Molecular Basis for the Different Activation Kinetics of the Pacemaker Channels HCN2 and HCN4

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Running title: Activation kinetics of HCN4
Abbreviations: HCN, hyperpolarization-activativated, cyclic nucleotide-gated cation channel, HEK, human embryonic kidney cells;
Summary

The pacemaker channels HCN2 and HCN4 have been identified in cardiac sino-atrial node cells. These channels differ considerably in several kinetic properties including the activation time constant ($\tau_{\text{act}}$) being fast for HCN2 (144 ms at $-140$ mV) and slow for HCN4 (461 ms at $-140$ mV). Here, by analyzing HCN2/4 chimeras and mutants we identified single amino acid residues in transmembrane segments 1 and 2 and the connecting loop between S1 and S2 that are major determinants of this difference. Replacement of leucine 272 in S1 of HCN4 by the corresponding phenylalanine present in HCN2 decreased $\tau_{\text{act}}$ of HCN4 to 149 ms. Conversely, activation of the fast channel HCN2 was decreased 3fold upon the corresponding mutation of F221L in the S1 segment. Mutation of N291T and T293A in the linker between S1 and S2 of HCN4 shifted $\tau_{\text{act}}$ to 275 ms. While residues 272, 291 and 293 of HCN4 affected the activation speed at basal conditions they had no obvious influence on the cAMP-dependent acceleration of activation kinetics. In contrast, mutation of I308M in S2 of HCN4 abolished the cAMP-dependent decrease in $\tau_{\text{act}}$. Surprisingly, this mutation also prevented the acceleration of channel activation observed after deletion of the carboxy-terminal cAMP binding site. Taken together our results indicate that the speed of activation of the HCN4 channel is determined by structural elements present in the S1, S1-S2 linker and the S2 segment.
Introduction

Hyperpolarization-activated, cyclic nucleotide-gated cation (HCN) channels are thought to underlie the native pacemaker current, termed $I_f$ or $I_h$, in the heart and brain where it contributes to the rhythmic activity of cardiac and neuronal pacemaker cells (1-4). All four members of the mammalian HCN channel gene family that have been cloned recently (5-9) share a highly preserved core region containing 6 transmembrane segments, including a voltage-sensing S4 segment and a pore region between S5 and S6, which is homologous to the S1-S6 region of voltage-gated potassium channels (Kv). The homology of the intracellular amino- and carboxy-termini within the HCN-subtypes is less pronounced than for the core region with the exception of the highly conserved 120 amino acid long cyclic nucleotide binding domain (CNBD) starting about 80 amino acids downstream of S6.

All four HCN-channels are expressed in cardiac tissue (5, 10), but with some variations in the expression intensities among different species. HCN4 is highly expressed in the sino-atrial node. It is generally assumed that the slowly activating HCN4 contributes to the pacemaker activity and the modulation of the heart rate by $\beta$-adrenergic stimulation, whereas the less expressed, faster activated HCN2 and HCN1 may have additional functions such as maintaining the resting potential of pacemaker and other cells (11-14).

All four HCN-channels are activated upon membrane hyperpolarization. Activation is voltage-dependent, i.e. the more hyperpolarized the membrane becomes, the faster the channels open. The HCN channels differ, however, greatly in their activation kinetics with HCN4 being the slowest, HCN1 the fastest, and HCN2 and 3 being intermediate types (8-10). Activation of HCN2, HCN4 and to a smaller degree that of HCN1 is accelerated by the binding of cAMP to the C-terminal cAMP binding domain (CNBD) (5-10, 15-17).
cAMP to the CNBD releases the inhibition that the C-terminal region exerts on the channel (18).

It has been suggested that crucial components for the activation of HCN channels are within the S1, S1-S2 linker, S2 and S6-C-terminal regions (19, 20). Exchanging these regions between HCN1 and HCN4 slows down and speeds up, respectively, the activation kinetics of these channels (19). To observe these effects, several parts of the channels had to be exchanged together, presumably because the difference between HCN1 and HCN4 is rather pronounced as can be seen not only from the basic activation kinetics but also from their different reaction to intracellular cAMP. The different responses to cAMP have been analyzed by HCN1/HCN2 chimeras (18, 20). Similarly to the HCN1/HCN4 chimeras, parts of the C-terminus and some – unknown – transmembrane elements accounted for the activation kinetic differences. The channel subtype-specific cAMP modulation of the activation kinetics was always transferred with subtype-specific parts of the C-terminus.

