Hyperpolarization-activated cyclic nucleotide-modulated (HCN) channels resemble Shaker K⁺ channels in structure and function. In both, changes in membrane voltage produce directionally similar movement of positively charged residues in the voltage sensor to alter the pore structure at the intracellular side and gate ion flow. However, HCNs open when hyperpolarized, whereas Shaker opens when depolarized. Thus, electromechanical coupling between the voltage sensor and gate is opposite. A key determinant of this coupling is the intrinsic stability of the pore. In Shaker, an alanine/valine scan of residues across the pore, by single point mutation, showed that most mutations made the channel easier to open and steepened the response of the channel to changes in voltage. Because most mutations likely destabilize protein packing, the Shaker pore is most stable when closed, and the voltage sensor works to open it. In HCN channels, the pore energetics and vector of work by the voltage sensor are unknown. Accordingly, we performed a 22-residue alanine/valine scan of the distal pore of the HCN2 isoform and show that the effects of mutations on channel opening and on the steepness of the response of the channel to voltage are mixed and smaller than those in Shaker. These data imply that the stabilities of the open and closed pore are similar, the voltage sensor must apply force to close the pore, and the interactions between the pore and voltage sensor are weak. Moreover, cAMP binding to the channel heightens the effects of the mutations, indicating stronger interactions between the pore and voltage sensor, and tips the energetic balance toward a more stable open state.

Hyperpolarization-activated cyclic nucleotide-modulated (HCN)
4 channels are similar in structure and function to Shaker K⁺ channels (1–3). As in Shaker, HCN channels are comprised of four subunits, which each consist of six predicted membrane-spanning segments (S1–S6). The S1–S4 segments form the voltage-sensing domain, and the S5 and S6 segments, the pore-forming domain. The S4 segment in both channels contains positive charges that move similarly in response to changes in membrane voltage (4–6), to then alter the pore structure at the intracellular side of the S6 segment; this region functions as a voltage-controlled gate to cation flow (7–10). Despite these similarities, HCN channels are opened by hyperpolarization of the membrane potential, whereas Shaker channels open in response to depolarization. Thus, the electromechanical coupling between the voltage sensor and the gate is reversed in these two channels.

A key determinant of this coupling is the intrinsic stability of the closed and open conformations of the pore. In Shaker channels, it has been proposed that the pore is intrinsically most stable when closed and that the voltage sensor works to open the pore during depolarization (11, 12). Results from an alanine/valine scan of residues across the entire Shaker pore, by single point mutation, showed that most mutations made the channel easier to open and steepened the response of the channel to changes in voltage. It was argued that, because most mutations likely destabilize protein packing, the closed conformation must be the stable state; this is consistent with the observed crystal structures of Shaker-related channels KcsA and MthK, in the closed and open states, respectively, wherein more optimally and tightly packed helices were seen in the closed conformation (13–15).

Because of presumed shared architecture of the gate between HCN and Shaker channels, HCN channels might also be most stable when closed, and thus the voltage sensor would work to open the pore upon hyperpolarization. To test this hypothesis, we performed an alanine/valine scan of the C-terminal 22 amino acids of the S6 segment in HCN2, used as a prototype, and examined pore energetics as described previously in Shaker (11). Choice of this region for mutation was based on: 1) in Shaker, the corresponding region harbors one of two clusters of gating-sensitive residues and 2) it contains the voltage-controlled gate. Surprisingly, the effects of the mutations on channel opening and on the steepness of the channel’s response to voltage are mixed and smaller than those in Shaker. These findings imply that, in HCN2, the stabilities of the open and closed pore are similar, the interactions between the pore and voltage sensor, both structural and functional, are weaker than in Shaker, and that the voltage sensor must apply force to the pore...
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to close it. Thus, Shaker is closed and HCN2 is open in the absence of input from the voltage sensor. Moreover, cAMP binding to the HCN2 channel heightens the effects of the mutations, indicating stronger interactions between the pore and voltage sensor, and tips the energetic balance toward a more stable open state.

EXPERIMENTAL PROCEDURES

Mutagenesis—Single-point alanine/valine mutant HCN2 channels were constructed in one of two ways. First, some mutants were constructed by overlapping PCR mutagenesis using a mouse HCN2 template in pcDNA3.1, as previously described (14). For remaining mutants, bp 1172–2216 of the mouse HCN2 template were amplified by PCR primers containing distal EcoRI and BamHI sites and subcloned into pBlue-script. QuikChange (Stratagene, La Jolla, CA) was then used to generate mutations in this amplified fragment. Next, BlpI- and AgeI-digested fragments were inserted into the mouse HCN2 template. All mutations were confirmed via DNA sequencing (Nucleic Acid Protein Service Unit facility, University of British Columbia).

