A Leucine Zipper Motif Essential for Gating of Hyperpolarization-Activated Channels*

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Background: It is poorly understood how hyperpolarization-activated cyclic nucleotide-gated channels (HCNs) function.

Results: We have identified a leucine zipper in the S5 segment of HCNs, regulating hyperpolarization-activated and instantaneous current components.

Conclusion: The leucine zipper is essential for HCN channel gating.

Significance: The identification and functional characterization of the leucine zipper is an important step towards the understanding of HCN channel function.

SUMMARY

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are pacemakers in cardiac myocytes and neurons. Although their membrane topology closely resembles that of voltage-gated K⁺ channels, the mechanism of their unique gating behavior in response to hyperpolarization is still poorly understood. We have identified a highly conserved leucine zipper motif in the S5 segment of HCN family members. In order to study the role of this motif for channel function, the leucine residues of the zipper were individually mutated to alanine, arginine or glutamine residues. Leucine zipper mutants traffic to the plasma membrane, but the channels lose their sensitivity to open upon hyperpolarization. Thus, our data indicate that the leucine zipper is an important molecular determinant for hyperpolarization-activated channel gating.

Residues of the leucine zipper interact with the adjacent S6 segment of the channel. This interaction is essential for voltage-dependent gating of the channel. The lower part of the leucine zipper, at the intracellular mouth of the channel, is important for stabilizing the closed state. Mutations at these sites increase current amplitudes or result in channels with deficient closing and increased min-Po. Our data are further supported by homology models of the open and closed state of the HCN2 channel pore. Thus, we conclude that the leucine zipper of HCN channels is a major determinant for hyperpolarization-activated channel gating.

Following an action potential, a slow spontaneous depolarization drives specialized pacemaking cells in cardiac and neuronal tissues towards another action potential. This pacemaker current, called Iᶠ in cardiac cells and Ih in the nervous system, is mainly carried by channels referred to as hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (1-2). The HCN channel family comprises four members (3-4). The membrane topology of HCN channels closely resembles that of voltage-gated K⁺ (Kv) channels, as they also have six transmembrane segments (S1-S6), a pore loop with a selectivity filter and a charged S4 segment acting as a voltage-sensor. In contrast to Kv channels, HCN channels open in response to hyperpolarization and are also permeable to Na⁺ (2). The mechanism for this uncommon gating has been the subject of many studies (5-9). However, the
inverse gating phenotype of HCN channels is still poorly understood.

Leucine zipper motifs are found in alpha-helical parts of proteins, mediating the interaction between two alpha-helical domains. Leucine zippers were initially described as highly conserved motifs involved in the interaction of transcription factors with DNA (10). As the interaction of two opposed leucine zippers leads to stabilized, more rigid proteins, the zippers can be of relevance for any form of protein-protein assembly. The motif is x-(Leu-x-x-Y-x-x-x-)n-Leu, where x can be any amino acid, Y is mostly an aliphatic residue and n is the number of repeats, with at least three. While the aforementioned motif is the classical leucine zipper, there is also a modified leucine zipper motif, where any leucine can be replaced by isoleucine or valine.

Leucine zippers in ion channels were previously identified to be of major relevance for several different functions. A leucine zipper in the S4-S5 linker of the drosophila Shaker channel is important for the coupling of S4 movement to opening of the inner pore helix (11). The Ca2+ activated hIK1 channels have a C-terminal leucine zipper essential for proper folding and trafficking of the channel (12). Calcium channels of the Ca1.x-family assemble with the A-kinase-anchoring protein AKAP15 depending on a leucine zipper in the distal C-terminus (13-14). The binding of AKAP15 scavenges protein kinase A to the channel, resulting in increased calcium current amplitudes (14). In CNG channels, which are closely related to HCN channels, a leucine zipper following the cyclic nucleotide binding domain (CNBD) is required for regulating the stoichiometry of CNG channel assembly (15).

Using a bioinformatic approach, we have identified a leucine zipper motif within the S5 segment of HCN channels. The leucine zipper is highly conserved and is present in all HCN channel family members and in spHCN channels. We used site-directed mutagenesis, electrophysiological recordings, surface expression analysis and molecular modeling to determine the structural and functional role of the leucine zipper motif in HCN2. Our data indicate that the leucine zipper is essential for an interaction of the S5 and S6 segments and that this interaction is important for proper gating of HCN channels.

EXPERIMENTAL PROCEDURES

Molecular biology— We used a mouse HCN2 clone with a deletion of the GC-rich N-terminus (amino acids 2-130), which we have previously described as ntHCN2 (5). HCN1 was studied using mouse HCN1 cloned in the pBF1 vector. QuikChange (Stratagene) site directed mutagenesis kit was used to introduce mutations into the ntHCN2 cDNA or mHCN1 cDNA. Complementary RNA (cRNA) for injection into *Xenopus* oocytes was prepared with mMessage mMachine® SP6 Kit (Ambion) after linearization with EcoRI (HCN2) or MluI (HCN1) (both Fermentas). To assess the surface expression of HCN channels, a modified hemagglutinin (HA) protein epitope was inserted in HCN2 as previously described (5).

Expression of HCN channels in oocytes— Stage IV and V oocytes from mature *Xenopus laevis* toads were isolated and injected with 3.5 ng of cRNA of either wild-type or mutant HCN2 channels for electrophysiological recordings or with 10 ng of cRNA for surface expression assay. For HCN1 7.5 ng of wild-type or mutant cRNA was injected. Isolated oocytes were stored at 18 °C in ND96 recording solution (containing in mM: NaCl 96, KCl 2, CaCl2 1.8, MgCl2 1, HEPES 5, pH 7.4) with added Na-pyruvate (275 mg/l), theophylline (90 mg/l) and gentamicin (50 mg/l).

