Functional Roles of Charged Residues in the Putative Voltage Sensor of the HCN2 Pacemaker Channel*

Jun Chen, John S. Mitcheson, Monica Lin, and Michael C. Sanguinetti†

From the Department of Medicine, Division of Cardiology and Eccles Program in Human Molecular Biology and Genetics, University of Utah, Salt Lake City, Utah 84112-5330

Received for publication, August 3, 2000
Published, JBC Papers in Press, August 28, 2000, DOI 10.1074/jbc.M007034200

Hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels contribute to pacemaking activity in specialized neurons and cardiac myocytes. HCN channels have a structure similar to voltage-gated K+ channels but have a much larger putative S4 transmembrane domain and open in response to membrane hyperpolarization instead of depolarization. As an initial attempt to define the structural basis of HCN channel gating, we have characterized the functional roles of the charged residues in the S2, S3, and S4 transmembrane domains. The nine basic residues and a single Ser in S4 were mutated individually to Gln, and the function of mutant channels was analyzed in Xenopus oocytes using two-microelectrode voltage clamp techniques. Surface membrane expression of hemagglutinin-epitope-tagged channel proteins was examined by chemiluminescence. Our results suggest that 1) Lys-291, Arg-294, Arg-297, and Arg-309 contribute to the voltage dependence of gating but not to channel folding or trafficking to the surface membrane; 2) Lys-303 and Ser-306 are essential for gating, but not for channel folding/trafficking; 3) Arg-312 is important for folding but not gating; and 4) Arg-309, Arg-315, and Arg-318 are crucial for normal protein folding/trafficking and may charge-pair with Asp residues located in the S2 and S3 domains.

Pacemakers of the heart and nervous system are comprised of specialized cells that exhibit spontaneous rhythmic firing of action potentials. A hyperpolarization-activated cation current underlies the slow and spontaneous depolarization of the membrane following an action potential of pacemaker cells. The pacemaker current is called I_{h} in nerves and I_{f} in atrial node cells of the heart (1). Recently, the genes that encode pacemaker channels were cloned from the mammalian brain (2, 3) and sea urchin sperm (4). The cloned pacemaker channels are collectively referred to as hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels (5–7). HCN channel subunits have an overall structure similar to voltage-gated K+ (Kv) channel subunits, including six transmembrane domains (S1–S6), a similar selectivity filter sequence containing the GYG motif, multiple basic residues in the S4 domain, and a few conserved acidic residues in the S2 and S3 domains. However, unlike Kv channels, HCN channels only open upon hyperpolarization and are permeable to both K+ and Na+ (2–4). The mechanism and structural basis of the gating behavior of HCN channels is unknown but has been the subject of considerable speculation (6, 7).

There are two alternative hypotheses to explain the gating of HCN channels. Channel opening in response to membrane hyperpolarization could be due to recovery of channels from an inactivated state. A competing hypothesis is that hyperpolarization-dependent channel opening represents activation from a normal closed state. In this model, the gating scheme for HCN channels is a mirror image of Kv channel gating. However, no experimental data using HCN channels exists to support either hypothesis. Indirect evidence in favor of the first hypothesis comes from the behavior of a mutant Shaker K+ channel. Introduction of three point mutations into the S4 domain of Shaker (R365N/L366A/R371I) caused a ~179 mV shift in the voltage dependence of channel activation (8). Because triple mutant Shaker channels were significantly inactivated at ~80 mV, further hyperpolarization permitted channels to recover from inactivation into the open state. This effectively converted the outward rectifier Shaker K+ channel into an inward rectifier channel, because the inactivation gate (N-terminal ball domain) acted like an activation gate. Indirect evidence for the second hypothesis comes from the gating behavior of a mutant HERG K+ channel. A single mutation in the S4–S5 linker of HERG (D540K) destabilized the closed state and permitted the channel to reopen upon hyperpolarization to membrane potentials negative to −90 mV (9). Hyperpolarization-dependent activation of D540K is still present in channels where inactivation has been removed by additional mutations (10). Whatever the specific mechanism, it is possible that only small changes in the Kv channel voltage sensor or associated structures would be sufficient to account for the unique gating properties of HCN channels.

