A Single Histidine Residue Determines the pH Sensitivity of the Pacemaker Channel HCN2*

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Hyperpolarization-activated cyclic nucleotide-gated (HCN) cation channels control the rhythmic activity of heart and neuronal networks. The activation of these channels is regulated in a complex manner by hormones and neurotransmitters. In addition it was suggested that the channels may be controlled by the pH of the cytosol. Here we demonstrate that HCN2, a member of the HCN channel family, is directly modulated by the intracellular pH in the physiological range. Protons inhibit HCN2 channels by shifting the voltage dependence of channel activation to more negative voltages. By using site-directed mutagenesis, we have identified a single histidine residue (His-321) localized at the boundary between the voltage-sensing S4 helix and the cytoplasmic S4-S5 linker of the channel that is a major determinant of pH sensitivity. Replacement of His-321 by either arginine, glutamine, or glutamate results in channels that are no longer sensitive to shifts in intracellular pH. In contrast, cAMP-mediated modulation is completely intact in mutant channels indicating that His-321 is not involved in the molecular mechanism that controls modulation of HCN channel activity by cyclic nucleotides. Because His-321 is conserved in all four HCN channels known so far, regulation by intracellular pH is likely to constitute a general feature of both cardiac and neuronal pacemaker channels.

Cationic inward currents activated upon hyperpolarization of the plasma membrane, termed $I_h$ (for hyperpolarization) or $I_f$ (for funny), are found in heart and in a variety of central and peripheral neurons (1–2). In spontaneously firing cells like sinoatrial node cells of the heart (3–5), thalamic relay neurons (6), and respiratory neurons of the brainstem (7), $I_h$ contributes to the pacemaker depolarization that generates rhythmic activity. In cell types that do not reveal pacemaker activity $I_h$ fulfills diverse functions. It helps to determine the resting potential (2), provides rebound depolarizations in response to pronounced hyperpolarizations (8–9), and is involved in the control of synaptic plasticity (10) and the integration of synaptic inputs (11).

Recently, a family of four $I_h$ channel genes, designated as HCN1–4 for hyperpolarization-activated cyclic nucleotide-gated cation channels, has been cloned (12–19). HCN1–4 are structurally related to voltage-dependent K⁺ channels and cyclic nucleotide-gated (CNG) channels (20–21). HCN channels contain six transmembrane helices (S1–S6), including a voltage-sensing S4 segment, a pore region between S5 and S6 and a cyclic nucleotide-binding domain (CNBD) in the C terminus.

$I_h$ channels are tightly regulated by neurotransmitters and metabolic stimuli. It is well known that cAMP enhances the activation of $I_h$ by directly binding to the CNBD of the channel and thereby shifting the voltage dependence of its activation curve to more positive values and speeding up its activation kinetics (22). Up-regulation of $I_h$ by cAMP underlies the positive chronotropic effect of catecholamines in heart as well as the regulation of oscillation patterns of neuronal networks by neurotransmitters that stimulate cAMP synthesis (1–2). Other factors including NO/cGMP (23), GTP-binding proteins (24) and Ca²⁺ (25–26) have also been reported to modulate $I_h$; however, in most cases the physiological relevance of these modulatory mechanisms is a matter of controversy.

Recently, Munsch and Pape (27–28) provided first evidence for an additional kind of $I_h$ regulation. They showed that $I_h$ of thalamocortical neurons is sensitive to shifts of the intracellular pH ($pHi$). Alkalization induced an increase of the $I_h$ amplitude, whereas acidification inhibited the current. Although it could not be unequivocally decided whether protons act directly on the $I_h$ channel or modulate $I_h$ via an indirect pathway, these findings suggested that pH-mediated modulation of $I_h$ may provide an important mechanism to control the activity of neuronal networks in vivo. A possible modulation of $I_h$ by protons would also have profound implications concerning the role of $I_h$ under pathophysiological conditions in a variety of cell types and tissues.