Two-electrode voltage clamp recordings— Standard two-electrode voltage-clamp experiments (TEVC) were performed at room temperature (21 – 22 °C) in ND66 recording solution (containing in mM: NaCl 66, KCl 32, CaCl2 1.8, MgCl2 1, HEPES 5, pH 7.4) 2 to 3 days after injection of oocytes with cRNA. Microelectrodes were fabricated from glass pipettes filled with 3 M KCl and had a resistance of 0.4 - 1.5 MΩ. Currents were elicited by 2 s pulses applied in 10 mV decrements to potentials ranging from +30 mV to -140 mV followed by a 750 ms test pulse to -130 mV. Normalized tail current amplitude taken from the 750 ms test pulse to -130 mV was plotted versus the test potential to obtain the relative conductance-voltage (G-V) relationship and fitted with a Boltzmann function:

\[ I_t = (1 - \min(P_o))[1 + \exp((V - V_{1/2})/k)] + \min(P_o) \]

where \( V_{1/2} \) is the voltage required for half-maximal activation, k is the slope factor, a measure of the voltage dependence of channel gating and \( \min(P_o) \) is the minimum open probability defined as the fraction of channels which are instantaneously open.

Surface expression assay in *Xenopus* oocytes— A chemiluminescence assay was performed as described by Zerangue et al. (16).
Briefly, oocytes were blocked with ND96/1% bovine serum albumin, labeled with anti-HA antibody, sequentially washed with ND96 solution (containing in mM: NaCl 96, KCl 2, CaCl2 1.8, MgCl2 1, HEPES 5; pH 7.4 with NaOH). For detection horseradish peroxidase-conjugated secondary antibody was used. Individual oocytes were placed in 20 µl SuperSignal Elisa Femto solution (Thermo Scientific, Rockford, IL, USA) and, after an equilibration period of 10 s, chemiluminescence was quantified in a luminometer (GloMax 20/20, Promega, Madison, WI, USA). The surface expression of 50 – 60 oocytes per construct (from three different batches of oocytes) was analyzed. Non-injected oocytes served as negative control. The difference between injected and non-injected oocytes was determined and the relative change in luminescence signal was compared for the different HA-tagged HCN2 mutant constructs.

**Western blot analysis**—Twenty oocytes per construct were homogenized in 400 µl lysis buffer (containing in mM: NaCl 150, Tris/HCl 20, pH 7.5, 1% Triton-X-100). Insoluble material was separated by centrifugation for 15 min at 4 °C. The protein content of the supernatants was quantified and subsequently probes were heat-denatured in sample buffer at 50 °C for 10 min. Similar amounts of total protein were separated for each probe on 12% SDS–polyacrylamide gels and visualized by immunoblotting with rat monoclonal anti-HA primary antibodies (clone 3F10, 1:1000 dilution; Roche Pharmaceuticals). The binding of the primary antibodies was detected using peroxidase-conjugated goat anti-rat antibodies (Jackson ImmunoResearch) and a chemiluminescent extended-duration substrate, Super Signal West Dura (Thermo Scientific).

**Homology modeling**—Modeller 9v9 (17) was used to create a closed homology model of the pore domain and S4-S5 linker of mouse HCN2 from the Mlo1tK1 crystal structure (18) [PDB ID: 3BEH]. The similarity between the two sequences was 29.2%. An open state model was also built, with the inner S6 helices modelled using the open state Kv1.2-Kv2.1 chimera (KvChim) [PDB ID: 2R9R] crystal structure (19) as a template. Four-fold symmetry was applied to both models. The final HCN2 models were compared to their crystal structure template to verify that the modeling step had not significantly altered backbone and side chain conformations.

**Data analyses**—Data are reported as means ± S.E.M. Statistical significance was calculated using unpaired Student’s t-test. All experiments were performed at room temperature (22 - 24 °C). *, indicates $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$. “n.s.” indicates non-significant changes.

**RESULTS**

A conserved leucine zipper motif in hyperpolarization-activated channels—We have identified a leucine zipper motif in the S5 segment of HCN channels (Fig. 1, A and B). The leucine zipper is conserved in hyperpolarization-activated cation channels from different species, including spHCN channels from sea urchin (Fig. 1A). Parts of the leucine zipper motif are also present in the closely related CNG channels (Fig. 1A). We have functionally analyzed the leucine zipper in mouse HCN2 channels, with zipper residues located at L343, L350, L357 and L364 (Fig. 1, A and B).

Alanine mutations at the leucine zipper motif of HCN2 lead to decreased current amplitudes due to altered channel gating—For the current study we used an HCN2 channel construct with an N-terminal truncation which was previously used in functional studies of HCN gating (5-6,9). Currents elicited by HCN2 expressed in *Xenopus* oocytes showed typical characteristics (Fig. 2A). We have analyzed the current amplitudes of channels with leucine zipper residues mutated to alanine. Therefore, we injected 3.5 ng cRNA of wild-type HCN2, L343A, L350A, L357A and L364A. The current amplitudes were measured 48 hours after injection. Currents of wild-type HCN2 channels were 7.2 µA ± 0.4 µA (n = 46; normalized 1.00 ± 0.05). Although the leucine zipper alanine mutants generated hyperpolarization-activated currents, the amplitude was strongly reduced for all zipper mutant constructs (Fig. 2, A and B and supplemental Fig. S1, A and B). The observed reduction of current amplitudes may be due to a reduced protein expression, a deficient channel trafficking or an altered channel gating. To further clarify the mechanism of current reduction, we performed wild-type HCN2 constructs and a chemiluminescence based surface expression assay. For these experiments an HA-tag was introduced in the extracellular S3-S4 loop between Gly-284 and Ile-285. Western blot analysis of oocytes injected with HCN2-HA constructs revealed that similar amount of channel protein was present for
all the zipper mutants (Fig. 2C). However, while current amplitudes are reduced, the chemiluminescence-assay revealed that the surface expression of mutant channels was similar as for wild-type channels (Fig. 2D). Thus, current reduction of the zipper mutants does not result from altered channel trafficking and must be caused by an altered conductivity or voltage dependence of HCN2 channels.

Changes in voltage dependence of activation by leucine zipper alanine mutations—The voltage dependence of channel activation was determined by analyzing the instantaneous tail current at -130 mV. The $V_{1/2}$ of activation for wild-type HCN2 was $-87.0 \pm 0.3$ mV and slope factor was $8.7 \pm 0.25$ (n = 10). G/V-curves for L343A, L350A and L357A were similar as for wild-type channels (Fig. 2E, Table 1 and supplemental Fig. S1C). All constructs (except L364A) had only minor shifts of the $V_{1/2}$ of activation (Fig. 2E, Table 1 and supplemental Fig. S1C). Due to a steeper slope factor k (Table 1), L350A reached its maximum current at around -100 mV, while the other mutants (except L364A) reached steady-state activation at about -120 mV (Fig. 2E). Slope factors were significantly changed for L350A (5.6 ± 0.2) and L364A (17.2 ± 1.3), indicating an altered voltage dependence of channel gating for these constructs (Table 1). In contrast to other leucine zipper alanine mutations L364A showed only a little hyperpolarization-activated current component, with a $V_{1/2}$ of activation that is more negative than -140 mV (Fig. 2E, Table 1 and supplemental Fig. S1C).