Extensive structure-function studies have established that the positively charged S4 domain is the voltage sensor of voltage-gated potassium, sodium, and calcium channels (11, 12). The S4 voltage sensor of Kv channel subunits contains 5 to 7 basic residues spaced at every third position. Compared with Kv channels, mammalian HCN channels have a much larger S4 domain, containing 9 basic (and 1 Ser) residues at every third position (Fig. 1). Counting in the N- to C-terminal direction, the Ser of HCN channels is located in what would otherwise be the 6th charged position of the putative S4 domain, and is followed by 4 additional Arg residues. The functional role of the Ser in HCN channels is unknown. The length of a membrane-spanning α-helix is about 20 amino acids. At a fixed
transmembrane voltage, the entire putative S4 domain (28 amino acids from Lys-291 to Arg-318 in HCN2) could not simultaneously traverse the membrane. Thus, it is likely that only a subset of the charged residues in the S4 domain of HCN subunits contribute to channel gating. Recent evidence suggests that the S2 and S3 domains of Kv channels are also crucial for channel function (13–16). These domains contain acidic residues in highly conserved positions, which interact specifically with basic residues in the S4 domain to mediate channel folding and gating (13, 17, 18). In HCN2 channel subunits, there are 4 Asp residues located in similar positions within the S2 and S3 domains. Although not yet investigated, mutation of the charged residues in the S2, S3, and S4 domains of HCN channels would be expected to have different functional consequences consistent with their role in channel gating and subunit folding.

To explore the structural and functional role of the charged residues in the HCN2 S4 domain, the 9 basic residues and the single Ser (Ser-306) were individually neutralized to Gln. In addition, 4 acidic Asp residues located in the S2 and S3 domains were individually mutated to Asn. The effects of each mutation on the amplitude and voltage dependence of HCN2 channel currents expressed in Xenopus oocytes were determined using two-microelectrode voltage clamp techniques, and the surface membrane expression of protein was determined by single cell luminesmetry.

EXPERIMENTAL PROCEDURES

Molecular Biology—The HCN2 channel cDNA was cloned from Marathon-Ready (CLONTECH) mouse brain cDNA into the pSP64T oocyte expression vector. The sequence of the clone was identical with the published HAC1 clone (3), except for a single nucleotide change causing a K283R substitution. To facilitate subcloning, the very GC-rich N terminus portion of the HCN2 channel (encoding amino acids 2–130) was amplified with the polymerase chain reaction-amplified segment. cRNA for injection construct was confirmed with restriction mapping and DNA sequencing prescribed previously (19). Before use in expression experiments, each ntHCN2. All mutations were introduced into ntHCN2 cDNA as described previously (19). Before use in expression experiments, each mutation was a negative shift in the voltage dependence of channel activation—was determined using two-microelectrode voltage clamp techniques (23). The voltage dependence of ntHCN2 channel activation was determined from a holding potential of −30 or 0 mV. Pulses of 3-s duration were applied to potentials ranging from 0 to −150 mV to activate current. Tail currents were usually measured at −130 mV. Normalized tail current amplitude was plotted versus test potential to obtain the relative conductance-voltage (G-V) relationship and fitted with a Boltzmann function. Data are expressed as mean ± S.E. (n = number of oocytes).

RESULTS

Comparison of Full-length and ntHCN2 Channel Currents—The biophysical properties of full-length HCN2 and ntHCN2 channel currents were relatively similar. Both currents activated slowly upon membrane hyperpolarization from a holding potential of 0 mV and had similar amplitudes when fully activated (Fig. 2, B and C). The G-V relationship was determined by plotting the relative amplitude of tail currents at −130 mV following 3-s pulses to potentials ranging from −20 to −140 mV (Fig. 2D). The V1/2 and slope factor for full-length HCN2 channel current was −73 ± 0.3 mV and 10 ± 0.2 mV, respectively (n = 7). The V1/2 of ntHCN2 channel current was −85 ± 0.2 mV, a shift of −12 mV compared with full-length HCN2. In addition, the slope of the activation curve was steeper (k = 7.7 ± 0.2 mV).

