Mutations in the S4 domain of a pacemaker channel alter its voltage dependence

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Received 13 June 2000; accepted 30 June 2000

Edited by Maurice Montal

Abstract In an attempt to study the functional role of the positively charged amino acids present in the S4 segment of hyperpolarization-activated cyclic nucleotide-gated cation (HCN) channels, we have introduced single and sequential amino acid replacements throughout this domain in the mouse type 2 HCN channel (mHCN2). Sequential neutralization of the first three positively charged amino acids resulted in cumulative shifts of the midpoint voltage activation constant towards more hyperpolarizing potentials. The contribution of each amino acid substitution was approximately −20 mV. Amino acid replacements to neutralize either the first (K291Q) or fourth (R300Q) positively charged amino acid resulted in the same shift (about −20 mV) towards more hyperpolarized potentials. Replacing the first positively charged amino acid with the negatively charged glutamic acid (K291E) produced a shift of approximately −50 mV in the same direction. None of the above amino acid substitutions had any measurable effect on the time course of channel activation. This suggests that the S4 domain of HCN channels critically controls the voltage dependence of channel opening but is not involved in regulating activation kinetics. No channel activity was detected in mutants with neutralization of the last six positively charged amino acids from the S4 domain, suggesting that these amino acids cannot be altered without impairing channel function.

Key words: HCN channel; Voltage sensor; Mutation; Electrophysiology

1. Introduction

Cationic currents activated upon cell membrane hyperpolarization modulate the intrinsic electrical activity in the heart [1] and in a variety of neurons [2]. The main conductance underlying this pacemaker activity was identified many years ago and named I_f (f for funny) or I_h (h for hyperpolarization) [3–5].

Recently, four members (HCN1–4) of a gene family of ion channels that carry I_h have been identified (for review, see [6]).

Hydropathicity and sequence analysis revealed that the primary structure of HCN channels is related to that of voltage-gated K^+ channels. Both types of channels are characterized by the presence of six putative transmembrane domains (S1–S6), including a positively charged voltage-sensing S4 segment and an ion-conducting pore between S5 and S6. Notably, the S4 segment of HCN channels contains 10 positively charged amino acids [7–10], whereas S4 segments of K^+ channels and other members of the voltage-gated cation channel superfamily have only 5–8 positive charges [11].

In spite of having the general motif (voltage sensor) found in the S4 domain of voltage-gated cation channels, HCN channels are activated by hyperpolarization, rather than by cell membrane depolarization. The mechanism underlying this difference remains largely unknown.

Undoubtedly, the activation of I_h by membrane hyperpolarization is an essential requirement for the slow depolarization phase observed at the end of action potentials in the heart [1], and for the firing properties of many neurons [2]. Therefore, understanding the mechanism of voltage activation in these channels would provide essential clues on how pacemaker activity is regulated.

We have produced single and sequential mutations in the S4 segment of the mouse HCN2 channel (mHCN2; [7]) in an attempt to identify the structural motifs responsible for its voltage dependence. Neutralization of the first three positively charged amino acids from this region resulted in an increment of the channel voltage dependence by shifting the midpoint activation constant to more negative values. Replacing positively charged amino acids with glutamine resulted in approximately −20 mV shift/amino acid towards more hyperpolarizing potentials.

The results presented here demonstrate that: (1) the S4 segment of HCN channels is part of the voltage-sensing machinery, (2) sequential neutralization of the first three positively charged amino acids from the S4 segment produce an additive −20 mV/amino acid shift towards more hyperpolarizing potentials in the midpoint activation of the channel, (3) mutations of the last six positively charged amino acids result in silent channels, (4) it is possible to affect dramatically the channel voltage dependence of HCN channels without altering the activation kinetics, indicating that the S4 domain does not contribute significantly to the modulation of these channel kinetics.

2. Materials and methods

2.1. Solutions and reagents

All salts used in this study were analytical grade (Sigma Chemical Co., St. Louis, MO, USA).

Ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) was purchased from Sigma. HEPES was obtained from Calbiochem.

The intracellular (pipette) solution contained (mM): 145 L-aspar-

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0014-5793/00/$20.00
PII: S0014-5793(00)01837-8

tatic acid potassium salt, 2 magnesium chloride, 2 EGTA and 10 HEPES.