Although HCN2 and HCN4 differ considerably in their activation kinetics, they share a relative high sequence homology and are both modulated by cAMP in the same way. In preliminary experiments, we found that the difference in the speed of activation between HCN2 and HCN4 was independent of the binding of cAMP to the C-terminal CNBD. This result suggested that just a short sequence may be responsible for this inherent difference between HCN2 and HCN4.

HCN2 and HCN4 consist of 889 and 1203 residues, respectively (8). The difference is primarily due to the significantly longer carboxy-terminus of HCN4. In the transmembrane region S1-S6, the homology of the human HCN2 and HCN4 is 90%, leaving only a few amino acids that could account for the differences in activation kinetics not modulated by the influence of the N- and C-terminal regions. We identified 5 amino acid residues in the S1 -
S2-region that contribute to the activation kinetics of HCN2 and HCN4. One of these amino acids is responsible for the difference in activation kinetics between HCN2 and HCN4.
Experimental Procedures

Molecular biology

Human HCN2 and HCN4 cDNAs were originally cloned from the atrioventricular node region of a human heart (8). HCN2/4-chimeric channel mutants and site-directed mutations were constructed in the pcDNA3 mammalian expression vector (Invitrogen) using polymerase chain reaction and restriction sites. Briefly, the Bsu36I – BspLU11I-fragment of HCN4 or HCN2 (a Bsu36I-restriction site had been introduced into the HCN2 sequence as a silent mutation at nt 537-543) was replaced by DNA-fragments, generated in several overlap-PCR steps, which contained the desired sequences and mutations. The correctness of the mutant channels and introduced point mutations was verified by DNA sequencing.

Nomenclature of mutant channels

Chimeras are named according to this nomenclature: H4 = HCN4-wild type; H2 = HCN2-wild type. First wild type in italics = backbone. Second wild type in italics = channel from which certain transmembrane segments or amino acids have been introduced into the first wild type. **Bold** numbers in between: Transmembrane segments that have been exchanged. *Small* numbers in between: Single amino acids (numbering from the "backbone"-wild type HCN) that have been exchanged. Thus, for example $H41-2H2$ is a chimeric HCN4-channel that contains the transmembrane segments 1 and 2 from HCN2. $H4267/272H2$ is the HCN4-channel in which the amino acids 267 and 272 (HCN4-numbering) have been replaced by the respective amino acids from HCN2, in this example the two leucines have been exchanged for
two phenylalanines. In Fig. 7, the classical nomenclature for point mutations is used since only leucine at position 272 in HCN4 has been exchanged for several other amino acids (L272X). Deletion of the C-terminal region of some channels (…ΔC) was achieved by exchanging the nucleotides encoding amino acid 535 for a STOP-codon (E535STOP, HCN4-numbering) leaving a C-terminus of only 14 amino acids which lacks the cyclic nucleotide binding domain (CNBD). Consult Supplement Table 1A to D for the exact crossover points and amino acid numbering for all chimeric channels.

Functional expression and electrophysiology

HEK293 cells were transiently transfected with expression vectors encoding either wild type or mutant HCN-channels using FuGENE6 transfection reagent (Roche) according to the manufacturer’s instructions (transfectant/DNA-ratio: 3/1 v/w). Cells were cultured in MEM supplemented with 10% fetal calf serum and kept at 37°C, 6% CO2. Currents were recorded 2-3 days after transfection with the whole cell patch recording technique at a temperature of 23 ± 1°C. The extracellular (bath) solution contained (in mM): 120 NaCl, 20 KCl, 1 MgCl2, 1.8 CaCl2, 10 HEPES, 10 Glucose, pH adjusted to 7.4 with NaOH. The intracellular (pipette) solution contained (in mM): 10 NaCl, 30 KCl, 90 K-Asp, 1 MgSO4, 5 EGTA, 10 HEPES, pH adjusted to 7.4 with KOH. For determination of the effect of cAMP on the wild type and mutant channels ("+ cAMP"), 100 µM cAMP (Boehringer, Mannheim) was added to the intracellular solution. Patch pipettes were pulled from borosilicate glass and had a resistance of 2-5 MΩ when filled with intracellular solution.