Summarizing, L343A, L350A and L357A have reduced current amplitudes with only minor changes in the voltage dependence of activation, while L364A currents are strongly reduced due to an uncoupling, as the channels open only upon very strong hyperpolarization of the membrane.

Double and triple alanine mutations lead to a strong reduction in current amplitudes and negative shifts in voltage dependence of activation—Next, we mutated multiple leucine residues at once and generated the double-mutations L343A/L350A, L343A/L357A, L350A/L357A and the triple mutant L343A/L350A/L357A. The uppermost S5 leucine zipper mutation L364A was not included in this multiple mutation approach as its current-voltage relationship was already shifted to very negative potentials. All two- and three-fold mutants showed functional hyperpolarization-activated HCN currents when expressed in Xenopus oocytes (Fig. 3A), but current amplitudes were strongly reduced (Fig. 3B). Again, current reductions were more pronounced for mutations in the upper part of the S5 segment. Current size was HCN2 > L343A/L350A > L343A/L357A > L350A/L357A (Fig. 3B).

Our chemiluminescence assays revealed that the double mutants L343A/L350A and L343A/L357A, efficiently traffic to the surface membrane (Fig. 3C), while TEVC recordings reveal that they have an altered channel gating, as they fail to open upon hyperpolarization (Fig. 3D). In contrast, the L350A/L357A double and the L343A/L350A/L357A triple mutants have a strongly reduced surface expression (Fig. 3C). Note that similar efficiency in protein expression was detected by western blot analysis for all constructs (Fig. 3C, inset). These data further highlight the relevance of the zipper residues in HCN channel gating, but also indicate that the leucine zipper might be important for the formation of a stable HCN channel complex, necessary for the subsequent trafficking to the surface membrane.

Of the single alanine mutants, only the uppermost S5 segment residue L364A showed a deficiency in opening upon hyperpolarization (Fig. 2E and supplemental Fig. S1C). While the single mutations L343A, L350A and L357A had only small changes in voltage dependence (Fig. 2E and supplemental Fig. S1C), the double mutations L343A/L357A and L350A/L357A showed drastic shifts in the voltage dependence of activation (Fig. 3D and Table 1). For the lower-most S5 double mutation, L343A/L350A, we observed only a minor change in the slope of the voltage dependence (Fig. 3D and Table 1), but a significantly higher min-Po (0.177 ± 0.013, n = 8) (Fig. 3D), indicating more instantaneous currents, as a measure of a closing deficiency. While this L343A/L350A mutant had no strong shift in the voltage dependence of activation, introducing a third alanine mutation (L357A) into this construct (L343A/L350A/L357A) resulted in channels that open only upon strong hyperpolarization of the plasma membrane (Fig. 3D).

Summarizing, current reduction and shift of the voltage dependence of activation are more pronounced for residues of the upper part of the leucine zipper. Multiple alanine mutations lead to an impaired voltage dependence of HCN channel gating and for the lower part of the leucine zipper we observed changes in the closing of the channel (increased min-Po of L343A/L350A).

Mutations of leucine zipper residues to glutamine or arginine strongly reduce current amplitudes and voltage dependence of HCN
As the leucine to alanine mutation is a conservative mutation, we studied the effects of mutating leucine zipper mutants to a bulky and charged (arginine) or to a bulky and aliphatic (glutamine) residue. Representative current traces of arginine and glutamine mutants of the leucine zipper are illustrated in Figure 4A and 4F. The S5 segment leucine zipper interacts with an opposing S6 segment. If the leucines in the S5 segment of HCN channels act as a zipper motif, these residues should interact with aliphatic side chains of an opposing helix to form a stable structure. To identify a domain and residues that interact with the leucine zipper in the S5 segment, we developed a closed state homology model of the mouse HCN2 channel based on the crystal structure of the closely related CNG channel Mlo1K1 (18). The pore homology model included the S4-S5 linker, the S5 and S6 segments (Fig. 5A). The model predicted an interaction of the leucine zipper residues with opposing aliphatic residues of the S6 segment from the same subunit (Fig. 5A). L343 was predicted to interact with L438, L350 with M430, L357 with M430, L364 with Y423, and L364 was predicted to

Experiments with HA-tagged and full-length HCN2 constructs confirm the functional role of the leucine zipper in HCN2 gating. Next, we performed control experiments to verify that the extracellular HA-tag introduced in HCN2 does not interfere with the gating phenotype of the mutants or the trafficking of the channels. Note that the HA-tagged wild-type HCN2 is activating at more hyperpolarized potentials and is slower in activation as the non-tagged HCN2 (supplemental Fig. S2A). However, the HA-tagged HCN2 constructs, used in Fig. 4, H and I revealed the same electrophysiological phenotypes (supplemental Fig. S2C, A-C) as the untagged HCN2 channel constructs (Fig. 4, F-G and J). The L343R mutant again shows a rightward shift in the voltage dependence of activation (supplemental Fig. S2C), an increased min-Po (supplemental Fig. S2, A and C) and a gain-of-function in the current amplitude (supplemental Fig. S2, A and B). In accordance to the untagged channels, the mutants L350R, L357R and L364R also show reduced current amplitudes in the HA-background and shifts of the voltage-dependence out of the physiological range (supplemental Fig. S2, A-C). As the relative current amplitudes also match with the untagged versions of the mutants, the HA-epitope most likely does not interfere with the trafficking of the channel.

To rule out that the N-terminal deletion of the ntHCN2 construct (5-6,9) used in the current study interferes with the channel gating of the zipper mutants, we studied the arginine mutants described in Figure 4 in the full-length HCN2 channel. The data obtained for the full-length HCN2 were very similar to that using the ntHCN2 (supplemental Fig. S2D).