The extracellular (bath) solution contained (mM): 145 sodium chloride, 5 potassium chloride, 2 calcium chloride, 2 magnesium chloride, 10 HEPES.

2.2. Preparation of mutants

Conventional PCR techniques with oligonucleotides purchased from MWG-Biotech GmbH (Ebersberg, Germany) were used to introduce mutations into the sequence of mHCN2 (original nomenclature: HAC1; [7]). Briefly, mutant clones were produced in the pcDNA3 vector (Invitrogen) by replacing the 390 bp *Acc*I–*Blp*I fragment of the wild type mHCN2 expression plasmid [7] by a PCR-generated fragment containing the respective mutations. The correctness of all mHCN2 mutants was verified by sequencing.

2.3. Cells

Human embryonic kidney (HEK293) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Gibco), 100 μg/ml streptomycin and 25 ng/ml amphotericin B (Gibco) at 37°C in a humidity-controlled incubator with 5% CO_2. Cells were used from passages 3–5. HEK cells between 20 and 40% confluency were transfected with the cDNA for either wild type or mutant expression plasmids using conventional calcium-phosphate transfection protocols.

2.4. Patch clamp experiments

Currents were recorded from transfected HEK293 cells with the patch clamp technique in the whole-cell configuration using an EPC-9 amplifier (HEKA Corporation, Germany), Patch pipettes were made of borosilicate glass (Clark Electromedical Instruments, Inc.) and pulled to obtain pipette resistances in the range of 4–6 MΩ when measured in experimental solutions. Voltage clamp data were stored on the computer hard disk for off-line analysis using Pulse software (HEKA Corporation, Germany). Data were acquired at 10 kHz with an 8-pole Bessel filter and digitized at 5 kHz. Conductance versus voltage plots were fitted to a Boltzmann equation of the form $y=((A1-A2)/(1+\exp(V-V_{1/2})/k))+A2$. Activation constants were obtained from voltage steps between −100 and −200 mV, the whole-cell currents were fitted to a mono exponential function using Igor 3.12 software (Wavemetrics, Inc.). The holding potential in all experiments was maintained at −40 mV. In all the experiments to be described, n = number of experiments performed for each condition. All experiments were performed at room temperature (22–25°C).

3. Results

3.1. Structure of HCN wild type channel and mutants

Fig. 1A illustrates a drawing with the putative transmembrane topology of HCN channels. The fourth transmembrane domain (S4) displays a sequence of orderly spaced positively charged amino acids that corresponds to the voltage sensor found in voltage-gated cation channels. All the mutations described in the present study were introduced in this domain. Fig. 1B illustrates the amino acid sequences of the S4 domain from the mHCN2 (WT) channel and all the mutants reported here. For simplicity sake, the letter M and a sequential number have been assigned to all mutants. This code will be used when referring to a mutant throughout the manuscript and in the figures.

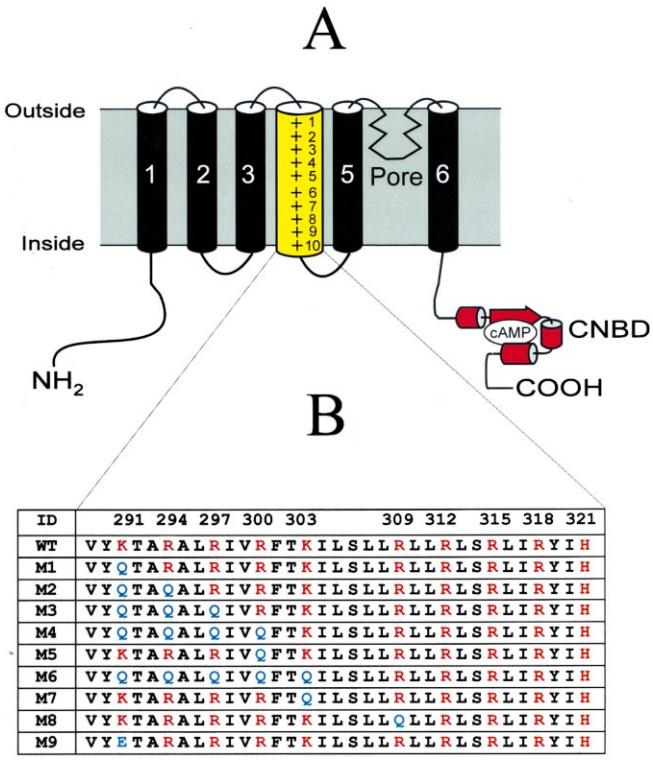
3.2. Neutralization of the first three positively charged amino acids in the S4 domain results in sequential shifts in the midpoint voltage activation constant