K291Q, R294Q, R297Q, and R300Q Mutations Cause Leftward Shift in Voltage Dependence of Channel Activation—Mutations of any one of the 4 basic residues located in the outermost positions of the putative S4 domain were well tolerated. Channels formed by subunits, where one of these residues (Lys-291, Arg-294, Arg-297, Arg-300) was neutralized to Q, produced currents with relatively normal amplitudes (Fig. 3, A and B). Current amplitudes at −130 mV, where channels were fully activated, were also similar for mutant and ntHCN2 channels (see Fig. 6). The only notable change, caused by all four point mutations, was a negative shift in the voltage dependence of activation (Fig. 3D, Table I). Neutralization of all 4 basic residues, K291Q/R294Q/R297Q/R300Q (quadruple mutant) induced a current with similar behavior (Fig. 3C) but also shifted the voltage dependence of activation by at least −49 mV relative to ntHCN2 channel current (Fig. 3D). This change in V1/2 was nearly the same as predicted by addition of the individual shifts caused by the 4 single mutations (−44.2 mV). However, the slope of the activation curve (k = 19.2 ± 1.7 mV) for the quadruple mutation was also less than measured for any of the single mutant channels (k = 8.5 to 9.5 mV; Table I).
The shift in the $V_{0.5}$ for the quadruple mutant channel current may be underestimated, because we could not pulse negatively enough to invoke full activation of these channels.

The shift in the voltage dependence of activation could be caused by a loss of surface charge screening normally contributed by the basic residues. This possibility was investigated by determining the shift in $V_{0.5}$ caused by addition of Mg$^{2+}$ to the extracellular solution for ntHCN2 and each mutant HCN2 channel. Elevating [Mg$^{2+}$] from 0.1 to 10 mM caused a progressive positive shift in the $V_{0.5}$ of activation for all channels. A greater shift was observed for K291Q HCN2 than ntHCN2 or any of the other channels containing a single charge neutralization (Fig. 4). Increasing [Mg$^{2+}$], to 10 mM eliminated the difference in $V_{0.5}$ between K291Q and ntHCN2 channels. This finding suggested that the $-7.5$-mV shift in $V_{0.5}$ caused by the K291Q mutation and part of the shift observed for the quadruple mutant channel (for [Mg$^{2+}$] = 1.8 mM) could be due to loss of a positive surface charge normally contributed by the ε-NH$_3^+$ of Lys-291. If this were so, then mutation of Lys-291 to an acidic group (e.g., Glu) would be expected to cause an even greater shift in $V_{0.5}$, because the −COO$^-$ group would effectively increase the negative surface charge near the voltage sensor. As predicted, K291E did produce an even greater shift in $V_{0.5}$ (−21.6-mV shift) than that caused by neutralization. However, elevation of [Mg$^{2+}$], did not overcome the voltage shift in gating for R294Q or R297Q, or R300Q or the quadruple mutant as would be expected if due only to a surface charge-screening mechanism.

Functional channels were also produced when the outermost basic residues were mutated to amino acids other than Glu (i.e., K291A, R294A, R297C, R300C; data not shown), suggesting that positive charges at these positions are not essential for channel folding or gating.

**K303Q, S306Q, and R312Q Mutations Reduce Current Amplitude**—Channels formed by subunits containing a neutralization of the fifth basic residue in the putative S4 domain (K303Q) activated more slowly and at potentials more negative than ntHCN2 channels (Fig. 5A). For example, at $-140$ mV, the time constants for activation of ntHCN2 channel current was 169 ± 13 ms ($n = 6$), but 1171 ± 128 ms ($n = 9$) for K303Q ntHCN2 channel current. Due to the presence of endogenous inward Cl$^−$ currents that also activated slowly and at very negative potentials, we could not pulse long enough at very negative potentials to obtain a reliable measure of the $V_{0.5}$ for the activation of K303Q ntHCN2 channel current. However, based on the I-V relationship following 3-s pulses to test potentials between 0 and $-150$ mV (Fig. 5B), the $V_{0.5}$ must be shifted by at least $-40$ mV. These findings indicate that Lys-303 contributes significantly to voltage sensing, and that the K303Q mutation stabilizes a closed state of the channel.

Ser-306 is located between the 5th and 6th basic residues of the S4 domain. It has been postulated that this Ser might contribute to the unusual voltage dependence of HCN channel gating (6, 7). S306Q ntHCN2 channel current was almost 9-fold smaller than ntHCN2 current. In addition, S306Q ntHCN2 channel current was largely instantaneous, with only a very small time-dependent component (Fig. 5C). Current activated by negative pulses was nearly all time-dependent for WT channel current, whereas the time-dependent component of S306Q ntHCN2 channel current accounted for only 10% of the total current. Substitution of Ser-306 with a relatively conserved residue (S306T) induced a current with properties similar to S306Q ntHCN2, whereas substitution with either an Arg or Tyr resulted in complete loss of function (not shown). These data indicate that Ser-306 is crucial for normal gating.