Thus, neither the N-terminal deletion nor the HA-epitope interfered with the results obtained with the ntHCN2 channel construct.
interact with L416. Therefore, we hypothesized that the leucine zipper is essential for the functional packing of channel proteins and that it might play a role in the conversion from the closed to the open state of HCN channels. To test for a putative interaction of the leucine residues with the residues in the S6 segment, we analyzed alanine mutations at the residues L416, V423, M430 and L438A (Fig. 5B). The current size was reduced in a similar manner as for the S5 segment alanine mutants. Meaning, the reduction in current amplitude was more pronounced for alanine mutations located in the upper S6 segment, similar as observed for the S5 segment (Fig. 5C). The current reduction by the S6 segment mutations L416A and L438A was also confirmed in the full-length HCN2 channel (supplemental Fig. S3). L438 which is located in the lower S6 segment, is supposed to interact with L343. Interestingly, L438A also shows an increased instantaneous current component (Fig. 5B and arrow in supplemental Fig. S3A), similar as L343R or L343A/L350A.

Taken together, these results support a possible interaction of the S5 and S6 domains via the leucine zipper and the residues proposed by our model.

**Mutations in HCN1 confirm the relevance of leucine zippers for the HCN channel family**—As the leucine zipper motif is highly conserved within the HCN channel family (Fig. 1A), we studied glutamine mutations in the leucine zipper of HCN1. Current amplitudes of the L290Q, L297Q, L304Q and L311Q HCN1 mutants were strongly reduced (Fig. 6, A and B and supplemental Fig. S4), similar as for the HCN2 glutamine mutants (Fig. 4, A and B). HCN1 leucine zipper mutants only open upon very strong hyperpolarization of the plasma membrane (Table 2). However, while the lowest S5 zipper mutation in HCN2 L343Q resulted in a gain-of-function (Fig. 4B), the lowest HCN1 zipper mutation L290Q resulted in a current reduction (Fig. 6, A and B). Thus, we analyzed the current amplitudes of different residues introduced at position L343 of HCN2. As the increased current amplitudes were specific for arginine and glutamine residues at this position (Fig. 6C), we conclude that the gain-of-function needs a large neutral or positively charged residue at position L343 of HCN2. In contrast, HCN1 currents were strongly reduced by arginine and glutamine residues introduced at the homologous site (L290). Thus, similar as for the leucine zipper in HCN2, the lowest zipper residue of HCN1 seems to be involved in regulating the open and closing properties of the channel, albeit in a different manner (see Discussion section).

Note that the lowest HCN1 zipper mutant, L290Q, has an increased min-Po (Fig. 6A, arrow). Similar effects were observed for the L343A and L343R mutations in HCN2 (Figs. 2A and 4F). The data obtained using the HCN1 channel further highlight the functional relevance of the leucine zipper for HCNs to function as hyperpolarization-activated channels and indicate that the lowest S5 zipper residue is involved in regulation the open state of the channel.

**The lowermost leucine zipper residue in the S5 segment regulates the open state of HCNs**—As we have observed increased instantaneous current amplitudes (increased min-Po) for mutations at the lowermost S5 leucine zipper residue, we studied the instantaneous current of channels mutated at this and the corresponding S6 site in more detail. Wild-type HCN1 currents have about 20 % of instantaneous currents when activated by a voltage step to -110 mV (Fig. 7A, grey trace and arrow). In contrast, for L290Q about 60 % of the current was already instantaneously activated (Fig. 7A, grey trace and arrow). For L290R 100 % of the channels were already in the open state when membrane potential was stepped to -110 mV (Fig. 7A). Wild-type HCN2 currents show about 8 % of instantaneous currents when activated by a voltage step to -110 mV (Fig. 7B). Similar to the HCN1 mutants, HCN2 channel mutants at the lowermost S5 zipper residue generate currents with an increased instantaneous component (Fig. 7B). The instantaneous current component was 35 % for L343A and 60 % for L343R (Fig. 7B). Introducing similar mutations at the corresponding S6 site L438, also results in increased instantaneous current components (Fig. 7B). The instantaneous current was 60 % for L438A and 70 % for L438R (Fig. 7B).

These data support our hypothesis that the first residue of the leucine zipper, the lowermost in the S5 segment, regulates the open state of HCN channels. In addition, it further supports an interaction of L343 of the S5 with L438 of the S6 segment. This interaction might stabilize the closed state of the channel as suggested by our closed state pore homology model (Fig. 5A).

**Instantaneously open mutants of HCN2 and HCN1 have unaltered ion selectivity**—As mutations at the lowermost leucine zipper residue and the pairing S6 segment site lead to currents with leak channel characteristics, we analyzed whether these mutations alter the ion selectivity of HCN2 and HCN1. Therefore, we recorded
fully-activated current-voltage relationships of HCN2 (L438R) and HCN1 (L290R) (Fig. 8, A and B). For both constructs the reversal potentials were similar to that of the respective wild-type channels, indicating that the ion selectivity was not changed for both channel isoforms. Thus, neither the S5 mutant (HCN1-L290R) nor the S6 mutant (HCN2-L438R) caused a major change in ion selectivity. For the L290R mutant however, we have observed a change in the rectification pattern of the fully-activated current-voltage relationship (Fig. 8B), further supporting our observation that mutations at the lowest leucine zipper residue can induce an altered channel gating. In addition, it was apparent from the fully-activated current-voltage relationship recordings that both mutants, L438R and L290R, have a slower rate of deactivation, indicating that the mutations interfere with proper closing of the channels.

The lowermost part of the leucine zipper stabilizes a salt-bridge and the closed state of HCN2. To analyze the role of an interaction of the lower leucine zipper with the late S6 segment during channel gating, we generated an open pore homology model of HCN2 based on the rKv1.2 crystal structure (19), for details see Methods section. In the closed state model the S5 residue L343 is in close proximity to L438 of the S6 segment (Fig. 9, A and C). In contrast, due to the movement of the S6 segment during channel opening, L438 dislodges from L343 (Fig. 9, B and D). Thus, an interaction of these residues might stabilize only the closed state of the channel. We have previously described that a salt-bridge between the S4-S5 linker residue R339 and the late S6 segment residue D443 stabilizes the closed state of the channel (9). Our closed state and open state homology models are in good agreement with these findings, as the salt-bridge is favored in the closed state model (Fig. 9, C and D). Opening of the channel leads to a sideward movement and rotation of the S6 segment, displacing D443 from R339 (Fig. 9, C and D). From our modeling data, we again conclude that the open state of HCN2 is regulated by a salt-bridge of the S4-S5 linker and the late S6 segment. We propose that this salt bridge is stabilized by an interaction of the lower leucine zipper (L343) interacting with the S6 segment (L438). Mutations at the zipper residue L343 or the S6 residue L438 might destabilize the closed state. In addition, bulky amino acids introduced at position L438 (Fig. 9D) might sterically hinder the downward movement of the S6 segment and the returning to the closed state of the channel.