Data were acquired using an Axopatch 200B amplifier and pClamp7-software (Axon Instruments) and low-pass filtered at 2 kHz with an 8-pole Bessel filter (LPBF-48DG, npi).
The membrane potential was held at –40 mV. To elicit inward currents, step pulses of 5 seconds duration were applied from –140 mV to –30 mV, followed by a step to –140 mV as described in Fig. 1. For some raw data displays, longer or shorter pulses were applied to more clearly demonstrate the differences in activation kinetics between the (mutant) channels, but these measurements were not included into the voltage-dependent steady-state evaluation to obtain $V_{\text{max}1/2}$.

Further analysis was done offline with the Origin 6.0-software (Microcal). Time constants of activation ($\tau_{\text{act}}$) were obtained by fitting the current traces of the –140 to –100 (-90) mV steps after the initial lag with the sum of two exponential functions $y = A_1 e^{(-x/\tau_1)} + A_2 e^{(-x/\tau_2)}$, where $\tau_1$ and $\tau_2$ are the fast and slow time constants of activation, respectively; $\tau_1$ is consequently referred to as $\tau_{\text{act}}$ since the slow component ($A_2$) of HCN-channel activation generally accounts for <10% of the current amplitude; thus, the fast component is the major if not even exclusively kinetics-determinating component of the HCN-channels as has been discussed in (9, 19). To obtain voltage dependent steady-state activation curves, tail currents measured immediately after the final step to –140 mV were normalized by the maximal current ($I_{\text{max}}$) and plotted as a function of the preceding membrane potential. The curves were fitted with the Boltzmann function: $(I-I_{\text{min}})/(I_{\text{max}}-I_{\text{min}}) = (A_1-A_2)/\left(1+e^{(V-V_{1/2})/k}\right) + A_2$, where $I_{\text{min}}$ is an offset caused by a nonzero holding current and is not included in the current amplitude, $V$ is the test potential, $V_{1/2}$ is the membrane potential for half-maximal activation, and $k$ is the slope factor. All values are given as mean ± s.e.m.; $n$ is the number of experiments. Statistical differences were determined using Student's unpaired t-test; p-values <0.05 were considered significant.
Results

HCN2 and 4 differ in their activation kinetics

For both human HCN2 and HCN4 channels expressed in HEK293-cells, non-inactivating inward currents upon hyperpolarizing steps can be measured (Fig. 1A). In addition, both channels are modulated by intracellular cAMP (Fig. 1B). Application of 100 µM cAMP shifted significantly the steady-state activation curves towards more positive potentials. The shift for both channels is about +13 mV resulting in $V_{\text{max}1/2}$ of –86.3 mV and –84.9 mV for HCN2 and HCN4, respectively, compared to –98.7 mV and –99.1 mV without cAMP. The differences in $V_{\text{max}1/2}$ between HCN2 and HCN4 are not significant. The slope factor k is generally larger for HCN4 (10.1 vs. 7.8 for HCN2, without cAMP) meaning that HCN4 is activated over a more extended range of potentials around $V_{\text{max}1/2}$.

Determination of the activation time constants ($\tau_{\text{act}}$) reveals a major difference between HCN2 and HCN4, as has been described earlier (8): At a potential range from –140 to –100 mV, HCN2 is activated 3fold faster than HCN4 (Fig. 1C). For both channels, $\tau_{\text{act}}$ is decreased by cAMP. At –140 mV, cAMP decreases $\tau_{\text{act}}$ for HCN4 from 461 to 165 ms, and $\tau_{\text{act}}$ for HCN2 from 144 to 91 ms. However, this difference becomes less dramatic if the relative change in $\tau_{\text{act}}$ induced by cAMP is evaluated. At –140 mV, $\tau_{\text{act}}$ decreases 2.8-fold and 1.6-fold for the HCN4 and HCN2 channel, respectively. This relation ($\pm 0.4$ for both) is maintained over the range of activation potentials from -140 to -100 mV. But even though $\tau_{\text{act}}$ for both channels are much faster in the presence of cAMP, there still remains an about 2-fold difference in $\tau_{\text{act}}$ between HCN2 and HCN4 that cannot be explained by a difference in the extend of the cAMP-induced conformational change.
The C-and N-terminal regions do not account for the inherent, cAMP-independent difference in activation kinetics of HCN2 and HCN4