Tissue Culture and Expression of HCN2 Constructs—Chinese hamster ovary (CHO-K1) cells (ATCC, Manassas, VA) were maintained in Ham’s F-12 media supplemented with antibiotics and 10% fetal bovine serum (Invitrogen) and maintained at 37 °C with 5% CO2. Cells were plated onto glass coverslips. Two days after splitting, mammalian expression vectors encoding C-terminal 22 amino acids of wild-type or mutant HCN2 channels (2 μg per 35-mm dish), were transiently cotransfected into the cells using FuGene6 transfection reagent (Roche Applied Science).

Whole Cell Patch Clamp Electrophysiology—Cells expressing green fluorescent protein were chosen for whole cell patch clamp recordings 24–48 h post transfection. The pipette solution contained (in mM): 130 potassium Asp, 10 NaCl, 0.5 MgCl2, 1 EGTA, and 5 HEPES with pH adjusted to 7.4 using KOH. At experiments at saturating levels of cAMP, 2 mM cAMP (sodium salt) was added to the pipette solution. Extracellular recording solution contained (in mM): 135 KCl, 5 NaCl, 1.8 CaCl2, 0.5 MgCl2, and 5 HEPES with pH adjusted to 7.4 using KOH. Whole cell currents were recorded using an Axopatch 200B amplifier and Clampex software (Axon Instruments, Union City, CA) at room temperature. Patch clamp pipettes were pulled from borosilicate glass and fire-polished before use (pipette R = 2.5–4.5 MΩ).

Data Analysis—Data were filtered at 2-kHz and were analyzed using Clampfit (Axon Instruments), Origin (MicroCal, Northampton, MA), and Excel (Microsoft, Seattle, WA) software. I1 activation curves were determined from tail currents at a 2-s pulse to −35 mV following 3- to 15-s test pulses ranging from −150 to −10 mV, in 20-mV steps. Single tail current test pulses were followed by a 500-ms pulse to +5 mV to ensure complete channel deactivation. The resting current was allowed to return to its baseline value before subsequent voltage pulses. I1 activation curves were determined by plotting normalized tail current amplitudes versus test voltage and fitting these with a single order Boltzmann function, to determine the midpoint of activation (V1/2) and slope factor (k). The effective charge (Z) was calculated using the equation Z = RT/kF, where T = 295 K and R and F have their usual thermodynamic meanings. Changes in free energy between open and closed states were given by −ΔF(V1/2). The perturbation in free energy produced by introduction of the point mutations (ΔF(V1/2)) was given by −ΔF(V1/2,mut) − ΔF(V1/2,wt). The standard errors for ΔF(V1/2) were calculated using α = (2F(V1/2,wt) + 2F(V1/2,mut))/2. Differences in values for V1/2, Z, and ΔF(V1/2) between the wild-type channel and mutant channels were determined independently using an unpaired t test (p < 0.05 was considered significant).

Western Blot Analysis—Each sample was derived from cells on 35-mm plates that had been lysed in 100 μl of lysis buffer containing 50 mm Tris at pH 8.0, 1% Nonidet P-40, 150 mm NaCl, 1 mm EDTA, 1 mm phenylmethylsulfonl fluoride, 2 μm each of Na3VO4 and NaF, and 10 μg/ml each of aprotinin, pepstatin, and leupeptin. Samples were left on ice for 30 min, during which time they were vortexed every 5 min for ~5 s. After centrifugation to remove cell debris (25,000 × g, 25 min), protein concentration of the supernatant was determined by Bradford assay. 20-μg samples of supernatant were fractionated by SDS-PAGE (8%) and electroblotted to polyvinylidene fluoride membrane (Bio-Rad, Mississauga, Ontario, Canada). Blots were washed three times in TBST (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and then blocked with 5% nonfat dry milk (Bio-Rad) in TBST for 1 h at room temperature. Blots were then incubated with a rabbit polyclonal antibody specific to the C terminus of HCN2 (Affinity Bioreagents, Golden, CO), at a dilution of 1:500 in TBST with 5% nonfat dry milk for 2.5 h at room temperature. Blots were washed in TBST for 10 min, three times, and then incubated with horseradish peroxidase conjugated to goat anti-rabbit 1:3000 dilution in 5% nonfat dry milk (Bio-Rad) in TBST for 1 h at room temperature. Blots were then incubated with a rabbit polyclonal antibody specific to the C terminus of HCN2 (Affinity Bioreagents, Golden, CO), at a dilution of 1:500 in TBST with 5% nonfat dry milk for 2.5 h at room temperature. Blots were washed in TBST for 10 min, three times, and then incubated with horseradish peroxidase conjugated to goat anti-rabbit 1:3000 dilution in 5% nonfat dry milk with TBST for 1 h at room temperature; they were subsequently washed three times in TBST. Signals were obtained with ECL Western blotting Detection Reagents (Amersham Biosciences). Protein loading was controlled by probing all Western blots with goat anti-glyceraldehyde-3-phosphate dehydrogenase antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS

Alanine/Valine Scanning of the Distal S6 Reveals Small Changes in Perturbation Energy—To determine the most stable conformation of the channel, we performed a single-point alanine/valine scan of the C-terminal 22 amino acids of the S6 segment in HCN2 (Ile422–Asp443) and examined channel opening, as described previously in Shaker (11). We hypothesized that, as for Shaker channels, the values for V1/2 would be shifted in the positive direction and Z would be larger, due to disruption of a more stable closed state by introduced alanine or valine residues. This assumes that the closed conformation of the channel is at an energetic minimum and that all of the mutations within the S6 will result in positive perturbation energies. The S6 sites involved in positive perturbations promote a more stable closed confor-
tion, whereas those that produce negative perturbations promote a more stable open conformation. The relative numbers that shift in the two directions give an approximation of the relative stability of the open versus the closed conformations, e.g. a larger number of negative perturbation energies would suggest a more stable open state, an equal number of positive and negative perturbation energies would suggest that the stabilities of the open and closed conformations are about equal. Finally, this assumes that each residue contributes equally to stability.

Wild-type and mutant channels were expressed independently in CHO cells from which \( I_f \) was recorded using the whole cell patch clamp approach. \( I_f \) activation curves were determined by plotting normalized tail current amplitudes versus test voltage and fitting these with Equation 1 (Experimental Procedures). From this fit, values for \( V_{1/2} \) and \( Z \) were determined to thereby allow calculation of perturbation energies (Table 1, A and B). Gating parameters and perturbation energies of wild-type channels were compared with those of the mutant channels using an unpaired \( t \) test. 18 of 22 single-point mutations expressed measurable levels of \( I_f \) from which activation curves could be derived (Fig. 1, A and B). Levels of \( I_f \) for G424A, A425V, T426A, and Y428A were not detectable. Unexpectedly, more mutants had a \( V_{1/2} \) value that were either significantly more negative (5/18) or unchanged (10/18) from that of wild type, than those which were more positive (3/18) (Fig. 1C, upper). With one exception, all \( V_{1/2} \) values of mutants were unchanged from that of wild type (Fig. 1C, lower). Finally, with the exception of three values, the free energies of mutants were unchanged from that of wild type (Fig. 1D). The mix of positive and negative shifts in \( V_{1/2} \) and lack of change in free energies in the mutant channels suggest that, contrary to our hypothesis, the stabilities of the open and closed conformations are similar. These data are in accordance with recent findings from an alanine/valine scan of S6 in HCN2 expressed in Xenopus oocytes, which showed that most mutations shifted the opening of the channel to more negative potentials or had no effect; however, the energetic repercussions of these changes on gating were not explored (16).

**cAMP Shifts the Balance of Perturbation Energies of the S6 Mutations toward Negative Values**—cAMP stabilizes the open conformation of HCN channels by removing a tonic inhibitory action of the cyclic nucleotide-binding domain (CNBD), located in the C terminus, on pore opening (17–22). Inhibition by the CNBD occurs by a coupled interaction with the C-linker, a structure that connects the CNBD to the S6 helices, which is thought to apply a force on these helices to inhibit pore opening (20, 23). cAMP binding reverses the coupled interaction, which then alleviates inhibition of pore opening thereby promoting a more stable open state. Given a more stable open conformation upon cAMP binding, we hypothesized that, in saturating levels of this cyclic nucleotide, the S6 mutations would produce more dramatic effects on \( V_{1/2} \) and \( Z \) and a shift in perturbation energies toward more negative values.

To test this hypothesis, identical experiments were conducted with all 22 mutant channels and the wild-type channel at saturating levels of cAMP (2 mM). All but one mutant (G424A) expressed measurable levels of \( I_f \) from which activation curves could be determined (Fig. 2, A and B). For the wild-type HCN2 channel, \( V_{1/2} \) was shifted +10.1 mV, and \( Z \) was decreased 0.4 compared with the values determined at basal cAMP (Table 1, A and B). The majority of \( V_{1/2} \) values in the mutant channels were more negative (6/21) or unchanged (9/21) compared with wild-type, whereas fewer values were more positive (6/21) (Fig. 2C, upper). The majority of \( Z \) values were larger (6/21) or unchanged (14/21) compared with wild type, whereas only one value was smaller (Fig. 2C, lower). A majority of free energies were more negative (9/21) or unchanged (11/21) compared with wild type, but only one value was more positive (Fig. 2D).