DISCUSSION

It is still not fully understood how voltage sensor movement in hyperpolarization-activated channels is coupled to channel opening and closing. For Kv channels a leucine zipper motif in the S4-S5 linker was described as an essential determinant for the transduction of charge movement into channel gating (11). Thus, when we observed a highly conserved leucine zipper motif in the S5 segment it was prompting to analyze its role for HCN channel function. Our data indicate that the leucine zipper is an important molecular determinant for hyperpolarization-activated channel gating. Our modeling data predicted an interaction of the leucine zipper in the S5 segment with the adjacent S6 segment of HCN channels. This interaction appears to be essential for voltage-dependent gating of hyperpolarization-activated channels. We found that alanine, glutamine and arginine mutations of the leucine zipper residues lead to a strong reduction in HCN current amplitudes (with the exception of L343Q/R). The higher the leucine zipper mutations were located in the S5 segment the more pronounced was the reduction in current amplitudes. When single or double alanine mutations were located high in the S5 segment, the channels were no longer able to open upon hyperpolarization. All arginine and glutamine mutations of the leucine zipper generated channels which did not open upon hyperpolarization, except for mutations in the lower part of the S5 segment. The lower part of the leucine zipper, at the intracellular mouth of the channel, appears to be important for the stabilization of the closed state of the channel. Arginine and glutamine mutations at these sites increased current amplitudes or resulted in channels with deficient closing and an increased instantaneous current component (min-Po). Summarizing, the upper part of the leucine zipper might be important for stabilizing a rigid S5 and S6 segment interaction which is necessary for a proper hyperpolarization-activated channel gating at the intracellular channel mouth. The lower part of the leucine zipper might be more flexible, as it is directly located at the inner channel gate formed by the S4-S5 linker, the early S5 and late S6 segment. Here leucine zipper residues might influence the open and closed state of the channels.
It is known that potassium channel gating needs global rearrangements of the S4, S5 and S6 domains. Voltage-dependent gating can be introduced into KcsA channels by constructing KcsA-Shaker chimeras (20). Here, voltage-dependent gating is critically dependent on the interaction of parts of the S4-S5 linker with residues in the late S6 segment (20). However, also concrete interactions or salt bridges between domains seem to be of major relevance for channel gating (8-9). In HERG channels which are more closely related to HCN than to Kv channels, a single charge-reversing mutation in the S4-S5 linker (D540K) is sufficient to prevent channel closure (21). In addition, the D540K mutant channels are able to open upon depolarization or hyperpolarization. The opening response to hyperpolarization can be abolished by introducing a second mutation at position R665 in the late S6 segment of HERG (8). The authors suggest a salt-bridge between D540 and R665 and that repulsion between K540 and R665 mediates the HCN-like channel gating in D540K HERG. A corresponding salt-bridge of the S4-S5 linker with the late S6 was proposed for HCN channels (9). An arginine residue in the S4-S5 linker of HCN2 (R339) was found to interact with an aspartate in the late S6 or early C-linker (D443). This interaction stabilizes the closed state of wild-type channels by an electrostatic interaction. Mutating R339 in the S4-S5 linker and D443 in the early C-linker de-stabilizes the closed state of the channel, resulting in instantaneous HCN currents. We have observed a similar phenotype for arginine and glutamine mutations at the lowermost S5 leucine zipper residue L343 and the S6 residue L438 which is predicted to interact with L343. We conclude that the lower part of the leucine zipper stabilizes the closed state of the channel. According to our state dependent HCN models, the residue R339 (S4-S5 linker) forms a salt-bridge with D443 (late S6 or C-linker) to stabilize the closed state. Thus, our models support the previously proposed electrostatic interaction (9). Most importantly, this salt-bridge might be stabilized by the lowest leucine zipper residue L343, which is preferentially interacting with the S6 residue L438 when the channels are in the closed state. Accordingly, mutations of either of the residues (R339 and D443 or L343 and L438) result in a de-stabilization of the closed state and in instantaneous currents. Our findings support the hypothesis of a concrete interaction of amino acids at the intracellular mouth to modulate HCN channel gating. Interactions of residues in the S4-S5 linker with the C-linker and of the early S5 with the late S6 stabilize the closed state of the channel.

Despite the lack of visible inactivation, both, spHCN channels and HCN2 channels, display some kind of inactivation (7,22). Shin et al., (2004) suggested that HCN2 channels display a small prepulse-dependent inactivation which is cAMP dependent (22). However, this physiological prepulse-dependence of HCN2 is minor and cannot attribute to the very pronounced loss-of-function for hyperpolarizing voltage steps ranging up to -140 mV. From a mechanistic view, if the zipper mutants caused a full conversion of a ‘cAMP shift-type’ response, as in HCN channels (22), to a ‘cAMP inactivation-type’ response, as in spHCN channels (22), then one might indeed observe a strong reduction in current amplitudes. However, the ‘cAMP inactivation-type’ of spHCN channels is not apparent in recordings from intact Xenopus oocytes, due to the relatively high intracellular levels of cAMP (7). Thus, even a full conversion to a spHCN-like inactivation cannot explain the almost complete loss-of-functions which we have observed for some of the HCN leucine zipper mutants. In addition, cAMP dependent inactivation of spHCN channels occurs without a shift in the voltage dependence of activation (22), whereas the leucine zipper mutants have a strong shift in the voltage dependence of activation.