Previously, it was reported that cAMP-dependent differences between HCN1 and HCN2 (17, 20) or HCN1 and HCN4 (19) depended on the nature of the C-terminus. To test this possibility, we successively exchanged the N- and C-termini between HCN2 and HCN4 and determined $\tau_{\text{act}}$ with and without cAMP. Figure 2A shows the main results for HCN4: $H4NC\text{CH}_2$, i.e. HCN4 with both N- and C-terminus including the CNBD from HCN2, is still activated slowly ($\tau_{\text{act}}$ at -140 mV = 466 ms, compared to 461 and 144 ms for HCN4 and HCN2, respectively). This chimera is still modulated 2.2 to 3.5-fold by cAMP. The same results were obtained when only the N-terminus ($H4N\text{H}_2$) or the C-terminus ($H4\text{C}\text{H}_2$) were exchanged (data not shown). In addition, deleting the whole C-terminus except for 14 amino acids adjacent to the S6-segments ($H4\Delta\text{C}$) results in a channel that is activated almost exactly as fast as the cAMP-modulated HCN4 ($\tau_{\text{act}}$ at -140 mV = 177 ms compared to 165 ms for HCN4 + cAMP). In accordance with the report of Wainger et al. (18) that cAMP releases the inhibition exerted by binding to the C-terminus, cAMP has no further stimulating effect on $\tau_{\text{act}}$ of the $H4\Delta\text{C}$ chimera. As for HCN4, the activation kinetics of HCN2 is not influenced by exchange of the N- or C-terminus (Fig. 2B) indicating that the slow activation kinetics of HCN4 is not transferred with its long C-terminus. Deletion of the HCN2 C-terminus 14 or 50 amino acids after S6 did not yield functional channels supporting the notion that the C-terminus of HCN2 is essential for basic channel activity in addition to mediating cAMP-dependent regulation of channel functions. From these results, we concluded that the inherent difference in $\tau_{\text{act}}$ has to lie somewhere in the transmembrane region.
The difference in activation kinetics lies in the S1-S2 transmembrane region

Next, we constructed chimeras in which we exchanged the transmembrane regions between HCN2 and HCN4 to find the regions responsible for the difference. As can be seen in Figure 3, replacing the S1-S2 region of HCN4 with the HCN2 counterpart (H41-2H2) results in a faster activating channel with $\tau_{\text{act}}$ matching $\tau_{\text{act}}$ of HCN2. Applying cAMP accelerates this mutant channel to the same extend as the HCN2 wild type. Indeed, all mutant HCN4 channels that contain at least the S1-S2 region of HCN2 (H41-6H2, H41-4H2, H41-2H2) show this fast activation. Interestingly, both H41H2 and H42H2 chimeras, in which only S1 or S2 plus the S1-S2 linker have been exchanged, also show faster activation kinetics. However, $\tau_{\text{act}}$ of H42H2 is significantly slower than $\tau_{\text{act}}$ of HCN2 wild type (198 vs. 144 ms at −140 mV, without cAMP; p<0.05). In fact, $\tau_{\text{act}}$ of H42H2 is within the cAMP-induced activation range of HCN4. H41H2 on the other hand is as fast as HCN2 ($\tau_{\text{act}}$ at −140 mV =135 vs. 144 ms, without cAMP) and is accelerated by cAMP to almost the same extend as HCN2 ($\tau_{\text{act}}$ at −140 mV = 103 vs. 91 ms; p>0.05). The S1-S2 reverse chimera H21-2H4 (Fig. 3, last row) in which the S1-S2 region of HCN4 has been introduced into HCN2 confirms the kinetic modulating function of this element: $\tau_{\text{act}}$ of H21-2H4 is slow and matches quite exactly $\tau_{\text{act}}$ of HCN4, (462 vs. 461 ms, and 194 vs. 165 ms at −140 mV, without and with cAMP, respectively).