Fig. 2A shows the whole-cell currents obtained from HEK293 cells expressing either the wild type mHCN2 (WT, left) or the M3 mutant (right). From experiments like these, a family of curves were obtained from the tail currents. Fig. 2B illustrates the normalized amplitude versus voltage obtained from the tail currents from WT channel and the three mutants M1–M3. All curves could be well fitted by a Boltzmann equation from which the midpoint activation constants were obtained.

The midpoint activation constant found for the WT channel from the Boltzmann fit was −99 mV ($n=15$), which is close to the value we had previously reported for this channel [7].

Sequential neutralizations of the first three positively charged amino acids found in the S4 segment resulted in subsequent shifts in the midpoint activation constants (Fig. 2B). Replacing the first lysine with glutamine (K291Q) in the M1 mutant shifted the midpoint activation constant to −118 mV ($n=21$). The double mutation (K291Q and R294Q) in M2 produced a shift to −143 mV ($n=24$). The triple mutation in M3 (K291Q, R294Q and R297Q) resulted in a midpoint activation constant of −169 mV ($n=20$). The sequential shift in the activation constants observed in the M1–M3 mutants when compared to WT indicated that each amino acid substitution contributed between 19 and 26 mV to the shift.

Neutralization of the next positively charged amino acid (R300Q) in the M3 mutant to produce the quadruple mutant M4 (K291Q, R294Q, R297Q and R300Q) resulted in loss of function. No channel activity could be detected in the M4 mutant in 12 independent transfections performed with this construct, indicating that the neutralization of the first four



ID	291	294	297	300	303	309	312	315	318	321
WT	V	Y	K	T	A	R	A	L	R	I	V	R	F	T	K	I	L	S	L	L	R	L	L	R	L	S	R	L	I	R	Y	I	H
M1	V	Y	Q	T	A	R	A	L	R	I	V	R	F	T	K	I	L	S	L	L	R	L	L	R	L	S	R	L	I	R	Y	I	H
M2	V	Y	Q	T	A	Q	A	L	R	I	V	R	F	T	K	I	L	S	L	L	R	L	L	R	L	S	R	L	I	R	Y	I	H
M3	V	Y	Q	T	A	Q	A	L	Q	I	V	R	F	T	K	I	L	S	L	L	R	L	L	R	L	S	R	L	I	R	Y	I	H
M4	V	Y	Q	T	A	Q	A	L	Q	I	V	Q	F	T	K	I	L	S	L	L	R	L	L	R	L	S	R	L	I	R	Y	I	H
M5	V	Y	K	T	A	R	A	L	R	I	V	Q	F	T	K	I	L	S	L	L	R	L	L	R	L	S	R	L	I	R	Y	I	H
M6	V	Y	Q	T	A	Q	A	L	Q	I	V	Q	F	T	Q	I	L	S	L	L	R	L	L	R	L	S	R	L	I	R	Y	I	H
M7	V	Y	K	T	A	R	A	L	R	I	V	R	F	T	Q	I	L	S	L	L	R	L	L	R	L	S	R	L	I	R	Y	I	H
M8	V	Y	K	T	A	R	A	L	R	I	V	R	F	T	K	I	L	S	L	L	Q	L	L	R	L	S	R	L	I	R	Y	I	H
M9	V	Y	E	T	A	R	A	L	R	I	V	R	F	T	K	I	L	S	L	L	R	L	L	R	L	S	R	L	I	R	Y	I	H

Fig. 1. A,B: Amino acid sequence of the S4 domain from mHCN2 channel and mutants. A illustrates a drawing representing the putative topology of HCN channels. Each of the six transmembrane helices is shown (1–6). The S4 domain is shown in yellow with the 10 positively charged amino acids (+1 to +10). The location of the cyclic nucleotide binding domain (CNBD) is also illustrated in red. B shows the amino acid sequences for the mHCN2 wild type (WT) channel and the mutants reported in this study (M1–M9). In red are shown the positively charged amino acids with the sequence number indicated above. In blue are shown the amino acid substitutions for the different mutants.