We have studied several mutations at the lowermost zipper position of HCN2 (L343) and HCN1 (L290) (Fig. 6C). These data indicate that mutations at the lowermost zipper residues (L343 or L290) primarily cause a reduction of $I_{\text{max}}$ (Fig. 6A). However, cAMP-induced tetramerization of the C-terminal domains of HCN channels removes the tonic inhibition of the pore gate by the C-linker/CNBD. In this context it is noteworthy, that HCN1 channels have a different gating than HCN2 channels, as HCN1 channels open at less hyperpolarized potentials and exhibit only a minor response to cAMP. Recently, these differences in HCN gating were explained by the
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effect that the C-linker/CNBD of HCN1 channels already form tetramers at basal cAMP concentrations (23-24); meaning that HCN1 channels are already disinhibited by the C-linker/CNBD. The difference between the interaction of the CNBD/C-linker with the late S5 and S6 of HCN2 and HCN1 might explain the apparent discrepancy of arginine und glutamine mutations at the lowest leucine zipper site. The glutamine and arginine mutations might exclusively lead to a gain-of-function in HCN2 by a disinhibition of the tonic block by the CNBD/C-linker, while HCN1 channels are already disinhibited. As for both HCN isoforms gain-of-functions or loss-of-functions were observed by glutamine and arginine mutations, the lowest zipper residue is a critical determinant for HCN1 and HCN2 channels to determine open-probability and the instantaneous current component.

HCN channels expressed in heterologous systems give rise to two different currents, a slowly activating current $I_h$ and an instantaneous voltage independent current (25). The amplitude of both is correlated and both currents are modulated by changes in intracellular chloride and elevation of cAMP (25). Nevertheless, the two components of HCN channel currents were previously proposed to flow through two distinct channel populations (26). We found that mutations in the upper part of the leucine zipper drastically alter the voltage-dependent state of HCN channels and that mutations in the lower part of the leucine zipper modulate the instantaneous voltage independent open state. We therefore support the idea that HCN channels have two different open states and that both open states are critically dependent on the leucine zipper.

Summarizing, we have observed that the leucine zipper plays a crucial role for HCN2 and HCN1 channel gating. The residues of the leucine zipper interact with the S6 segment and they are important molecular determinants for regulating the current amplitudes, voltage dependence and the closed state of hyperpolarization-activated cation channels.
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**FOOTNOTES**

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**FIGURE LEGENDS**

FIGURE 1. Leucine zipper in HCN channels. *A*, The positions of the four leucine residues of the zipper motif in HCN channels and the closely related spHCN channel are highlighted in bold with boxes. The leucine zipper is not present in CNG and Kv channels. The numbers below the sequence provide the position of the leucine residues in mouse HCN2. *B*, Cartoon of a HCN2 channel indicating the position of the leucine zipper residues in the S5 segment.

FIGURE 2. Electrophysiological properties of leucine zipper alanine mutants. *A*, Representative current traces for wild-type HCN2 and alanine mutants recorded during 2 s pulses from a holding potential of -30 mV to potentials ranging from -30 to -140 mV in 10 mV decrements. Tail currents were measured at -130 mV. The dotted line represents zero current. The arrow indicates the instantaneous current component. *B*, Relative currents measured at the end of a 2 s activating pulse to -130 mV, recorded two days after injection. Currents were normalized to wild-type HCN2 amplitude of every recording day. Wild-type HCN2 channel currents were 7.2 ± 0.4 µA (n = 46). The mean currents of L343A, L350, L357 and L364A were 2.5 ± 0.1 µA (n = 42), 1.3 ± 0.1 µA (n = 42), 0.8 ± 0.1 µA (n = 39) and 0.6 ± 0.1 µA (n = 36), respectively. *C*, Western blot of HCN2 channel proteins expressed in *Xenopus* oocytes after injection of the same amount of wild-type or mutant HCN2 cRNAs. HCN channels with an extracellular HA-epitope were detected by an HA-antibody and a peroxidase-linked secondary antibody. *D*, Relative surface expression determined by single cell luminometry. Surface expression was normalized to wild-type surface expression (1.00 ± 0.08). E,
Activation curves for wild-type HCN2 and alanine mutants of the leucine zipper. Relative currents were fitted to a Boltzmann equation. The $V_{1/2}$, slope factor and $\min-Po$ values are given in Table 1. For L364A currents were normalized to wild-type HCN2 currents at -130 mV.

FIGURE 3. Electrophysiological properties of double and triple alanine mutants in the leucine zipper of HCN2. A, Representative current traces for wild-type and double/triple alanine mutants recorded during 2 s pulses from a holding potential of -30 mV to potentials ranging from -30 to -140 mV in 10 mV decrements. Tail currents were measured at -130 mV. The dotted line represents zero current. B, Relative currents measured at the end of a 2 s activating pulse to -130 mV, recorded two days after injection. Currents were normalized to wild-type HCN2 amplitude of every recording day. Mean currents were: wild-type HCN2 7.2 ± 0.4 µA (n = 46), L343A/L350A 2.5 ± 0.3 µA (n = 14), L343A/L357A 1.2 ± 0.1 µA (n = 10), L350A/L357A 0.4 ± 0.1 µA (n = 20), L343A/L350A/L357A 0.4 ± 0.1 µA (n = 9). C, Relative surface expression determined by single cell luminometry after injection of equal amounts of cRNA. Surface expression was normalized to wild-type surface expression (1.00 ± 0.18). Inset displays the western blot to show total HCN2 expression for wild-type and double/triple alanine mutants in Xenopus oocytes, after injection of equal amounts of cRNA. The western blot was performed as described above. D, Activation curves for wild-type and mutant HCN2 channels. Relative currents were fitted to a Boltzmann equation. The $V_{1/2}$, slope factor and $\min-Po$ values are given in Table 1. For L343A/L357A, L350A/L357A and L343A/L350A/L357A currents were normalized to wild-type HCN2 currents at -130 mV.