Comparing free energies in saturating cAMP with those in basal cAMP (Figs. 1D and 2D), there was a lower proportion of more positive free energies (1/21 versus 2/18), a lower proportion of unchanged free energies (11/21 versus 15/18), and a

**TABLE 1**

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|------------------------------------|
| **A** | **B** |
| **HCN2 channel** | **baxal cAMP** | **2 mM cAMP** | **2 mM cAMP** |
| | \( V_{1/2} \) (mV) | \( Z \) | \( \Delta Z \) (cal/mole) | \( \Delta Z \) (cal/mole) |
| Wild type | 9 | -108.9 ± 1.8 | 2.24 ± 0.18 | -5.47 ± 0.36 |
| A422A | 6 | -111.4 ± 3.3 | 2.03 ± 0.40 | -6.98 ± 1.03 | -1.40 ± 1.09 |
| A423A | 6 | -104.7 ± 2.9 | 2.03 ± 0.40 | -6.98 ± 1.03 | -1.40 ± 1.09 |
| G424A | no expression | no current | no current | no current |
| T426A | 7 | -96.4 ± 2.4* | 2.85 ± 0.28 | -6.20 ± 0.63 | -0.73 ± 0.73 |
| Y428A | 9 | -115.0 ± 2.1* | 2.61 ± 0.13 | -8.06 ± 0.44* | -1.33 ± 0.37 |
| A425V | 4 | -116.3 ± 2.7* | 2.92 ± 0.42 | -8.71 ± 0.74* | -2.09 ± 0.82 |
| G424A | 7 | -99.4 ± 4.9* | 2.00 ± 0.17 | -8.12 ± 0.43* | 1.35 ± 0.61 |
| G424A | 7 | -105.8 ± 1.9 | 1.99 ± 0.17 | -8.49 ± 0.47 | 0.57 ± 0.61 |
| A422A | 7 | -105.5 ± 2.2 | 2.20 ± 0.19 | -6.57 ± 0.48 | 0.19 ± 0.60 |

*Z values are from fits of activation curves with Equation 1 for each data set. Values are reported as the mean ± S.E. Asterisks represent significant differences from wild type.
Basal cAMP

A.

HCN2

Q440A

1 nA

3 s

0.5 nA

3 s

C.

ΔV_{1/2}

B.

C427A

L438A

1 nA

3 s

ΔZ

D.

normalized current

-150 -130 -110 -90 -70 -50 -30 -10 10

voltage (mV)

ΔZ_{1/2}
higher proportion more negative free energies (9/21 versus 1/18). For one site (G433A), free energy was significantly positive in basal cAMP but, in saturating concentrations of cAMP, it was not altered significantly. The shift of perturbation energies toward the negative, when assayed at saturating levels of cAMP, suggest that the open conformation becomes more stable as a result of cAMP binding.

Three of the mutants that were not functional in basal cAMP recovered function in saturating levels cAMP (A425V, T426A, and Y428A), which may have been due to one or both of the following reasons. First, in basal cAMP levels, the mutations may have shifted the range of current activation to very negative voltages at which function cannot be reliably ascertained (i.e. more negative than −150 mV). In elevated cAMP, the activation range would have moved to less negative voltages where the likelihood of detecting channel activity is increased using our protocols. Second, the number of functional channels at the cell surface or single channel conductance may have been reduced by the mutations. For HCN2 channels, cAMP has been suggested to increase open probability in addition to shifting reduced by the mutations. For HCN2 channels, cAMP has been suggested to increase open probability in addition to shifting the pore showed that this mutant did not undergo the identical mutant when expressed in the pore showed that three mutations is supported by the significantly lower levels of functional channels or single channel conductance by these single channel conductance. A reduction in the number of functional channels or single channel conductance may have been reduced by the mutations. For HCN2 channels, cAMP has been suggested to increase open probability in addition to shifting the activation curve to more positive voltages (20), which could have overcome reductions in number of functional channels or single channel conductance. A reduction in the number of functional channels or single channel conductance by these three mutations is supported by the significantly lower levels of current they produce compared with the wild-type channel (wt HCN2, −421 ± 98 pA/picofarad (pF), n = 8; A425V, −71 ± 8 pA/pF, n = 3; T426A, −116 ± 22 pA/pF, n = 4; Y428A, −100 ± 16 pA/pF, n = 5; all of the mutants are significantly different from wild-type HCN2, p < 0.05).