R312Q ntHCN2 channel current was similar to ntHCN2 channel current, but had an amplitude that was $-5$-fold less. This reduction was not caused by a shift in the voltage dependence of channel activation, because the $V_{0.5}$ for activation of R312Q HCN2 channels ($-74.6 ± 1.2$ mV) was shifted by $+10$ mV compared with ntHCN2 (Table I).

**Mutation of Arg-309, Arg-315, or Arg-318 Causes Loss of Channel Function**—In contrast with the mutations discussed above, mutation of Arg-309, Arg-315, or Arg-318 to Gln caused a complete loss of channel function, defined as lack of measurable current in response to a hyperpolarizing pulse to $-140$ mV. Other amino acid substitutions for some of these residues (R315A, R315C, R318A, R318W) also caused loss of channel function (not shown). These mutations might disrupt subunit folding, prevent normal gating, or shift the voltage dependence of activation to very negative potentials.

**Surface Expression of S4 Mutants with Partial or Complete Loss of Channel Activity**—Partial or complete loss of function in mutant HCN2 channels could result from altered gating properties (e.g., lack of channel opening, reduction in single channel conductance, or open probability), or defects in folding and/or trafficking that cause reduced surface expression of mature protein. To help distinguish between these possibilities, the surface expression of WT and mutant ntHCN2 channels containing a HA-epitope tag was determined using a single oocyte chemiluminescence technique (22). Luminometry experiments were performed on oocytes expressing mutant ntHCN2 constructs that either poorly expressed (K303Q, S306Q, R312Q) or exhibited complete loss of function (R309Q, R315Q, R318Q).

The HA epitope was inserted into the S3–S4 linker of ntHCN2 channels as described under "Experimental Procedures." This construct (ntHCN2-HA) functionally expressed in oocytes, but had a shifted voltage dependence of activation. The $V_{0.5}$ and slope factor for activation was $-114 ± 0.7$ mV and 6 ± 0.3 mV, respectively, when determined with 6-s pulses ($n = 9$). Although $V_{0.5}$ was shifted by $-29$ mV, the amplitude of fully activated current at $-130$ mV for ntHCN2-HA was not changed compared with untagged ntHCN2 (Fig. 6). Thus, addition of the HA epitope altered the gating of ntHCN2 but did not significantly change surface expression of the channel.
The chemiluminescence signal (in RLU) of oocytes expressing nHCN2-HA was more than 1000-fold the signal from oocytes expressing untagged nHCN2 channels. The surface expression of HA-tagged K303Q or S306Q mutant channels was only partially reduced when compared with nHCN2-HA (Fig. 7A). The relative surface expression of protein was reduced 28% for K303Q channels and 14% for S306Q channels. These data suggest that channels containing either mutation are able to fold and traffic to the surface membrane relatively normally but that gating associated with channel opening is dysfunctional.

The surface expression of HA-tagged R312Q channels was reduced by ~4-fold, consistent with the current reduction caused by this mutation. This correlation indicates that the reduction of current amplitude for R312Q was due to the partial loss of surface expression and not an alteration in single channel properties. Mutant channels that exhibited complete loss of functional expression (R309Q, R315Q, and R318Q) all had reduced levels of surface expression (Fig. 7A). The large reduction (94%) in surface expression of R309Q channels sufficiently explains the lack of measurable currents. However, there was a reduction in surface expression for R315Q (75%) and R318Q (54%) was not enough to explain the absence of currents in oocytes expressing these mutant channels. Current at −130 mV averaged 20 μA in oocytes injected with 75 ng of cRNA encoding nHCN2 channels. Even a reduction of 75% of this current would be easily measurable. Thus, neutralization of Arg-315 or Arg-318 prevents normal channel opening, in addition to reducing surface expression of protein.

**Mutation of Asp Residues Located in S2 or S3 Domains Cause Loss of Channel Function**—It has been proposed that interaction of the S2 and S3 domains with the S4 domain of Kv channels facilitates subunit folding and modulates voltage sensing (13, 17, 18). The S2 and S3 domains of HCN2 channel subunits each contain 2 Asp residues, Asp-225 and Asp-231 in S2, and Asp-267 and Asp-275 in S3. The position of these residues is conserved in HERG and similar to the relative location of Asp and Glu residues in Shaker channels (Fig. 1). The Asp residues of nHCN2 were mutated to Asn to neutralize, but not significantly alter size, of the amino acid. Neutralization of any Asp residue caused loss of channel function, defined as lack of measurable currents in response to a hyperpolarizing pulse to −140 mV.