Figure 4 compares the electrophysiological behavior of the H41-2H2 and H21-2H4 chimeras. Both mutants are yielding inward currents comparable in size to the wild type channels, but with activation kinetics of H41-2H2 resembling HCN2 and H21-2H4 resembling HCN4. The difference in $\tau_{\text{act}}$ between the chimeras and their respective wild type channels is present over the whole range of activation potentials (-140 to -100 (-90) mV). The
changes in $\tau_{\text{act}}$ are not the result of a changed steady-state voltage dependence (Fig. 4C). There is no shift in the activation curves. The $V_{\text{max}}1/2$ do not differ significantly between any of the mutant and wild type channels.

**A single amino acid in S1 accounts for the inherent kinetic difference of HCN2 and HCN4**

The sequence comparison of the S1-S2 segment of HCN2 and HCN4 revealed five differing amino acids: two in S1, two in the S1-S2 linker and 1 in S2 (Fig. 5A). These five amino acids were replaced singly or together in the HCN4 channel by the corresponding amino acids from the HCN2 channel (Fig. 5B, 5C). Leucine 272 plays a major role in controlling the activation kinetics. Replacing leucine 272 by phenylalanine ($H4_{272}H2$, Fig. 5C, 6th row) results in a HCN2-like, fast-activating channel whereas replacing leucine 267 by phenylalanine ($H4_{267}H2$, 8th row) has no effect on activation kinetics. The double mutant that is identical to $H4_{1}H2$ has the kinetics as chimera $H4_{272}H2$ (Fig. 3). $\tau_{\text{act}}$ of $H4_{272}H2$ is 149 ms at -140 mV compared to 144 ms for HCN2. In addition, the mutant channel is modulated by cAMP like HCN2 with a $\tau_{\text{act}}$ of 106 ms at -140 mV compared to 91 ms for the wild type channel ($p>0.05$). Deletion of the C-terminus of $H4_{272}H2$ results in a functional, fast activating channel with an activation time comparable to that of HCN2 plus cAMP, suggesting that the element for the inherent activation kinetics has been transferred with the mutation L272F. The importance of this amino acid is also shown by the reverse chimera. Replacing the phenylalanine 221 in HCN2 by leucine - amino acid 272 in HCN4 corresponds to amino acid 221 in HCN2 - results in a significantly slower activating channel ($\tau_{\text{act}} = 353$ ms for $H2_{221}H4$ vs. 144 ms for HCN2). This value is close to the activation range of HCN4 being 461 ms in the absence of cAMP (Fig. 5C, 9th row and Fig. 6A). In addition, activation of $H2_{221}H4$ is accelerated 2.5- to 3-fold by 100 µM cAMP, a characteristic feature of the wild type HCN4.
channel (see Fig. 1 and above). As already shown for the S1-S2 chimeras (Fig. 4), exchanging the amino acids of S1 did not result in a left or right shift of the steady-state activation curves that could simply account for the changes in activation kinetics at a given potential (data not shown).

The relevance of the three amino acid exchanges in the external S1-S2-linker and the S2-segment was already analyzed by the chimera H42H2. This chimera is identical with the mutations of H4291/293/308H2 (see Fig. 3). This exchange results in a faster, but not quite HCN2-like fast activating channel. Replacing both amino acids of the linker together (H4291/293H2, Fig. 5C) yields a channel activating still faster than HCN4, but slower than H4291/293/308H. Replacing amino acids 291 and 293 individually (H4291H2=N291T and H4293H2=T293A) results in channels with activation kinetics not much different from HCN4. Finally, replacing amino acid 308 (H4308H2) results in a channel apparently activating even slower than the wild type HCN4 channel (τact 507 ms at −140 mV, p=0.06).

Interestingly, all the chimeras in which parts of the S1-S2-linker and/or S2 were mutated show an altered cAMP response. Most notably, the H4308H2-chimera can no longer be modulated by cAMP (τact 507 ms vs. 520 ms at −140 mV without and with cAMP, respectively).