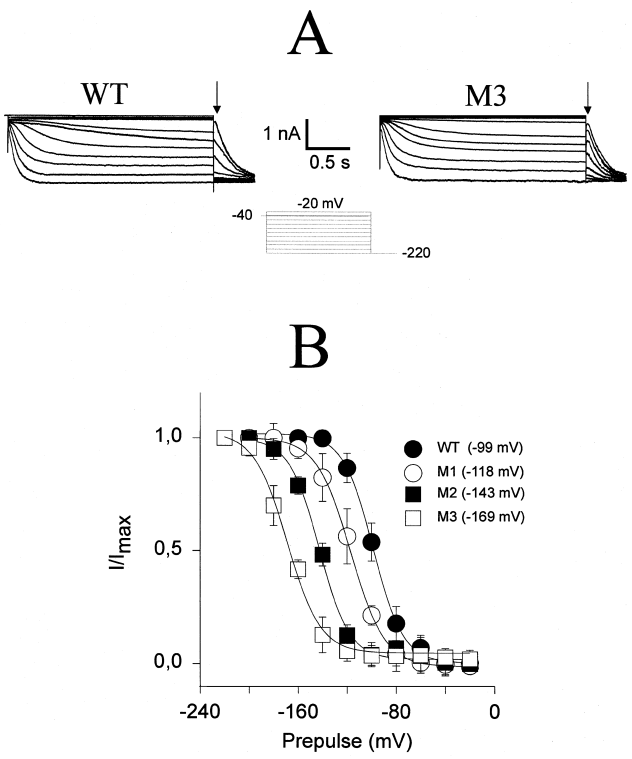


Fig. 2. A,B: Mutations on the first three positively charged amino acids shift the activation curves. A shows representative examples of currents from HEK293 cells expressing the mHCN2 channel (WT) or the M3 mutant (K291Q, R294Q and R297Q). The vertical arrow shows the point at which the amplitude of the tail currents was measured. Currents were elicited with the voltage protocol shown in the inset. The holding potential was −40 mV and the voltage was switched for 2 s from −220 mV to −20 mV. The tail currents were obtained after returning to −220 mV. B illustrates the normalized current versus voltage plot for the WT, M1 (K291Q), M2 and M3 mutants. The solid line shows the fit to a Boltzmann function with midpoint activation constants shown in parentheses for each construct. The steepness of the curve obtained from the fit was WT = 12.23 ($n = 15$), M1 = 13.74 ($n = 21$), M2 = 13.48 ($n = 24$) and M3 = 13.68 ($n = 20$).

positively charged amino acids results in a silent channel. However, a single substitution of the fourth positively charged amino acid in the M5 mutant (R300Q) resulted in a functional channel (Fig. 3A), indicating that channel function is lost only when the first four amino acids are simultaneously neutralized.

Further substitutions of positively charged amino acids resulted also in mutants with no detectable channel activity. In over 25 attempts, no channel activity was detected in the M6 mutant in which the first five positively charged amino acids were replaced with glutamines, or the M7 mutant in which only the fifth positively charged amino acid was replaced (K303Q). Similar results were obtained after replacing only the sixth positively charged amino acid in the M8 mutant (R309Q). These results indicate that mutations of lysine 303 or arginine 309 resulted in non-functional channels. Sequential mutations of the remaining positively charged amino acids from the S4 domain resulted also in mutants with no discernible channel activity. These results indicated that only the first four positively charged amino acids could be neutralized without losing channel activity. Mutations on any of the last six positively charged amino acids resulted in loss of channel function. We cannot discard the possibility, however, that the silent mutants had voltage dependence shifted out of the range of our recording system.

3.3. Neutralization of the first or the fourth positively charged amino acid produces a similar shift in the midpoint voltage activation constants

Replacing the first lysine with an glutamine in the mutant M1 produces a shift in the midpoint activation constant to −118 mV as indicated above (Fig. 2B). A similar shift was obtained after replacing the fourth positively charged amino acid (R300Q) in the M5 mutant, which had a midpoint activation constant of −117 mV (Fig. 3A; $n = 22$). In this mutant, the contribution of R300 to the shift in the midpoint activation constant was −18 mV compared to the contribution of −19 mV observed from K291Q in the M1 mutant.