The G424A mutant did not yield current in either basal or elevated cAMP. A lack of function has also been reported for the identical mutant when expressed in Xenopus oocytes (16). Western blotting showed that this mutant did not undergo complex glycosylation, unlike the wild-type channel but like a channel in which the N-glycosylation site has been mutated (N380Q) (Fig. 3). These data suggest that G424 is important for plasma membrane localization of functional channels.

**Effects of S6 Mutations on Z Are Consistent with an Altered Closed to Open Transition**—In Shaker, an alanine/valine scan of the pore showed that Z values increased as V½ values became more negative (11). This relationship is consistent with effects on the final closed to open step in a linear gating scheme in which each of the four voltage sensors moves independently, and, once all sensors reach the permissive state, the pore opens by a voltage-independent concerted transition (24, 25).

For HCN2, we were struck by the mutation-induced changes in Z, because they were very small compared with those in Shaker. To determine whether the comparatively small changes in Z are still consistent with an altered closed to open step in HCN2, we applied an allosteric model that captures most aspects of HCN channel behavior (26) (Scheme 1).

In this model, the voltage sensor in each of the four monomeric subunits moves from reluctant to willing states (C to C4) independently to then allosterically trigger closed to open transitions. Successive engagement of each subunit enhances the probability of channel opening (P o) given by

\[
P_o = \frac{1}{1 + P(L(V)) (1 + 1/K(V))^{3/2}}
\]

(Eq. 2)

where K(V) and L(V) are the equilibrium constants for voltagensensor movement and the closed to open step, respectively. One important way in which this model differs from the scheme used to describe Shaker is that the closed to open step is dependent upon voltage. Using this model, Altomare et al. (26) showed that HCN-mediated currents were well fitted and that isoform-specific positions of the activation curves and delays in both current activation and deactivation could be predicted.

We used this allosteric model to generate hypothetical values of Z and V½ by varying the rate of either the closed to open step (L(V)) or voltage-sensor movement (K(V)) to assess which change could best predict the effects of the S6 mutations on Z. Because the HCN2 S6 mutations are in a region of the pore that contains the gate, an effect on the closed to open transition, and thus on L(V), would be expected. Z values derived from model P o curves by varying L(V), but not by varying K(V), should then approximate our experimental Z values.

To test this, P o curves were generated using Equation 2 with a range of L(V) and K(V) values and model parameters specific for either basal or 2 mM cAMP. Model parameters were determined by best fitting and are shown in Table 2. Select P o curves that spanned a similar range of voltages as those determined experimentally were then fitted with Equation 1 to yield theoretical values for Z and V½, which were then plotted in Fig. 4 (A and B). Both the Z values obtained by varying L(V) and those observed experimentally do not vary greatly with V½; this held true at basal and at saturating levels of cAMP (in Fig. 4, compare the experimentally determined Z values with those determined from the model using a range of L(V) values, represented by the individual symbols and black lines, respectively). In contrast, the Z values obtained by varying K(V) in the model increase at negative voltages and plateau in the range of voltages separated from that in which most of the experimentally determined Z values are found, in both basal and saturating levels of cAMP (in Fig. 4, compare the experimentally determined Z values with those determined from the model using a range of K(V) values, represented by the individual symbols and gray lines, respectively). Furthermore, when K(V) was...
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2 mM cAMP

A.

HCN2

Q440A

1 nA

1 nA

3 s

3 s

T426A

A437V

0.5 nA

1 nA

3 s

3 s

B.

normalized current

voltage (mV)

HCN2

Q440A

A437V

T426A

C.

ΔV_{1/2}

ΔZ

D.

ΔZFV_{1/2}
FIGURE 3. Glycine 424 is critical for the expression of cell surface HCN2 channels. A, current traces elicited from cells expressing wild-type HCN2 (upper trace) or HCN2-G424A (lower trace) in response to hyperpolarizing voltage pulses to \(-150\) mV from a holding potential of \(-35\) mV. B, Western blot probed with a rabbit polyclonal antibody directed against the C terminus of HCN2. Lane 1, untransfected cells (UT); lane 2, wt HCN2; lane 3, HCN2 N380Q (N-glycosylation mutant); lane 4, HCN2-G242A. The arrows indicate the presence of mature (M, \( \sim 136\) kDa), immature (I, \( \sim 114\) kDa) protein forms. These data are representative of three independent experiments. Note the absence of a mature form of HCN2 in lanes containing HCN2 N380Q (as demonstrated previously (37, 38)) and HCN2 G424A.