As a first step to address this important issue, we have investigated the molecular basis of pH sensitivity of the heterologously expressed HCN2 channel. Within the HCN channel family HCN2 is the most abundantly expressed member. It is found in heart cells (14–15) and in most parts of the brain including the thalamus (29–30). In particular, the heterologously expressed HCN2 channel reveals all functional properties attributed to native $I_h$, i.e. activation by hyperpolarization, permeation of Na⁺ and K⁺, block by Cs⁺, and activation by cAMP (14–15, 30–31). In the present study, we have demonstrated that protons reversibly inhibit the HCN2 current by shifting the activation curve of the channel to more hyperpolarizing membrane potentials. We identified a highly conserved
histidine residue localized at the C-terminal end of the S4 segment that confers most if not all of the pH effect.

**EXPERIMENTAL PROCEDURES**

Construction of HCN2 Mutants and Functional Expression in HEK293 Cells—All channel mutants were constructed in the expression vector for murine HCN2 (mHCN2, former designation HAC1 (14)). Point mutations were introduced with polymerase chain reaction. The correctness of the introduced mutations and all sequences amplified by polymerase chain reaction were verified by sequencing. HEK293 cells were transiently transfected with expression vectors encoding either wild-type or mutant HCN2 channels using conventional calcium-phosphate transfection methods.

Electrophysiology—Currents were measured at room temperature 2–3 days after transfection using either whole cell, outside-out, or inside-out patch clamp techniques. The extracellular solution was composed of (in mM): 110, NaCl; 1.5, MgCl₂; 1.8, CaCl₂; 5, HEPES; 30, KCl. The intracellular solution contained (in mM): 130, KCl; 10, NaCl; 0.5, MgCl₂; 1, EGTA; 5, HEPES. The pH of intracellular (pHi) and extracellular (pHₑ) solutions was adjusted to various pH values by using KOH for pHₑ and NaOH for pHi.

Data were acquired at 10 kHz using an Axopatch 200B amplifier and pClamp 7 (Axon Instruments) and were low-pass filtered at 2 kHz with an 8-pole Bessel filter (LPBF-48DG, npi). Voltage clamp data were stored on the computer hard drive and analyzed off-line by using Clampfit (Axon Instruments). Steady-state activation curves were determined by hyperpolarizing voltages (−150 to −56 mV for inside-out and excised inside-out patch measurements, respectively; −150 to −30 mV for whole cell recording) from a holding potential of −40 mV for 2.8 s followed by a step to −150 mV. Tail currents measured immediately after the final step to −150 mV were normalized by the maximal current (I_max) and plotted as a function of the preceding membrane potential. The data points were fitted with the Boltzmann function: (I-I_min)/(I_max-I_min) = 1/(1+exp((V-V_0.5)/k)), where I_max is the test current, V is the membrane potential for half-maximal activation, and k is the slope factor. As has been described earlier for expressed HCN2 channel (14) and native I_HCN (32), V_0.5 was dependent on the configuration of the electrophysiological measurement. At pH 7.4, V_0.5 of HCN2 was −104 ± 1.6 mV (n = 6; see Fig. 5B), −117 ± 1.5 mV (n = 6; see Fig. 4B) and −125 ± 1.3 mV (n = 16; see Fig. 5C) in whole-cell, outside-out, and inside-out mode, respectively. Similarly, in inside-out patches, V_0.5 of mutant channels was also shifted by about 20–25 mV to more hyperpolarizing voltages with respect to the V_0.5 values measured in whole cell mode (see Fig. 5, B and C).

Time constants of channel activation (τ_act) of wild-type and mutant HCN2 channels were determined in excised inside-out patches by fitting the current evoked during hyperpolarizing voltage pulses of 2.8-s duration with monoexponential functions. As has been described earlier (15), the initial lag in the activation of HCN channel was excluded from the fitting procedure. All values are given as mean ± S.E.; n is the number of experiments.

**RESULTS**

**HCN2 Current Is Modulated by Internal pH in the Physiological Range**—To test for a possible modulation of HCN channels by shifts in intracellular pH (pHi), we transiently expressed the murine HCN2 channel in HEK293 cells. Inward currents were activated from excised inside-out patches of transfected cells by subsequently stepping from a holding potential of −40 mV to −130 mV, a voltage at which the channel is about half-maximally activated, and then to −150 mV, a voltage at which the channel is fully activated. Fig. 1A shows current traces obtained from an inside-out patch measured at different pH valeurs in the bath solution. Whereas the maximal current amplitude at −150 mV was clearly not altered by switching between pH 6.4 and pH 8.4; the current amplitude measured at −130 mV was strongly dependent on pHi, being smaller under acidic conditions and bigger at alkaline pH. The effect of pHₑ on the current amplitude was fully reversible and not because of a time-dependent increase or rundown of the current (Fig. 1B).