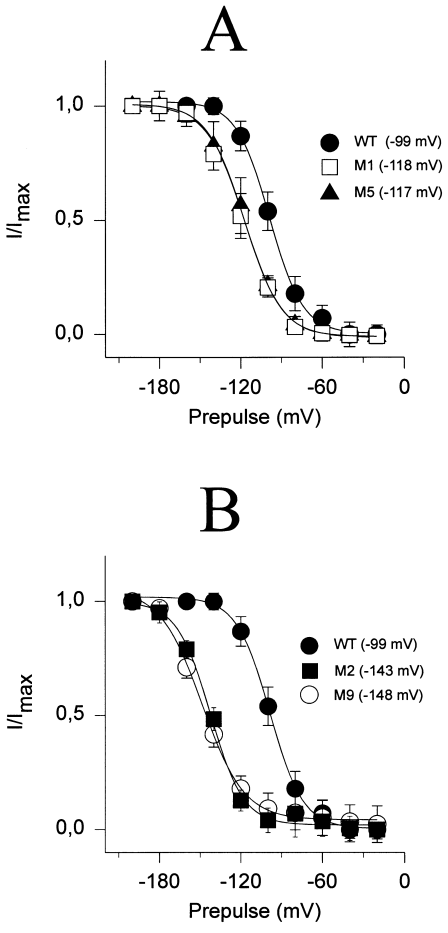


Fig. 3. A,B: Neutralization versus negative charged replacements. A compares the normalized current versus voltage plot for the WT and the M1 (K291Q) and M5 (R300Q) mutants. The midpoint activation constants for the M1 (−118 mV, $n = 21$) and M5 (−117 mV, $n = 22$) mutants were not significantly different from each other ($P > 0.1$). B compares the M2 and M9 (K291E) mutants. The midpoint activation constants for these mutants were similar (shown in parentheses), indicating that replacing the first positively charged amino acid with the negatively charged glutamic acid is equivalent to neutralizing the first two positively charged amino acids with glutamines. The solid line shows the fit to a Boltzmann function with midpoint activation constants shown in parentheses for each construct. The steepness of the curve for both mutants was similar (M9 = 14.04, $n = 25$; M2 = 13.48, $n = 24$; $P > 0.1$).