The surface expression of the S2 and S3 mutant nHCN2 channels was also determined using the single cell luminometry technique. The D225N mutation reduced surface expression by 98% (Fig. 7B), fully explaining the inability to record currents in oocytes injected with cRNA encoding this mutant channel. HA-tagged channels containing the other single mutations (D231N, D267N, and D275N) reduced surface expression by 58–75% (Fig. 7B), not enough to explain the lack of

**Table I**

| Construct | $V_{0.5}$ | $k$ | $n$ | Shift |
|----------|----------|-----|-----|-------|
| HCN2     | -73.0 ± 0.3 | 10.0 ± 0.2 | 7 | +11.6 |
| nHCN2    | -84.6 ± 0.2 | 7.2 ± 0.2 | 5 |        |
| K291Q    | -92.1 ± 0.3 | 8.5 ± 0.2 | 10 | -7.5  |
| K291E    | -103.9 ± 0.7 | 9.1 ± 0.3 | 7 | -19.3 |
| R294Q    | -85.4 ± 0.2 | 8.8 ± 0.3 | 6 | -13.3 |
| R297Q    | -101.4 ± 1.2 | 9.5 ± 0.5 | 6 | -16.8 |
| K303Q    | ND        |       |    |       |
| R309Q    | No expression | | | |
| R315Q    | No expression | | | |
| R318Q    | No expression | | | |
| R315Q/A  | No expression | | | |
| S360Q/T  | ND        |       |    |       |
| R309Q/T  | ND        |       |    |       |
| Quad mut | -133.7 ± 1.2 | 19.2 ± 1.7 | 8 | -49.1 |

$V_{0.5}$, half-point of activation curve; $k$, slope of activation curve; $n$, number of oocytes; Shift, change in $V_{0.5}$ relative to nHCN2 channel current; ND, not determined, because construct was too far shifted (R309Q) or tail currents were too small (S306Q).
FIG. 5. Mutation of Lys-303 or Ser-306 in the S4 domain of ntHCN2 channels causes marked changes in gating. A, K303Q ntHCN2 channel current in response to 3-s pulses from −150 to −70 mV, followed by a 1-s pulse to −130 mV. The holding potential was −30 mV. The lower panel shows currents in response to the same pulse protocol in an uninjected oocyte. The dotted line represents zero current level. B, current-voltage relationships for ntHCN2 (n = 6), K303Q ntHCN2 (n = 10), and uninjected oocytes (n = 4) isolated from the same frog. Currents were measured at the end of the 3-s activating pulse. C, S306Q ntHCN2 channel current in response to 3-s pulses from −140 to −40 mV, followed by a 1-s pulse to −130 mV. The holding potential was −30 mV. The lower panel shows currents in response to the same pulse protocol in an uninjected oocyte. D, current-voltage relationships for S306Q (n = 11) and uninjected oocytes (n = 11) isolated from the same frog. Currents were measured at the end of the 3-s activating pulse.

FIG. 6. Relative current amplitude for mutant and HA-tagged ntHCN2 channels. Currents were measured at −130 mV following a 3-s pulse to −140 mV. For comparative purposes, current amplitude for each mutant channel was normalized to that measured for ntHCN2 channel current. *, significantly different from ntHCN2, p < 0.001.

FIG. 7. Effects of point mutations in the S2, S3, and domains on surface expression of ntHCN2 channels determined by single cell luminescence. A, relative chemiluminescence of ntHCN2 S4 domain mutant channels. Relative light units (RLU) for each mutant were normalized to the signal obtained with oocytes from the same frog expressing HA-tagged ntHCN2 channels (RLU = 280,100 ± 20,700; n = 23). B, relative chemiluminescence of S2 and S3 domain HA-tagged ntHCN2 mutants. RLUs were normalized to the value for HA-tagged ntHCN2 channels (RLU = 183,480 ± 10,850; n = 24). *, significantly different from ntHCN2-HA, p < 0.001.

measurable currents. Thus, Asp-231 in the S2 domain, and Asp-267 and Asp-275 in the S3 domain, must have important roles in folding and gating of the channel.