FIGURE 4. Electrophysiological properties of arginine and glutamine mutations in the leucine zipper of HCN2. A and F, Representative current traces for wild-type HCN2, glutamine (A) and arginine (F) mutants recorded during 2 s pulses from a holding potential of -30 mV to potentials ranging from -30 to -140 mV in 10 mV decrements. Tail currents were measured at -130 mV. The dotted line represents zero current. The arrow indicates the instantaneous current component. B and G, Relative currents of glutamine (B) and arginine (G) mutants measured at the end of a 2 s activating pulse to -130 mV, recorded two days after injection. Currents were normalized to wild-type HCN2 amplitude of every recording day. Current amplitudes were as follows: wild-type HCN2 7.2 ± 0.4 µA (n = 46), L343Q 43.5 ± 4.4 µA (n = 13), L350Q 0.2 ± 0.02 µA (n = 22), L357Q 0.3 ± 0.04 µA (n = 22) and L364Q 0.6 ± 0.1 µA (n = 24), L343R 14.8 ± 1.4 µA (n = 16), L350R 0.3 ± 0.1 µA (n = 24), L357R 0.5 ± 0.1 µA (n = 24) and 1.3 ± 0.2 µA (n = 23). C and H, Western blot of HCN2 channels expressed in Xenopus oocytes, after injection of equal amounts of cRNA encoding glutamine (C) and arginine (H) HCN2 mutants of the leucine zipper. Proteins with an introduced extracellular HA-tag were detected by an HA-antibody with a luminometric western blot. D and I, Relative surface expression of glutamine (D) and arginine (I) mutants determined by single cell luminometry. Surface expression was normalized to wild-type surface expression (1.00 ± 0.08). E and J, Activation curves for wild-type HCN2 glutamine (E) and arginine (J) mutants. Relative currents were fitted to a Boltzmann equation. The $V_{1/2}$, slope factor and $\min-Po$ values are given in Table 1. For L357Q, L364Q, L357R and L364R currents were normalized to wild-type HCN2 currents at -130 mV.

FIGURE 5. A closed state pore homology model predicts S6 segment residues to interact with the S5 segment leucine zipper. A, A closed state homology model of HCN2, based on the MlotiK1 crystal structure. Depicted in yellow are the residues of the leucine zipper and in magenta the interacting residues of the opposing S6 segment. B, Representative current traces of alanine mutations at S6 segment residues which were predicted to interact with the leucine zipper. The arrow indicates the instantaneous current component. C, Current amplitudes of alanine mutants in the leucine zipper of Figure 2 were compared to the opposing S6 segment residues, recorded two days after injection and analyzed at -130 mV. Currents were normalized to wild-type HCN2 amplitude of every recording day. Wild-type current amplitude was 8.0 ± 1.3 µA (n = 33) and mutant current amplitudes were: L416A 0.8 ± 0.1 µA (n = 39), V423A 2.2 ± 0.3 µA (n = 36), M430A 3.3 ± 0.4 µA (n = 36) and L438A 3.4 ±
0.3 µA (n = 31). The reduction in current amplitudes of the leucine zipper mutants correlates with the reduction in current amplitudes of the corresponding S6 segment mutants.

**FIGURE 6. Electrophysiological properties of HCN1 leucine zipper mutants.** A, Representative current traces for HCN1 leucine zipper alanine mutants recorded during 2 s pulses from a holding potential of -30 mV to potentials ranging from -30 to -140 mV in 10 mV decrements. Tail currents were measured at -130 mV. The dotted line represents zero current. The arrow indicates the instantaneous current component. B, Currents measured at the end of a 2 s activating pulse to -130 mV, recorded two days after injection. Currents were normalized to wild-type HCN1 of every recording day. The mean HCN1 amplitude was 13.3 ± 1.6 µA (n = 39). The mean current amplitudes of the mutants L290Q, L297Q, L304Q and L311Q were, 0.5 ± 0.1 µA (n = 24), 0.4 ± 0.1 µA (n = 24), 1.9 ± 0.3 µA (n = 15) and 1.0 ± 0.1 µA (n = 15), respectively. The $V_{1/2}$, slope factor and min-$P_{o}$ values are provided in Table 2. C, Relative current amplitudes of mutations at position L343 of HCN2 and at position L290 of HCN1. Currents were measured at the end of a 2 s activating pulse to -130 mV, recorded two days after injection. Currents were normalized to the respective wild-type amplitudes of every recording day.

**FIGURE 7. Mutations at the lowermost leucine zipper residue and the interacting S6 residue lead to instantaneous currents in HCN1 and HCN2.** A, Representative HCN1 currents of L290 mutations with an increased instantaneous current, see grey arrows at grey traces at -110 mV, to see the fraction of instantaneous current. The right panel illustrates the relative instantaneous current at -110 mV. B, Representative HCN2 currents of L343 and L438 mutations, displaying an increased relative instantaneous current, see arrows at grey traces at -110 mV, to see the fraction of instantaneous current. Right panel illustrates the relative instantaneous current at -110 mV.

**FIGURE 8. Fully-activated current-voltage relationships reveal no changes in ion selectivity for instantaneously open HCN2 and HCN1 mutants.** A, Fully-activated current-voltage relationships for wild-type HCN2 and the S6 mutant L438R. For the representative current traces only the voltage steps around the reversal potential (-0 to -60 mV) are illustrated. The inset illustrates the reversal potentials for wild-type HCN2 and L438A. B, Fully-activated current-voltage relationships for wild-type HCN1 and the S5 leucine zipper mutant L290R. For the representative current traces only the voltage steps around the reversal potential (0 to -60 mV) are illustrated. The inset illustrates the reversal potentials for wild-type HCN1 and L290Q. Grey arrows in (A) and (B) indicate the fraction of instantaneous current.

**FIGURE 9. The leucine zipper stabilizes a salt-bridge of the S4-S5 linker and the C-linker, which is only present in the closed state of the channel.** A, Closed state pore homology model of HCN2 based on the MlotiK1 crystal structure, with leucine zipper residues (yellow) and corresponding S6 segment sites (magenta). B, Depicted is an open state homology model of HCN2 based on the rKv1.2 crystal structure. The same color code as in (A) was used. R339 is illustrated in brown and D443 in pink. C, A single subunit of the closed state homology model of HCN2 and D, open state homology model, using the same color code as in (A and B).
### TABLES