Our findings explain the presence of an “instantaneous” current at all voltages in wild-type HCN channels (3, 27–30) and the frequent observation that artificial perturbations to HCN lead to even larger constitutively active currents. A resting conductance of \( \sim 2\% \) has been estimated for HCN2 channels, whereas a value between 4 and 8% has been estimated for sea urchin HCN channels, without and with cAMP, respectively (28). Our data imply that the channel open probability does not reach zero, yielding a significant resting conductance, and that the voltage sensor is unable to exert sufficient force to realize this end. The production of greater constitutive current seen with a number of single-point mutations in the S4-S5 and C-linkers (30–33), and upon cadmium binding to cysteine substitutions near the intracellular side of the pore (8), when understood in the context of a naturally open pore, suggests that these perturbations weaken the link between the voltage sensor and pore. Alternatively, residual current through a channel in the closed state may contribute to a resting conductance, but this would not depend upon the energetic balance between the

depicting the changes in \( \Delta Z \) (upper) and \( Z \) (lower) values for each mutant channel relative to wild type. D, bar graph depicting change in perturbation of free energy, \( \Delta(ZF'/S) \), in mutant channels relative to wild type. One mutant channel did not yield measurable levels of \( I_0 \) (solid line through numbered residue, x axis).

TABLE 2
Allosteric model parameters at basal and saturating (2 mM) levels of cAMP

| Parameter | Basal cAMP | Saturating cAMP |
|-----------|------------|-----------------|
| L, \( L' \) | \( \alpha \) 0.0001594 | \( \alpha \) 0.0003785 |
| \( \beta \) | 1198 | 208.4 |
| K, \( K' \) | \( \gamma \) 1068 | \( \gamma \) 13.33 |
| \( \delta \) | 106.4 | 86.86 |
| \( z_0 \) | 1.123 | 0.5874 |
| \( z_1 \) | 0.1437 | 0.9621 |
| a | 3.2 | 0.2 |
| r | 0 | 0 |

**DISCUSSION**

The mixed effects on the voltage dependence of channel opening and very small perturbation energies produced by the majority of S6 mutations in basal levels of cAMP, and an abundance of mutations with negative perturbation energies in saturating levels of cAMP, suggest that the stability of the open and closed states are similar, and that cAMP binding shifts the energetic balance toward a more stable open state. This implies that the voltage sensors must apply force upon the HCN2 pore to close. This is unlike Shaker channels, which are most stable in the closed conformation and in which the voltage sensor works to open the pore (11). Thus, voltage-dependent channel gating in both HCN and Shaker channels is constrained such that the force exerted by the voltage sensor on the gate occurs during depolarization of the membrane potential.

Our findings explain the presence of an “instantaneous” current at all voltages in wild-type HCN channels (3, 27–30) and the frequent observation that artificial perturbations to HCN lead to even larger constitutively active currents. A resting conductance of \( \sim 2\% \) has been estimated for HCN2 channels, whereas a value between 4 and 8% has been estimated for sea urchin HCN channels, without and with cAMP, respectively (28). Our data imply that the channel open probability does not reach zero, yielding a significant resting conductance, and that the voltage sensor is unable to exert sufficient force to realize this end. The production of greater constitutive current seen with a number of single-point mutations in the S4-S5 and C-linkers (30–33), and upon cadmium binding to cysteine substitutions near the intracellular side of the pore (8), when understood in the context of a naturally open pore, suggests that these perturbations weaken the link between the voltage sensor and pore. Alternatively, residual current through a channel in the closed state may contribute to a resting conductance, but this would not depend upon the energetic balance between the
open and closed states. Nevertheless, a constitutively open channel may not necessarily be an inevitable consequence of a pore that is more stable when open. At more positive voltages, the voltage sensor could actively keep the channel shut. This is the opposite of what happens in a channel with a pore that is more stable when closed, like Shaker, in which the voltage sensors work to keep the channel open.

Perturbation energies induced by the S6 mutations in HCN2 were smaller than those in Shaker (11), which suggest weaker interactions between the voltage-sensing elements and the pore. Loose coupling between the voltage sensor and pore, as might be expected from a weak structural interaction, has been proposed recently for HCN channels (34). These authors showed that the energetics of voltage-sensor movement is little affected in sea urchin HCN channels that have been “locked open,” as opposed to the energetics of voltage-sensor movement in locked open Shaker channels, which are significantly affected. The lack of apparent coupling in a locked open HCN channel is completely consistent with the notion that the pore is naturally open without input from the voltage-sensing elements.