**DISCUSSION**

The 4 Basic Residues Located at the N-terminal End of the Putative S4 Domain Play No Role in Channel Folding and a Limited Role in Gating—Lys-291, Arg-294, Arg-297, and Arg-300 are located in the outermost portion of the putative S4 domain. Substitutions of these basic residues with Gln produced functional channels with whole-cell current amplitude similar to WT ntHCN2. Individual mutation of these 4 basic residues to Gln caused a hyperpolarizing shift in the voltage dependence of channel opening with a trend toward a more pronounced shift for residues further away from Lys-291. More interestingly, combining all four mutations in a single channel had a cumulative effect. The shift in voltage dependence for activation of the quadruple mutant ntHCN2 channel (−49 mV) was similar to the addition of the shifts caused by each single mutation. The relative positions of the G-V relationships along the voltage axis reflects the stability of closed and open states and does not indicate a change in net gating charge. Further studies are required to examine the effect of these mutations on gating valence (z_g). Even if neutralization of Lys-291, Arg-294, Arg-297, or Arg-300 does not reduce z_g, the observed shift in the charge hypothesis was the finding that mutation of Lys-291 to Gln shifted the G-V relationship by −7.5 mV, whereas mutation to a negatively charged Glu shifted the relationship by −19 mV. However, we found that elevation of [Mg^{2+}], caused similar shifts in the V_{0.5} of WT and mutant HCN2 channel currents. Although screening of excess negative surface charge with Mg^{2+} did not cause the expected shifts in the G-V relationship, this may be because Mg^{2+} could not...
Charged Residues of HCN2

substitute for the positively charged residues of the protein, which may be shielded from the extracellular bulk solution by hydrophobic residues in the S3–S4 linker. Another possibility is that all 4 basic residues are situated outside the membrane field and mutation alters the voltage dependence of gating by an unknown mechanism. Whatever the mechanism for the shift in the G-V curves by mutation of the most N-terminal basic residues of the putative S4 domain, it seems reasonable to conclude that these residues play no role in subunit folding and only limited roles in voltage-dependent gating. By contrast, in Shaker channels, individual neutralization of each of the first 4 residues produced a variable effect on the voltage dependence of activation, with leftward shifts for some and rightward shifts for other residues (24, 25).

SPIH is a hyperpolarization-activated cation channel with a much more positive range of activation than HCN channels. The S4 domain of SPIH channels has an acidic residue (Glu) in a position equivalent to Lys-291 in HCN2 channels. However, this substitution does not account for the much more positive range of activation for SPIH, because K291E HCN2 channels activated at even more negative potentials than WT HCN2.

Ser-306 and 5 Basic Residues in the S4 Domain Are Critical for Normal Gating of HCN2 Channels—HCN channels are unusual in having a Ser residue (Ser-306 in HCN2) located in a position that is otherwise a basic residue in Kv channels. The surface expression of S306Q nHCN2 channels was relatively normal compared with WT channels, but the current magnitude was reduced 9-fold. Moreover, current activated by hyperpolarization was nearly instantaneous. These data suggest that Ser-306 is essential for channel gating but not for subunit folding.

The surface expression of K303Q nHCN2 channels was similar to WT nHCN2 channels, but the voltage-dependent activation was very slow and shifted in the hyperpolarized direction. Because of interference from endogenous Ca²⁺-activated Cl⁻ currents, we could only accurately record currents for this mutant channel at potentials positive to -150 mV. Thus, Lys-303 may be an important contributor to voltage sensing and may carry a significant amount of gating charge. Comparison of gating currents for WT and K303Q HCN2 channels will be required to confirm this possibility.

Mutations of three basic residues (Arg-309, Arg-315, and Arg-318) in the S4 domain caused loss of channel function. The loss of channel activity for Arg-309 results largely from a folding and/or trafficking defect as indicated by the 94% reduction in surface expression. However, loss of channel function by the R315Q and R318Q HCN2 mutations cannot be explained by the same mechanism, because these mutations only reduced surface expression of protein by 74% and 54%, respectively. These findings indicate either that these mutant channels were incapable of opening or that the voltage dependence of their activation was far shifted in the negative direction.

State-dependent salt bridge formation between Lys-374 and Arg-377 in S4 and Glu-283 and Glu-293 of S2 or Asp-316 of S3 in Shaker was proposed to be important for channel stabilization. Similar electrostatic interactions between Arg-315 and Arg-318 and 1 or more acidic residues of the S2 and S3 domains of HCN2 may also be important for stabilization of folded subunits and channel gating. Further studies are required to define the mechanisms of channel dysfunction caused by mutation of these basic residues. However, it is clear that Arg-315 and Arg-318 are crucial residues for normal function of HCN2 and that Arg-309 has a critical role in channel folding and may also be important in gating.