#### Table 1: Properties of HCN2 leucine zipper mutations

| Mutations           | min-$P_o$     | $V_{1/2}$ in mV | $k$    | $n$  |
|---------------------|---------------|-----------------|--------|------|
| HCN2 wild-type      | 0.053 ± 0.005 | -87.0 ± 0.3     | 8.7 ± 0.3 | 10   |
| L343A               | 0.128 ± 0.005 | -80.1 ± 0.3     | 8.9 ± 0.3 | 12   |
| L350A               | 0.082 ± 0.005 | -81.5 ± 0.2     | 5.6 ± 0.2 | 15   |
| L357A               | 0.099 ± 0.006ns | -83.1 ± 0.3     | 8.0 ± 0.3 | 5    |
| L364A               | 0.059 ± 0.001ns | < -140         | n.a.   | 3    |
| L343A/L350A         | 0.177 ± 0.013 | -85.7 ± 0.7     | 6.8 ± 0.6 | 8    |
| L343A/L357A         | 0.043 ± 0.010 | < -140         | n.a.   | 12   |
| L350A/L357A         | 0.020 ± 0.002 | < -140         | n.a.   | 8    |
| L343A/L350A/L357A   | 0.029 ± 0.001 | < -140         | n.a.   | 6    |
| L343R               | 0.558 ± 0.017 | -70.5 ± 1.5     | 8.3 ± 1.2 | 6    |
| L357R               | 0.033 ± 0.001 | < -140         | n.a.   | 4    |
| L364R               | 0.071 ± 0.001ns | < -140         | n.a.   | 6    |
| L343Q               | 0.088 ± 0.006ns | -85.5 ± 0.3    | 6.2 ± 0.2 | 5    |
| L357Q               | 0.018 ± 0.001 | < -140         | n.a.   | 4    |
| L364Q               | 0.030 ± 0.001 | < -140         | n.a.   | 9    |

min-$P_o$ = minimum open probability defined as the minimum value of relative tail current, $V_{1/2}$ = voltage required for half-maximal activation, $k$ = slope of current voltage relationship, n.a. = not applicable.

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*min-$P_o$ = minimum open probability defined as the minimum value of relative tail current, $V_{1/2}$ = voltage required for half-maximal activation, $k$ = slope of current voltage relationship, n.a. = not applicable.*
Table 2: Properties of HCN1 leucine zipper mutations

|               | min-$P_o$       | $V_{1/2}$ in mV | k       | n   |
|---------------|-----------------|-----------------|---------|-----|
| HCN1 wild-type| 0.249 ± 0.014   | -66.2 ± 0.8     | 11.3 ± 0.5 | 10  |
| L290Q         | 0.586 ± 0.029***| -67.5 ± 2.3     | 8.8 ± 1.5 | 3   |
| L304Q         | 0.094 ± 0.006***| < -140 mV       | n.a     | 8   |
| L311Q         | 0.067 ± 0.004***| < -140 mV       | n.a     | 5   |
| L290R         | 0.147 ± 0.004***| < -140 mV       | n.a     | 6   |

min-$P_o$ = minimum open probability defined as the minimum value of relative tail current, $V_{1/2}$ = voltage required for half-maximal activation, k = slope of current voltage relationship, n.a = not applicable.
Figure 1

A

LxxYxxxLxxYxxxLxxYxxxLxxYxxx
mHCN1 ASAVVRIFNL IGMMLL LCHWDGCL QFLVPL LQDFPPDCWVSLHE 324
mHCN2 ASAVMRICNL ISMMLL LCHWDGCL QFLVPM LQDFPRNCWVSING 377
hHCN2 ASAVMRICNL ISMMLL LCHWDGCL QFLVPM LQDFPRNCWVSING 404
mHCN3 ASAVVRIFNL IGMMLL LCHWDGCL QFLVPM LQDFPRNCWVSING 287
mHCN4 ASAVVRINNL IGMMLL LCHWDGCL QFLVPM LQDFPRNCWVSING 455
spHCN ANAVIRICNL VCMMLL LGHWNGCL QYLVPM LEYPDQSWVAING 405
mCNGA1 YPNIFRISNL VMYIVL LNNAGCWSYISHEIGFHNHTWVPDV 329
mCNGA2 YPNIFRISNL VLYILV LNNACWYTAISHEIGFGVDTWVPNI 314
mKv1.5 LGFLFFLFLGFVILFSAVYFAMADNQGDFPSISIPDAFWAVVT 466
mKv4.3 LGFLLFLGLATIYFAYMVYTAMQAPSSASASFSIPASFWYTVT 364

B

S1 S2 S3 S4 S5 S6

343 350 357 364
Figure 2

A) 

B) 

C) 

D) 

E)
Figure 3

A. HCN2 WT

B. Relative current

C. Relative light units

D. Normalized current vs. Voltage (mV)
Figure 4

A. HCN2 WT

B. Graph showing relative current for WT, L343Q, L350Q, L357Q, and L364Q.

C. Western blot and bar graph comparing relative light units for WT, L343Q, L350Q, L357Q, and L364Q.

D. Bar graph showing normalized current for WT, L343R, L350R, L357R, and L364R.

E. Graph showing normalized current against voltage for WT, L343Q, L357Q, and L364Q.

F. HCN2 WT

G. Graph showing relative current for WT, L343R, L350R, L357R, and L364R.

H. Western blot and bar graph comparing relative light units for WT, L343R, L350R, L357R, and L364R.

I. Bar graph showing normalized current for WT, L343R, L350R, L357R, and L364R.

J. Graph showing normalized current against voltage for WT, L343R, L357R, and L364R.
Figure 5

A

B

C

L416A, V423A

L438A

L364A, L357A, L350A, M430A

L438A

WT

Relative current
Figure 6

A

B

C

1.0

L290Q

L297Q

L304Q

L311Q

Relative current

0.0

0.5

1.0

HCN1 WT

HCN1 L290Q L297Q L304Q L311Q

HCN2

HCN1

Relative current

0.0

0.5

1.0

Control

HCN2

HCN1

HCN1 L290Q L297Q L304Q L311Q

HCN2

HCN1

500 ms

μA
Figure 7

A

HCN1 WT

L290Q

L290R

B

HCN2 WT

L343A

L343R

L438A

L438R
Figure 8

A

- HCN2 WT
- HCN2 L438R

500ms

B

- HCN1 WT
- HCN1 L290R

500ms

Relative current

Voltage (mV)

-60 -40 -20 20 40

-1.5 -1.0 -0.5

Relative current

Voltage (mV)

-60 -40 -20 20 40

-1.5 -1.0 -0.5

Reversal potential
Figure 9
A Leucine Zipper Motif Essential for Gating of Hyperpolarization-Activated Channels
Konstantin Wemhoener, Nicole Silbernagel, Stefanie Marzian, Michael F Netter, Susanne Rinne, Phillip J. Stansfeld and Niels Decher

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