We next determined the voltage dependence of the HCN2 activation for a range of pHi values (Fig. 2). Acidic pHₑ (6.0) led to a leftward shift of the half-maximal activation voltage (V_0.5) to about 10 mV more negative potentials (V_0.5,pHi 6.0) = −134 ± 1.3 mV; n = 11), whereas alkaline pHₑ (9.0) resulted in a shift of V_0.5 to 10 mV more positive potentials (V_0.5,pHi 9.0) = −114 ± 2.9 mV; n = 6), as compared with V_0.5, at the control pHₑ of 7.4 (V_0.5,pHi 7.4) = −125 ± 1.6 mV; n = 12). In contrast, shifts in pHₑ had no major influence on the steepness of the activation curve (kₐ_HCN 6.0 = 4.3 ± 0.4 mV; kₐ_HCN 7.4 = 4.4 ± 0.5 mV; kₐ_HCN 9.0 = 6.3 ± 0.8 mV). By plotting V_0.5 versus pHₑ and fitting the data with a sigmoidal equation, a titration curve for the pHₑ dependence of HCN2 was obtained (Fig. 2B). The pHₑ effect on V_0.5 was most pronounced in the range between pHₑ 7.0 and pHₑ 8.0 whereas further acidification or alkalization had significantly less effect. The calculated pKₐ value was 7.6, which is within the range of normal cellular pHₑ. This finding indicated that modulation of HCN channel by protons may be physiologically important.

Single-exponential fits to current traces recorded at different pHₑ values indicated a strong dependence of the kinetics of channel opening on pHi (Fig. 2C). At −130 mV, the time constant (τ_act) of channel activation decreased significantly with an increase in pHi. At pHₑ 6.0, the time constant was 6.8 ± 1.6 s
pHi affects voltage dependence and kinetics of HCN2 channel activation. A, voltage dependence of HCN2 channel activation measured from inside-out patches at pH 6.0 (circles), pH 7.4 (squares), and pH 9.0 (triangles). The solid lines represent fits to the Boltzmann function (see "Experimental Procedures") with the following factors: pH 6.0, $V_{0.5} = -134$ mV, $k = 4.8$ mV; pH 7.4, $V_{0.5} = -125$ mV, $k = 5.2$ mV; pH 9.0, $V_{0.5} = -114$ mV, $k = 7.2$ mV. Number of experiments ($n$) $= 11, 12, 6$ for pH values of 6.0, 7.4, and 9.0, respectively. B, proton titration curve of $V_{0.5}$. $V_{0.5}$ was determined for various pH values from Boltzmann fits as described in A and plotted as a function of pH. Solid line is a fit to a sigmoidal function with a $p_K$ value of 7.58; $n = 3–12$. C, dependence of current activation kinetics on voltage and pH. Current traces were evoked at different pH values of 6.0, 7.4, and 9.0, respectively. $\tau_{act}$ was obtained by fitting the current traces with a monoexponential function (see "Experimental Procedures"). The number of experiments is indicated on top of the bars.

(n = 12), whereas it was $2.2 \pm 0.6$ s ($n = 12$) at pH 7.4 and $3.0 \pm 0.2$ s ($n = 6$) at pH 9.0. The pHi effect on activation kinetics was profoundly influenced by the membrane potential. It was very pronounced in the voltage range of $V_{0.5}$, but disappeared completely at $-150$ mV when the channel was fully activated ($\tau_{act(pHi \text{ 6.0})} = 0.28 \pm 0.02$ s; $\tau_{act(pHi \text{ 7.4})} = 0.28 \pm 0.02$ s; $\tau_{act(pHi \text{ 9.0})} = 0.26 \pm 0.05$ s).

