turn between the A‘- and B‘-helices, did not

**Fig. 4. Mutations in the B‘-helix of HCN2 do not alter channel gating.** a, currents are shown for the mutant channels P466Q and Y481A. b, $P_o$ values are shown of mutations studied in the B‘-helix. The voltage dependence of activation for these mutants was: P466Q, $V_0 = -92.6 \pm 1.7$ mV and $k = 16.7 \pm 0.6 (n = 11)$; P481A, $V_0 = -80.3 \pm 0.5$ mV and $k = 9.5 \pm 0.3 (n = 10)$; Y476A, $V_0 = -86.2 \pm 3.9$ mV and $k = 15.9 \pm 1.4 (n = 7)$; Y477A, $V_0 = -82.1 \pm 3.1$ mV and $k = 16.3 \pm 1.3 (n = 7)$; and Y481A, $V_0 = -85.4 \pm 2.9$ mV and $k = 18.1 \pm 0.5 (n = 6)$. c, ribbon diagram of the C-linker and the CNBD of HCN2 showing four of the residues (Asp-443, Arg-447, Tyr-449, and Tyr-453) identified in the Ala scan (white). Residues mutated in the B‘-helix are indicated in red (Pro-466) and yellow (Tyr-476, Tyr-477, and Tyr-481). d, ribbon diagram of C-linker (16) indicating the cutting sites used to delete residues Arg-447–480. The lower panel shows the currents conducted by this deletion mutant.

**Fig. 5. Interaction between Arg-339 of the S4–S5 linker and Asp-443 of the C-linker probed by analysis of double mutations.** a, single mutations were introduced into Arg-339 and Asp-443, and the currents were recorded using the same pulse protocol described in the legend of Fig. 2. b, R339D rescues gating disrupted by D443R or D443N mutations. Scale bars indicate 2 $\mu$A (or 0.5 $\mu$A for R339D/D443R currents) and 0.5 s. c, R339E and R339Q do not restore channel gating disrupted by charge-reversal mutations of Asp-443. d, $P_o$ values are shown for channels containing single or double mutations.
appreciably increase \( P_{\text{a}} \) (Fig. 4, a–c) or cause a shift \( V_{1/2} \) of the voltage dependence of activation (not shown).

We next determined the functional consequences of a partial deletion of the C-linker. We deleted 33 amino acids (Arg-447–480) of HCN2, a region that included most of the A'-helix and the entire B'-helices (Fig. 4d, upper panel). This deletion removed three of the residues identified in our Ala scan (Arg-447, Tyr-449, and Tyr-453), yet it did not shift the voltage dependence of activation \( (V_{1/2} = -85.0 \pm 1.9 \text{ mV}) \) and only mildly reduced channel closure \( (P_{\text{a}} = 0.12; \quad n = 13) \) (Fig. 4d, lower panel).

This finding indicates that although mutations of Arg-447, Tyr-449, and Tyr-453, yet it did not shift the voltage dependence of activation \( \quad (\text{not shown}) \).

**Table II**

| Single mutation | \( P_{\text{a}} \) values | n | Double mutation | \( P_{\text{a}} \) values | n |
|-----------------|--------------------------|---|-----------------|--------------------------|---|
| R339D           | 0.13 ± 0.02              | 10| R339D/S441A     | 0.23 ± 0.003             | 10|
| S441A           | 0.51 ± 0.02              | 15| R339D/Y453L     | 0.30 ± 0.01              | 8 |
| Y453L           | 0.67 ± 0.03              | 8 | R339D/Y331S     | 0.90 ± 0.01              | 5 |
| Y331S           | 0.78 ± 0.02              | 6 | R339D/D443Y     | 0.95 ± 0.01              | 5 |
| Y331D           | 0.93 ± 0.01              | 5 | R339D/D443K     | 0.93 ± 0.004             | 8 |
| D443Y           | 0.82 ± 0.01              | 5 | Y331D/D443Y     | 0.95 ± 0.01              | 5 |
| D443K           | 0.69 ± 0.01              | 8 | R339D/D443K     | 0.93 ± 0.004             | 8 |
| Y331R           | 0.63 ± 0.03              | 7 | Y331D/D443Y     | 0.95 ± 0.01              | 5 |
| R447Y           | 0.52 ± 0.01              | 20| Y331R/R447Y     | 0.82 ± 0.02              | 11|
| Y331K           | 0.78 ± 0.03              | 5 | Y331KY449D      | 0.55 ± 0.02              | 5 |
| Y469D           | 0.08 ± 0.001             | 15| Y331KY453D      | 0.83 ± 0.02              | 6 |
| Y453D           | 0.21 ± 0.01              | 3 | Y331KY453D      | 0.83 ± 0.02              | 6 |
Arg-339 forms a salt bridge with Asp-443 in wild-type HCN2 channels. We previously proposed an analogous role for an electrostatic interaction between Asp-540 of the S4–S5 linker and Arg-665 of the S6 domain in HERG (14). In addition to Arg-339, the mutation of Tyr-331 in the S4–S5 linker disrupted HCN2 channel closure (15). Does Tyr-331 also interact with a specific residue in the C-linker? We could find no evidence for an interaction between Tyr-331 and the specific residues identified by the Ala scan of the C-linker. Reciprocal mutations of Tyr-331 and Asp-443 or Arg-447 did not rescue function disrupted by single mutations (Table II), nor did double mutations to residues that could potentially form a salt bridge (i.e. Y331D/D443K) (Table II). A reciprocal mutation approach obviously could not be used to test for possible interaction of Tyr-331 with Tyr residues in the C-domain. Instead, mutation of Tyr-331 to Lys and Tyr-449 or Tyr-453 to Asp was examined in an attempt to induce formation of a salt bridge. However, these pairings also did not restore normal channel closure (Table II). Together, these findings support a key role for a specific electrostatic interaction between single residues in the S4–S5 linker (Arg-339) and the A’-helix of the C-linker (Asp-443).