A difference in gating dynamics of HCN2 from Shaker is also suggested by our finding that the effective charge $Z$, determined from the slope of the activation curve, was changed only minimally by the single-point S6 mutations. In contrast, single-point mutations in the S6 of Shaker altered $Z$ and perturbation energy to a much greater extent, and the $Z$ values increased as $V_{1/2}$ values became more negative (11). This difference in observed $Z$ between these two channels may arise from the fact that, in HCN2, the closed to open transition as well as the movement of the voltage sensor may be voltage-dependent (11, 26). Thus, the slope of the HCN2 activation curve would reflect contributions from both processes, whereas that of Shaker would reflect a contribution primarily from voltage-sensor movement. It should be noted that in 2007 a study on HCN2 channels suggested that the closed to open transition may instead be voltage-independent (21). It will be interesting to determine whether the gating model developed in that study predicts the small changes in $Z$ seen in our study.

cAMP has been proposed to stabilize the HCN open state by removing an inhibitory action of the CNBD on pore opening. In the absence of cAMP, inhibition by the CNBD occurs by a coupled interaction with the C-linker region that is thought to apply a force on the S6 helices to actively inhibit pore opening (20, 23). Our data showing a significant shift of perturbation energies to more negative values by mutations in the S6 are consistent with this proposed action of cAMP and identify a cluster of residues around the proposed activation gate (35) that are modified by the inhibitory action of the CNBD (supplemental Fig. S1). Our data are also consistent with previous work in sea urchin HCN wherein mutation of a single residue in S6 (F459L) produced an equivalent effect to cAMP on gating (36). The corresponding site in mouse HCN2 (F431) is one of the ten cAMP-sensitive sites identified in our study.

Our data suggest that the primary effect of the S6 mutations is on the closed to open step, the final step of the activation process, which seems reasonable for several reasons. First, the mutations that are energetically sensitive cluster in a region of the S6 that likely forms the activation gate (7, 8, 35). Second, the small effects of the mutations on effective charge can be mostly, although not completely, explained by effects on the pore-opening step. Third, cAMP, which releases the inhibitory influences on pore opening, significantly shifts perturbation energies toward the negative, suggesting that both the mutations and the CNBD target the same region. Nevertheless, an allosteric effect of the mutations on voltage-sensor movement could have contributed to the observed alterations in gating. We found that the significant effects on the effective charge ($Z$) produced by some of the mutations could not be explained by an allosteric model in which only the pore-opening step, or only the voltage-sensor movement, was altered. Other strategies are required to determine whether the voltage-sensing elements of HCN channels contribute to the observed effects of the S6 mutations on gating. It is important to note that the perturbation energies of the S6 mutations in HCN2 are small relative to those in the prototypical Shaker channel, especially at basal levels of cAMP; therefore, neither the pore or voltage sensor are apparently affected despite mutations in and around the activation gate. These small perturbation energies, along with their shift toward the negative by cAMP, are strong support for both a weak interaction between the pore and voltage sensor, compared with Shaker, and a pore that is not at its energetic minimum when closed. The evidence demonstrating that the effects of the mutations on perturbation energy in saturating cAMP levels are larger, and shifted toward negative, greatly strengthens this conclusion.
A naturally open pore in HCN2 has important implications for the structural orchestration of gating. The direction of charge and voltage-sensor movement is similar between HCN and Shaker-related channels, despite the inverted dependence of HCN channel opening to voltage, which implies that the coupling of voltage-sensor movement to channel opening is inverted (4–6). We suggest that positive force is applied by the voltage sensor to the C-terminal region of the S6 helices during depolarization to cause the gate to close in HCN2, rather than to open as in Shaker. The structural details of this action will have to await more sophisticated analyses such as the determination of HCN crystal structure, but we believe our present findings provide a glimpse into a fundamentally different way of cycling between open and closed states in the Kv superfamily of voltage-gated channels.