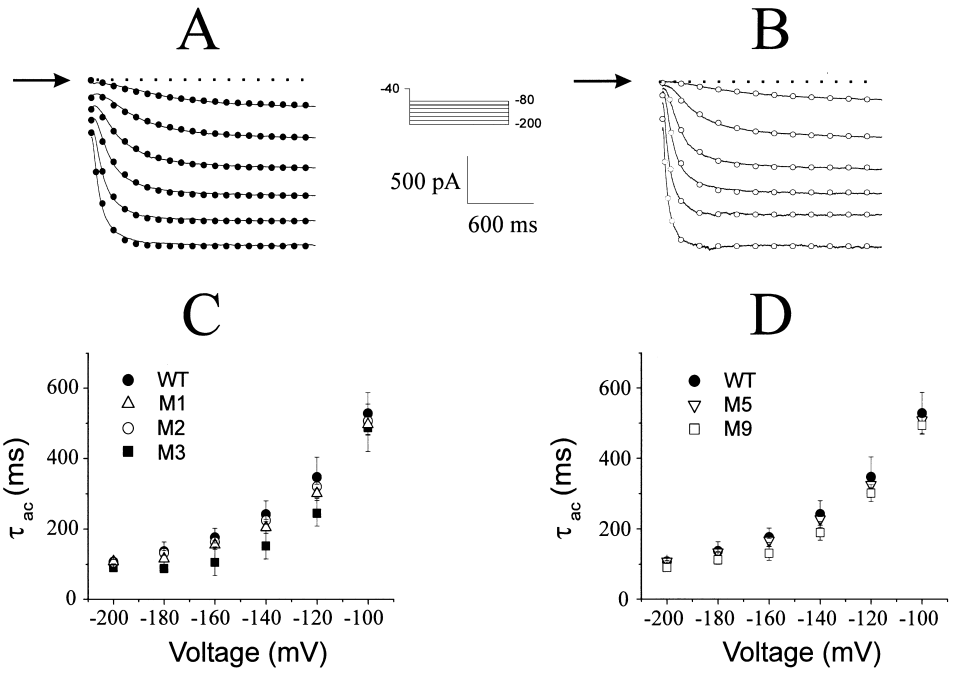


Fig. 4. A–D: The activation constants of all mutants are similar to mHCN2 wild type channel. The currents obtained from voltage pulses between −200 and −100 mV (holding potential = −40 mV) were fitted to mono exponential functions to obtain the activation constants as previously described [12]. A shows the currents from the WT and B from the M3 mutant. The symbols show the fit and the solid lines the current. C plots the activation constants obtained between −200 and −100 mV for the WT ($n = 12$), M1 ($n = 6$), M2 ($n = 8$) and M3 ($n = 7$) mutants. D shows the plots obtained for the WT, M5 ($n = 11$) and M9 ($n = 8$) mutants. No significant differences between the WT and the mutants activation constants were observed ($P > 0.07$).

3.4. *Replacing the first positively charged amino acid with a negatively charge amino acid is equivalent to neutralizing the first two positively charge amino acids*

Fig. 3B illustrates the activation curves obtained from the tail currents of the mutant M9 (K291E) and the double mutant M2 (K291Q and R294Q). The midpoint activation constant for the mutant M9 was −148 mV ($n = 25$), which is close to the value obtained for M2 (Fig. 1C; −143 mV). Replacing the lysine 291 with the negatively charged glutamic acid (M9 mutant) produced a shift in the midpoint activation constant equivalent to neutralizing two consecutive positively charged amino acids (M2 mutant). The difference in the midpoint activation constants from the two mutants is not statistically significant ($P > 0.1$).

3.5. *HCN channel activation kinetics are not affected by mutations in the S4 segment*

Activation kinetics were assessed for the WT and all mutants showing channel activity. The activation constants were obtained after fitting the currents from experiments like those shown in Fig. 4A–B with mono exponential functions as previously described [12].

Fig. 4C shows a family of curves with the activation constants obtained between −100 and −200 mV for WT ($n = 12$) and M1 ($n = 6$), M2 ($n = 8$) and M3 ($n = 7$) mutants, while Fig. 4D shows the curves for WT, M5 ($n = 11$) and M9 ($n = 8$) mutants. All mutants had activation constants which were statistically indistinguishable from the constants obtained with the WT channel.

These results indicate that mutations on the S4 segment of mHCN2 have profound effects on the channel voltage dependence without affecting channel activation, therefore, channel activation is likely to be controlled by a different structure in the channel.

4. Discussion

The fourth transmembrane domain (S4) of voltage-gated channels contains between four and eight positively charged residues (lysines or arginines) separated from each other by two hydrophobic amino acids [11]. This type of structure allows the formation of an α helix in which the positively charged amino acids are lining next to each other. It is well established that in voltage-gated K^+, Na^+ and Ca^{2+} channels, the S4 domain constitutes the main part of the voltage sensor [11,13–15]. The S4 segment found in HCN channels is unique among voltage-gated channels because it contains 10 basic residues, two more than the maximal number found in other channels.

A wealth of information obtained from many studies performed with fluorescence labels, neutralization of positively charged amino acids and histidine-scanning mutagenesis reveals a clear picture of how the S4 domain may sense and move upon a change in transmembrane voltage [11]. A model elaborated from these studies suggests that the first four positively charged amino acids in *shaker* are lying in a narrow aqueous crevice accessible to the intracellular medium. Membrane depolarization would force these charges to move outwardly or to rotate to an extracellularly accessible aqueous crevice without a very large movement of the S4 segment [14]. The translocation of the S4 domain produces a gating current and would result in the opening of the channel [11,13–15].

Studies performed over the last few years measuring the

gating current of *shaker* type K^+ channels have shown that neutralization of the first four positively charged amino acids results in reduced gating currents [11,13]. All the first four amino acids appear to contribute equally to the gating current [11,13–15]. Neutralization of the fifth or sixth positively charged amino acid results in loss of function [11,13,15].