The substantial decrease in current magnitude caused by neutralization of Arg-312 could be explained by a concomitant reduction in surface expression of protein. The remaining current had relatively normal gating properties, suggesting that this residue may have a key role in stabilization of subunit folding but probably is not critical for gating.

KAT1 is a K⁺ channel cloned from the plant Arabidopsis thaliana (26). These channels open slowly in response to hyperpolarization and also have overall structural similarity to the HCN channels. However, in contrast with HCN, neutralization of two charged residues in the S4 domain of KAT1 (R176L, R177Q) shifted the voltage dependence of activation by +89 and +54 mV, respectively (27). Another difference in the gating of KAT1 and HCN channels is the role of the N terminus. Deletion of half of the N terminus (Δ2–34) of KAT1 eliminated currents, and a smaller deletion (Δ20–34) caused a -65 mV shift in the voltage dependence of activation (28). It was proposed that the N terminus contributes to the voltage sensitivity of KAT1 by contributing to the electric field sensed by the voltage sensor (29). By contrast, we found that deletion of two-thirds of the N terminus (Δ2–130) of HCN2 caused only a -12 mV shift. Thus, the N-terminal domain does not appear to have a crucial role in the gating of HCN2 channels as proposed for KAT1 channels.

Acidic Residues Located in S2 and S3 Domains Are Required for Proper Channel Function—D225 located in the S2 domain of HCN2 is required for normal channel folding and/or trafficking. Neutralization of this residue to Asn reduced surface expression of the protein by 98%. In contrast, mutations of Asp-231 in S2 and the two Asp residues in S3 (Asp-267, Asp-275) to Asn only partially reduced surface expression of mutant protein, despite a total loss of channel function. The homologous acidic residues in voltage-gated K channels are more tolerant of mutation. In these channels, mutation interferes with protein folding and therefore reduces channel expression, but channels that reach the membrane appear to have relatively normal gating properties. For example, channel function was maintained after neutralization of all 3 of the acidic residues located in the S2 and S3 domains (E283Q, E293Q, and D316N) of Shaker (13). Neutralization of all the acidic residues in the S2 and S3 domains was well tolerated in drk1 channels (16), as were two of four negative charges (Glu-235 and Glu-272 but not Glu-225 and Asp-258) in Kv.1.1 channels (14). These findings indicate that acidic residues located in the S2 and S3 domains are more important for normal channel gating and/or folding and trafficking to the surface membrane in HCN2 channels than in Kv channels. Further experiments are needed to determine if the loss of channel function caused by these mutations in HCN2 results from disruption of charge pairing between these acidic residues and basic residues in the S4 domain, as previously reported for Shaker channels (13, 17, 18).

A Topology Model for the Voltage-sensing Domains of HCN2—HCN channels have a similar overall structure to the voltage-gated potassium channels, including six transmembrane domains, a similar selectivity filter, and the presence of multiple basic residues in S4. However, HCN channels have a greater number (9) of basic residues (plus 1 Ser) located at every third position in the putative S4 domain compared with Kv channels that have only 5 to 7 basic residues. Assuming the S4 of HCN2 is an α-helix, then the entire domain is too long to fit within the plane of the plasma membrane. Based on the discussion above, we propose that the region containing Lys-291 to Arg-300 is located outside of the membrane, while the lower portion of S4 (from Lys-303 to Arg-318) lies within the membrane field when the channel is in the closed state. Channel activation in response to membrane hyperpolarization is likely to involve a twisting and/or translocation of the S4 domain across the electric field of the membrane (30, 31). This
proposed topology for the S4 domain of HCN channels predicts potential electrostatic interactions between the basic residues of the S4 and S2/S3 domains. The relative position of the negatively charged residues in S2 and S3 are conserved among HCN2, HERG, and Shaker B channels (Fig. 1), suggesting these residues may have a conserved role in subunit folding and/or channel gating.