The modulation of HCN current by shifts in pHi resembled the cAMP-mediated modulation of $I_h$ in two major characteristics. 1) It affected the voltage dependence of channel activation, and 2) it affected the speed of channel activation. We therefore tested whether both kinds of channel modulation interfered with each other. Fig. 3 shows activation curves determined at different pHi in the absence and presence of a saturating cAMP concentration (10 $\mu$M) in the internal solution. At each pHi, cAMP induced a profound shift of the $V_{0.5}$ to more positive values. The shift was strongest at acidic pHi ($\Delta V_{0.5 \text{ (pHi 7.0)}} = +22$ mV, shift from $-132 \pm 1.8$ mV ($n = 3$) to $-110 \pm 1.0$ mV ($n = 3$)) and became increasingly weaker when pHi was altered to physiological pHi ($\Delta V_{0.5 \text{ (pHi 7.4)}} = +18$ mV, shift from $-128 \pm 1.1$ mV ($n = 4$) to $-110 \pm 3.8$ mV ($n = 3$)) and to alkaline pHi ($\Delta V_{0.5 \text{ (pHi 7.9)}} = +12$ mV, shift from $-118 \pm 1.2$ mV ($n = 5$) to $-106 \pm 2.6$ mV ($n = 4$)). On the other hand, although the channel was very sensitive to shifts in pHi under control conditions (shift of $V_{0.5}$ of $+14$ mV between pHi 7.0 and pHi 7.9 in the absence of cAMP), it became rather insensitive to pHi when it was fully activated by a saturating cAMP concentration (shift of $V_{0.5}$ of $+4$ mV between pHi 7.0 and pHi 7.9 at 10 $\mu$M cAMP). This result suggested that modulation of the channel by intracellular protons critically depends on the basal activity of the channel.

Finally we tested whether HCN2 currents could be modulated by shifts in external pH (pHe). Fig. 4A shows current traces evoked at three different pHi values from the same outside-out patch by stepping to $-125$ mV and $-150$ mV. It is evident that both current amplitudes and kinetics were unaffected by acidification or alkalization. Similarly, the activation curves determined at different pHi values (Fig. 4B) revealed no shift in $V_{0.5}$ or alteration of the slope factors (pHe 6.0: $V_{0.5} = -116 \pm 1.7$ mV, $k = 3.9 \pm 0.5$ mV, $n = 6$; pHe 7.4: $V_{0.5} = -117 \pm 1.5$ mV, $k = 5.1 \pm 0.8$ mV, $n = 6$; pHe 9.0: $V_{0.5} = -119 \pm 4.1$ mV, $k = 3.4 \pm 0.7$ mV, $n = 4$). Thus, modulation of the HCN2 channel by pH revealed a profound site preference being restricted to shifts in internal pH.

Histidine 321 Is a Crucial Determinant of Sensitivity to pHi—Our experiments suggested that protons inhibit HCN2 by binding to a titratable acceptor side of the channel, which faces the intracellular environment. Because the $p_K$ deduced from the HCN2 titration curve was 7.6, we postulated that a histidine residue ($p_K = 6.0$) would be the most likely candidate for providing a proton binding site. By contrast, the $p_K$ values of lysine ($p_K \text{ - 10.5}$) and arginine ($p_K \text{ - 12.5}$) are far away from the observed range of pH regulation. HCN channels contain several histidine residues that principally could confer the pH effect. The finding that protonation induced a shift in the activation curve prompted us to focus on residues in the cytoplasmic S4-S5 linker, which has been shown to control together with the S4 segment the activation of K+ channels (33–34). The sequence of the S4 segment and the S4-S5 linker is completely conserved in HCN1-4 (Fig. 5A). A histidine residue in this sequence, His-321, is positioned “in-frame” with the nine regularly spaced arginines and lysines of the S4 segment suggest-
ing that it may be part of the voltage-sensing and/or gating machinery of the channel. To investigate the role of His-321, we replaced the residue with either a positively charged arginine (H321R), a neutral glutamine (H321Q), or an acidic glutamate (H321E). Replacement by arginine did not significantly alter the voltage dependence of activation at physiological pH, as determined in whole cell mode (Fig. 5B) or in excised inside-out patches (Fig. 5C). Similarly, mutation of His-321 to glutamine only weakly affected the activation curve. It induced a slight shift of the $V_{0.5}$ to about a 5 mV more positive voltage with respect to the wild-type channel (Fig. 5, B and C). By contrast, the $V_{0.5}$ was profoundly shifted to the right ($\Delta V_{0.5} = +20$ mV) when His-321 was exchanged by the negatively charged glutamate residue (Fig. 5, B and C). Determination of reversal potentials from current/voltage relationships of the fully activated channels (14) revealed that mutations at position 321 did not alter the ion selectivity of the channels with respect to wild-type HCN2 (not shown).