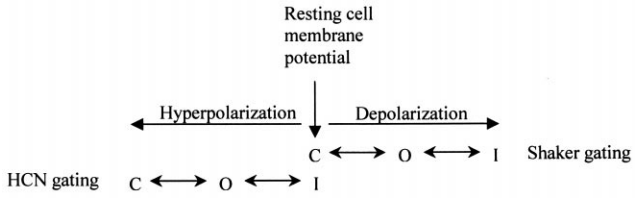
Although we have not been able to measure thus far gating currents from HCN channels, the results presented here support the notion that the S4 domain is part of the voltage sensor in these channels. Neutralization of the first three positively charged amino acids results in sequential shifts in the channel voltage dependence towards more hyperpolarized potentials. Each amino acid contributes equally to the shift in voltage dependence (about −20 mV/amino acid), similarly to the results obtained from measuring gating currents of *shaker* K^+ channels. Furthermore, in our studies, neutralization of the fifth and sixth positively charged amino acids resulted also in channels with no measurable activity in agreement with the studies performed on *shaker* K^+ channels.

Neutralization of positively charged amino acids from the S4 domain in mHCN2 channel resulted in shifts of the midpoint activation constant towards more hyperpolarized potentials without a significant change in the steepness of the curve. The steepness of a Boltzmann curve reflects the number of charges involved in sensing the transmembrane voltage. These numbers can only be interpreted unequivocally in channels having two transitional states. Unfortunately, it is not possible to correlate directly the steepness of the activation curve with the number of charges involved in gating in the case of HCN channels because these channels reveal complex gating kinetics with multiple transitional states [16].

The results presented here indicate that when the net charge in the S4 from mHCN2 is reduced, a stronger cell membrane hyperpolarization is required to open the channel. Interestingly, single, double and triple mutations on the S4 domain of *shaker* K^+ channels progressively shifted the activation curve to more hyperpolarized potentials so that the triple mutant behaved like an inward rectifier K^+ channel [17]. In the same study, the single mutant R365N had a shift in the activation curve of −20 mV towards more hyperpolarized potentials when compared to wild type *shaker*, this value is similar to the value we obtained with the M1 mutant (K291Q), which had a midpoint activation constant shifted −19 mV to more hyperpolarized values when compared to mHCN2 wild type channel.

The conversion from an outwardly rectifying to an inwardly rectifying channel in *shaker* does not appear to involve a change of direction in the movement of the voltage sensor, but rather a change in the resting state of the channel [17]. At rest, *shaker* wild type channels remain in the closed state (C). Membrane depolarization drives the channels to the open state (O) and later to the inactive state (I). The *shaker* mutants described above are apparently resting in the I state. Membrane hyperpolarization drives the *shaker* mutants to the O state and later to the C state [17].

This type of kinetics may also explain the gating and activation properties of inward rectifier K^+ channels, in particular a similar gating model has been proposed for the HERG cardiac K^+ channel [18]. Although there is no experimental evidence indicating that HCN channels have this type of gating scheme, the model described above may accommodate HCN channel gating as well. If such is the case, then the mutations on the S4 domain that we have reported here may stabilize the channel in the I state, which would require a more pronounced hyperpolarization to drive the channels into the O state. The scheme shown below compares the gating of *shaker* with the gating proposed in this study for HCN channels:



In spite of resulting in dramatic changes in the voltage dependence of mHCN2, mutations on the S4 domain did not alter significantly the activation properties of all mutants when compared to WT. This result indicates that the first four positively charged amino acids from the S4 domain do not contribute significantly to the activation of HCN channels. The contribution of the rest of the positively charged amino acids from the S4 domain could not be assessed because the resulting mutants did not show any discernible channel activity.

Taken together, the data indicate that the positively charged S4 segment of HCN channels constitutes a major part of the voltage-sensing machinery of these channels. Although the mechanism of voltage-dependent activation of HCN channels still remains to be elucidated, this study gives a first clue into this issue. Our results favor a mechanism in which the HCN channel is in the inactivated state at resting potentials. Upon hyperpolarization of the plasma membrane, the channel would recover from the inactivated state and switch to the ion-conducting open state. Further studies including investigations into the complex modulation of voltage-dependent channel activation by cyclic AMP will be necessary to verify this putative mechanism.

Acknowledgements: This work was supported by grants from Deutsche Forschungsgemeinschaft und Fond der Chemie. L.V. is the recipient of an Alexander von Humboldt fellowship.

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