**DISCUSSION**

We previously used Ala-scanning mutagenesis to identify several residues of the S4–S5 linker of HCN2 that are critical for normal channel gating and hypothesized that these residues might interact with the activation gate to mediate coupling of voltage sensing to channel opening (15). In the present study, Ala-scanning mutagenesis of the C-linker was used to identify residues near the putative activation gate (10, 26) that might participate with the S4–S5 linker in this coupling process. Based on our findings we propose that an interaction between Arg-339 in the S4–S5 linker and Asp-443 in the C-linker stabilizes the closed state of wild-type channels. An important role for electrostatic interactions in activation gating, which also involve specific residues in the C-linker, was previously proposed for cyclic nucleotide-gated channels. Replacement of three amino acids of the cone cyclic nucleotide-gated channel, including two Asp residues, with the corresponding residues of the olfactory channel caused an increase in cAMP efficacy (27). It is unknown whether these Asp residues of cyclic nucleotide-gated channels interact with charged residues in the S4–S5 linker as we propose for HCN channels.

The effects of single or double point mutations in the C-linker on HCN2 channel gating were quantified by changes in min-Po. Most of the findings (Fig. 3) were consistent with the strict physicochemical requirements of amino acid side groups to maintain normal voltage-dependent gating. For example, residue 443 must be an acidic amino acid; however, several findings were not consistent with such simple assumptions. First, the charge-reversal mutation R339E disrupted channel gating, but R339D did not. Second, charge-reversal mutations of Arg-447 permitted normal (R447E) or enhanced (R447D) channel closure, and deletion (along with 33 additional residues) had almost no effect on min-Po. Third, disrupted gating caused by D443R could be rescued by a combination with mutation of the nearby residue Arg-447 to Asp (D443R/R447D) (Fig. 7, last panel). The functional consequences of single or double charge-reversing mutations on channel gating can be reconciled if it is assumed that 1) a salt bridge must be formed between residue 339 of the S4–S5 linker and an oppositely charged residue at position 443 or 447 of the C-linker to maintain normal gating; 2) electrostatic repulsive forces can disrupt channel closure; and 3) the spacing between residues 339 and 443 is critical (i.e. functional effects of R339D versus R339E). These ideas are illustrated in Fig. 7 where an electrostatic attraction is indicated by a dotted line and repulsion is indicated by a double-headed arrow. Further studies using double Cys mutations and cross-linking agents or fluorescence resonance energy transfer are needed to further probe the importance of direct coupling between the S4–S5 and the C-linker of HCN channels.

In summary, we used Ala-scanning mutagenesis to identify residues located near the putative activation gate of HCN2 that were important for normal voltage-dependent gating. Functional analyses of a deletion mutant and channels with double mutations suggest a specific electrostatic interaction between Arg-339 of the S4–S5 linker and Asp-443 of the C-linker that stabilizes the closed state of the HCN2 channel. In response to membrane hyperpolarization or specific mutations that disrupt the Arg-339-Asp-443 interaction, the open state of the channel is energetically favored. Together with our previous studies with HERG (14), these findings provide further support for the importance of interactions between specific residues in the S4–S5 linker and the intracellular activation gate in the coupling of voltage sensing and channel opening and closing.

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