We next asked whether HCN2 mutants were sensitive to alterations of pH, (Fig. 6). Activation curves determined from inside-out patches revealed that pH sensitivity was almost completely lost in H321R (Fig. 6A), H321Q (Fig. 6B), and H321E (Fig. 6C). Again, the activation curves of the H321E mutant were significantly shifted to more depolarizing voltages when compared with H321R, H321Q, and wild-type channels. The loss of pH-dependent shifts in $V_{0.5}$ of mutant channels was mirrored by a loss of pH effect on activation kinetics (Fig. 6D). Whereas in wild-type channel alkalization led to a pronounced decrease of $\tau$ values at $-130$ mV (Fig. 2C), activation constants of H321R and H321Q were not significantly altered by shifts in pH under the same conditions. However, as in wild-type, channel activation constants of HCN2 mutants were dependent on the membrane potential reaching a minimal value at voltages that fully activated the channel ($-150$ mV) and increasing with membrane depolarization. Similar results were obtained with the H321E mutant (not shown).

We finally investigated whether the replacement of His-321 in HCN2 interfered with the cAMP modulation of the channels. Activation curves were measured at pH 7.4 for wild type (Fig. 7A), H321R (Fig. 7B), H321Q (Fig. 7C), and H321E (Fig. 7D) in whole cell mode under control conditions and after intracellular perfusion with 1 mM cAMP. Cyclic AMP shifted the $V_{0.5}$ of all three mutants to 20–25 mV more positive values. The $V_{0.5}$ values for H321R, H321Q, and H321E were $-102 \pm$
Mutant channels that had lost sensitivity to shifts in pH$_i$ could still be activated by cAMP indicating that cAMP-mediated modulation of voltage dependence of channel activation is not controlled by His-321. In addition, wild-type HCN2 is up-regulated by cAMP over a broad range of pH$_i$, supporting the notion that cAMP-mediated modulation works independent of pH$_i$. On the other hand, pH$_i$-mediated shifts in V$_{0.5}$ are profoundly influenced by the intracellular cAMP concentration being significantly larger in the absence than in the presence of the cyclic nucleotide. This finding could be explained by the presence of allostERIC interactions between the molecular pathways that confer proton- and cAMP-mediated channel modulation.

**Discussion**

Modulation of HCN2 by Shift in pH$_i$—This study demonstrates for the first time the modulation of a heterologously expressed HCN channel by protons. Like native I$_h$, HCN2 is specifically modulated by shifts in cytosolic pH$_i$ but is not sensitive to changes in extracellular pH. The modulation by internal protons refers to two aspects of HCN2 channel activation, namely its voltage dependence and its kinetics. Intracellular acidification induces a down-regulation of the receptor by shifting the activation curve to the left and also slows down the speed of activation. Alkalization enhances the current by shifting the activation curve to more depolarized voltages and in addition accelerates opening kinetics. Our results strongly support the hypothesis that protons modulate I$_h$ by directly binding to the HCN channel molecule. By contrast, there is no evidence that additional factors or auxiliary channel subunits are necessary to confer this effect.

**Site of Action of Internal Protons and Mechanism of Modulation**—By using site-directed mutagenesis we have identified a single amino acid in the HCN2 primary sequence (His-321) that is a major determinant of the pH$_i$ modulation. Replacement of this histidine residue by a nontitratable residue (H321Q) or by residues that at physiological pH$_i$ are either positively (H321R) or negatively (H321E) charged almost completely abolished sensitivity to pH$_i$. By contrast, these mutations did not alter the ion selectivity and the kinetics of the fully activated channels. Whereas all three mutants lacked pH$_i$ sensitivity, they also differed from each other in their respective V$_{0.5}$ values. The activation curve was shifted to more and more positive values in the order of H321R, H321Q, and H321E. This finding suggests that the electrical charge at position 321 may be involved in the mechanism that regulates HCN channel activity. However, charge effects alone are not sufficient to explain the inhibitory effect of protons. If the electrical charge at position 321 would be the major determinant of V$_{0.5}$ one would expect the V$_{0.5}$ of the H321R mutant to be more negative than the V$_{0.5}$ of the wild-type channel. This is clearly not the case. In contrary, protons even shift the V$_{0.5}$ of the wild-type channel to values that are significantly more negative than the V$_{0.5}$ of the H321R mutant.