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REFERENCES
1. Santoro, B., Liu, D. T., Yao, H., Bartsch, D., Kandel, E. R., Siegelbaum, S. A., and Tibbs, G. R. (1998) Cell 93, 717–729
2. Ludwig, A., Zong, X., Jeglitsch, M., Hofmann, F., and Biel, M. (1998) Nature 393, 587–591
3. Gauss, R., Seifert, R., and Kaupp, U. B. (1998) Nature 393, 583–587
4. Mannikko, R., Elinder, F., and Larsson, H. P. (2002) Nature 419, 837–841
5. Bell, D. C., Yao, H., Saenger, R. C., Riley, J. H., and Siegelbaum, S. A. (2004) J. Gen. Physiol. 123, 5–19
6. Vemana, S., Pandey, S., and Larsson, H. P. (2004) J. Gen. Physiol. 123, 21–32
7. Shin, K. S., Rothberg, B. S., and Yellen, G. (2001) J. Gen. Physiol. 117, 91–101
8. Rothberg, B. S., Shin, K. S., and Yellen, G. (2003) J. Gen. Physiol. 122, 501–510
9. Macri, V., Proenza, C., Agranovich, E., Angoli, D., and Accili, E. A. (2002) J. Biol. Chem. 277, 35939–35946
10. Giorgetti, A., Carloni, P., Mistrik, P., and Torre, V. (2005) Biophys. J. 89, 932–944
11. Yifrach, O., and MacKinnon, R. (2002) Cell 111, 231–239
12. Hackos, D. H., Chang, T. H., and Swartz, K. J. (2002) J. Gen. Physiol. 119, 521–532
13. Doyle, D. A., Morais Cabral, J., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) Science 280, 69–77
14. Jiang, Y., Lee, A., Chen, I., Cadene, M., Chait, B. T., and MacKinnon, R. (2002) Nature 417, 515–522
15. Jiang, Y., Lee, A., Chen, I., Cadene, M., Chait, B. T., and MacKinnon, R. (2002) Nature 417, 523–526
16. Cheng, L., Kinard, K., Rajamani, R., and Sanguinetti, M. C. (2007) J. Pharmacol. Exp. Ther. 322, 931–939
17. DiFrancesco, D., and Tortora, P. (1991) Nature 351, 145–147
18. DiFrancesco, D. (1999) J. Physiol. 515, 367–376
19. Wainger, B. I., DeGenarro, M., Santoro, B., Siegelbaum, S. A., and Tibbs, G. R. (2001) Nature 411, 805–810
20. Craven, K. B., and Zagotta, W. N. (2004) J. Gen. Physiol. 124, 663–677
21. Chen, S., Wang, J., Zhou, L., George, M. S., and Siegelbaum, S. A. (2007) J. Gen. Physiol. 129, 175–188
22. Barbuto, A., Baruscotti, M., Altmare, C., Moroni, A., and DiFrancesco, D. (1999) J. Physiol. 520, 737–744
23. Zhou, L., and Siegelbaum, S. A. (2007) Structure 15, 655–670
24. Zagotta, W. N., Hoshi, T., and Aldrich, R. W. (1994) J. Gen. Physiol. 103, 321–362
25. Schoppa, N. E., and Sigworth, F. I. (1998) J. Gen. Physiol. 111, 313–342
26. Altmare, C., Buschi, A., Camatini, E., Baruscotti, M., Visconi, C., Moroni, A., and DiFrancesco, D. (2001) J. Gen. Physiol. 117, 519–532
27. Proenza, C., Angoli, D., Agranovich, E., Macri, V., and Accili, E. A. (2002) J. Biol. Chem. 277, 5101–5109
28. Proenza, C., and Yellen, G. (2006) J. Gen. Physiol. 127, 183–190
29. Ishii, T. M., Takano, M., Xie, I. H., Noma, A., and Ohmori, H. (1999) J. Biol. Chem. 274, 12835–12839
30. Chen, J., Mitcheson, J. S., Tristani-Firouzi, M., Lin, M., and Sanguinetti, M. C. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11277–11282
31. Macri, V., and Accili, E. A. (2004) J. Biol. Chem. 279, 16832–16846
32. Decher, N., Chen, J., and Sanguinetti, M. C. (2004) J. Biol. Chem. 279, 13859–13865
33. Chen, J., Mitcheson, J. S., Lin, M., and Sanguinetti, M. C. (2000) J. Biol. Chem. 275, 36465–36471
34. Bruening-Wright, A., Pandey, S., and Larsson, P. (2008) Biophys. J. 94, 119
35. Rothberg, B. S., Shin, K. S., Phale, P. S., and Yellen, G. (2002) J. Gen. Physiol. 119, 83–91
36. Shin, K. S., Maertens, C., Proenza, C., Rothberg, B. S., and Yellen, G. (2004) Neuron 41, 737–744
37. Nazzari, H., Angoli, D., Chow, S. S., Whitaker, G., Leclair, L., McDonald, E., Macri, V., Zahynacz, K., Walker, V., and Accili, E. A. (2008) Am. J. Physiol. 295, C642–C652
38. Much, B., Wahl-Schott, C., Zong, X., Schneider, A., Baumann, L., Moosmann, S., Ludwig, A., and Biel, M. (2003) J. Biol. Chem. 278, 43781–43786