In conclusion, our findings suggest that the charged residues of the S2, S3, and S4 domains have distinct functional roles in the structure and gating of HCN2 channels. Lys-291, Arg-294, Arg-297, and Arg-300 play a relatively minor role in gating, and are not important for channel folding or trafficking to the surface membrane. Lys-303 and Ser-306 are essential for normal gating but not for subunit folding or channel trafficking. Arg-312 is important for folding but not gating. The charged residues located at the C-terminal end of the S4 domain (Arg-309, Arg-315, and Arg-318) and the S2 and S3 domains (Asp-225, Asp-231, Asp-267, Asp-275) are crucial for normal protein folding and/or gating. Further studies will determine if some of these basic residues charge-pair with Asp residues located in the S2 and S3 domains.

Acknowledgments—We thank Mike Martinez for technical assistance and Dr. N. Zerangue for sharing details of the chemiluminescence technique.

REFERENCES
1. DiFrancesco, D. (1993) Annu. Rev. Physiol. 55, 455–472
2. Santoro, B., Liu, D. T., Yao, H., Bartsch, D., Kandel, E. R., Siegelbaum, S. A., and Tibbs, G. R. (1998) Cell 93, 717–729
3. Ludwig, A., Zong, X., Jeglitsch, M., Hofmann, F., and Biel, M. (1998) Nature 393, 587–591
4. Gauss, R., Seifert, R., and Kaupp, U. B. (1998) Nature 393, 587–591
5. Biel, M., Ludwig, A., Zong, X., and Hofmann, F. (1999) Rev. Physiol. Biochem. Pharmacol. 136, 165–181
6. Clapham, D. E. (1998) Neuron 21, 5–7
7. Santoro, B., and Tibbs, G. R. (1999) Ann. N. Y. Acad. Sci. 868, 741–764
8. Miller, A. G., and Aldrich, R. W. (1996) Neuron 16, 853–858
9. Sanguinetti, M. C., and Xu, Q. P. (1999) J. Physiol. 514(Pt 3), 667–675
10. Miteson, J. S., Chen, J., and Sanguinetti, M. C. (2000) J. Gen. Physiol. 115, 229–240
11. Sigworth, F. J. (1993) Quart. Rev. Biophys. 27, 1–40
12. Bezanilla, F. (2000) Physiol. Rev. 80, 555–592
13. Papazian, D. M., Shao, X. M., Sech, S.-A., Mock, A. F., Huang, Y., and Wainstock, D. H. (1995) Neuron 14, 1293–1301
14. Planells-Cases, R., Ferrer-Montiel, A. V., Patten, C. D., and Montal, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9424–9426
15. Seoh, S.-A., Sigg, D., Papazian, D. M., and Bezanilla, F. (1996) Neuron 16, 1159–1167
16. Li-Smerin, Y., Hackos, D. H., and Swartz, K. J. (2000) J. Gen. Physiol. 115, 33–49
17. Tiwari-Woodruff, S. K., Schultheis, C. T., Mock, A. F., and Papazian, D. M. (1997) Biophys. J. 72, 1489–1500
18. Tiwari-Woodruff, S. K., Lin, M.-c. A., Schultheis, C. T., and Papazian, D. M. (2000) J. Gen. Physiol. 115, 123–138
19. Sarkar, G., and Sommer, S. S. (1996) BioTechniques 8, 404–407
20. Goldin, A. L. (1991) Methods Cell Biol. 36, 487–509
21. Goldin, A. L., and Sumikawa, K. (1992) Methods Enzymol. 207, 279–296
22. Zerangue, N., Schwappach, B., Jan, Y. N., and Jan, L. Y. (1999) Neuron 22, 537–548
23. Stuhmer, W. (1992) Methods Enzymol. 207, 319–339
24. Papazian, D. M., Timple, L. C., Jan, Y. N., and Jan, L. Y. (1991) Nature 349, 305–310
25. Liman, E. R., Hess, P., Weaver, F., and Koren, G. (1991) Nature 353, 752–756
26. Anderson, J. A., Huprikar, S. S., Kochian, L. V., Lucas, W. J., and Gaber, R. F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3736–3740
27. Zet, P. C., and Aldrich, R. W. (1998) J. Gen. Physiol. 112, 679–713
28. Marten, I., and Hoshi, T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3448–3453
29. Marten, I., and Hoshi, T. (1998) Biophys. J. 74, 2953–2962
30. Cha, A., Snyder, G. E., Selvin, P. R., and Bezanilla, F. (1999) Nature 402, 809–813
31. Glauer, K. S., Mannuzza, L. M., Gandhi, C. S., and Isacoff, E. Y. (1999) Nature 402, 813–817