How might His-321 control the voltage dependence of channel activation? His-321 is separated by two neutral amino acid residues from the last positive charged residue of S4. Thus, His-321 could either constitute the C terminus of the S4 helix or the N terminus of the S4-S5 linker. Recent studies indicate that the C-terminal end of S4 of Shaker K$^+$ channel subunits is accessible by thiol-reactive reagents from the cytoplasm making it impossible to precisely define the boundary between S4 helix and S4-S5 linker (35). In any case, the localization of His-321 is very consistent with a possible role in modulating channel activation. The S4 segment has been demonstrated to constitute the main component of the voltage sensor in calcium, sodium, and potassium channels (36). Recently, it was shown that replacement of charged residues in the S4 of HCN channels profoundly influences the voltage dependence of channel activation (37–38). Thus, conformational changes induced by the protonation or deprotonation of His-321 could well interfere with the voltage-dependent movement of the S4 helix. There is also evidence that the S4-S5 linker is an important determinant of voltage-dependent gating in other channels. Mutations in the S4-S5 linker of the KCNQ1 channel associated with long QT syndrome induce shifts in the voltage dependence of channel activation (33). Similarly, the S4-S5 linker was shown to be a crucial component of the activation gate of HERG K$^+$ channel (34). It is thus tempting to speculate that as in these K$^+$ channels, the S4-S5 linker of HCN channels transduces movement of the voltage-sensing S4 helix into opening of the channel. Our data indicate that His-321 could play a key role in this transduction process.
Physiological Implications of HCN Channel Modulation by pH—Given the very abundant expression of HCN2 in most parts of brain and heart, modulation of this channel by pH is of significant physiological importance. Because the S4–S5 region including the His-321 residue is completely conserved in all other mammalian HCN channels cloned so far, sensitivity to pH presumably is a general feature of HCN channels.

Thalamic neurons produce a range of complex firing patterns such as tonic bursts and slow 6 oscillations that control brain functions like the phases of sleep and the information flow through the thalamus toward the cerebral cortex (2, 21). It is well established that modulation of Ih by neurotransmitters that up- or down-regulate cellular cAMP or cGMP concentrations plays a key role in the control of thalamic oscillations (23, 39–40). Our data indicate that shifts in pH may provide an additional regulatory pathway of neuronal network activity. The sensitivity of Ih to shifts in pH may also be important in neurological diseases. Acidification as occurring during epileptiform discharges (41) could inhibit Ih, thereby counteracting the Ca2+-mediated positive shift in the Ih activation curve (40) and, hence, prolonging the duration of burst activity. In accordance with this model, it was recently postulated that the anti-convulsant action of acetazolamide is related to an intracellular alkalinization of thalamic neurons causing an up-regulation of Ih (28).

It is well known that the respiratory frequency is increased in response to acidosis and hypoxia. Recently, it was shown in inspiratory neurons of the brainstem that an increase in the respiratory frequency can be induced by the blockade of Ih by Cs+ or the specific Ih blocker ZD7288 (7). Thus, it is tempting to assume that protons may control respiratory frequency by controlling the activity of HCN channels.

Modulation of HCN channels by pH may also be important in non-neuronal cell types. It was speculated that the motility of sperm cells may be controlled by up-regulation of the HCN channel because of intracellular alkalinization (18). Although we have not tested whether or not HCN4 is sensitive to shifts in pH, the presence of a residue equivalent to His-321 in HCN4 is in favor of such a modulation.

Finally, pH may control the activity of HCN channels in heart. Such a regulation could be of particular importance under pathological conditions such as heart failure and myocardial infarction. During acute ischemia accompanying these diseases, CO2 production and the net production of protons shift pH to values as low as 6.0 (42). Our data suggest that inhibition of HCN channels by increases in H+ concentration could disturb pacemaker activity in sinoatrial node and other parts of the cardiac conduction tissue and, hence, contribute significantly to the generation of arrhythmia.

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