Furthermore, deleting the C-terminus of this mutant (H4308H2ΔC, Fig. 5C, second but last row and Fig. 6B) does not yield a fast activating channel as can be observed after deletion of the C-terminus of the HCN4-wild type channel (H4ΔC, Fig. 2A, 5C, last row and 6B). These phenotypes were observed for all chimeras at a voltage range from -140 mV to -100 (-90) mV (Fig. 6) ruling out that the differences in activation time constants were caused by an alteration of the voltage dependence of these channels.

These results suggest, that isolated mutations in the S1-S2 linker and S2 segment interfere with the proper activation of HCN4 channels, independently from the presence or absence of cAMP or a C-terminus. However, these effects are not present, if the corresponding
mutation(s) is done in the S1 segment. All mutant channels show inward currents comparable in size to the wild type HCN channels (50-250 pA/pF). The exchange of amino acid 272 between HCN2 and HCN4 changes HCN4 into an HCN2-like channel and vice versa with respect to the activation kinetics (Fig. 6A). Exchanging amino acid 308 in HCN4 for the respective amino acid of HCN2 channel does not alter significantly the basic activation kinetic of the channel, but renders it unresponsive to cAMP.

**Structural requirements determining the effect of amino residue 272 of HCN4**

In a last set of experiments, we examined to which extent other amino acids can mimic the effect of phenylalanine at position 272 in HCN4. We replaced leucine 272 with methionine, tryptophan and alanine. Methionine is the amino acid found in HCN1 at the respective position, tryptophan is an aromatic, strongly hydrophobic amino acid like phenylalanine and alanine is a neutral amino acid with a short side chain. Methionine at position 272 is not able to speed up the activation of HCN4 (Fig. 7) supporting the previous notion (19) that the activation speed of HCN1 depends on several factors including the S1-S2 transmembrane region and part of the S6-C-terminal region.

A tryptophan at position 272 was able to accelerate the activation of the HCN4 channel significantly ($\tau_{\text{act}}$ at $-140 \text{ mV}$: 186 vs. 461 vs. 144 ms, $H4272W$ vs. HCN4 vs. HCN2, respectively) (Fig. 7B and C). However, introduction of tryptophan flattened the activation curve ($k = 15.9$ vs. 10.1 for HCN4) and shifted the steady-state activation curve towards more positive potentials ($V_{\text{max}1/2} = -90.8$ vs. $-99.1 \text{ mV}$ for HCN4). These changes could at least partly account for a faster $\tau_{\text{act}}$ at a potential of $-140 \text{ mV}$ (Fig. 7D). Replacement of leucine 272 by alanine had only a minor effect on $\tau_{\text{act}}$ (345 vs. 461 ms, $H4272A$ vs. HCN4) and induced a less pronounced shift of the steady-state activation curve without changing the slope factor ($V_{\text{max}1/2} = -94.0 \text{ mV}$, $k = 9.9$). Both, the $H4272F$ ($=H4272H2$) and $H4272M$-
mutations did not result in significant changes of $V_{\text{max}}^{1/2}$ (~99.1 mV and ~101.4 mV, respectively). The slope factor of H4272M is 10.2, thus equaling that of HCN4. The $k$ value of $H4272F$ is shifted towards the HCN2 value (8.4 vs. 7.8).
Discussion

HCN2 and HCN4 are structurally closely related pacemaker channels that are distributed partly in the same, partly in different tissues where they may have different functions (10,14, 21-24). A characteristic difference of the two channels is their different speed of activation. Part of this difference is carried by their different responsiveness to intracellular cAMP, but apart from this, there is also an inherent difference in activation kinetics rendering HCN4 the slower activating channel. It has been suggested that the molecular basis for this inherent difference resides somewhere in the transmembrane region. In order to find this region, we constructed chimeric HCN4/2-channels. As a basic principle, we tried to turn HCN4 into a mutant channel with the activation kinetics of HCN2, i.e. we tried to speed up the activation of HCN4. We were able to demonstrate that several residues in the S1-S2 transmembrane region determine the activation kinetics of HCN2 and HCN4 channels.

A single residue in the S1-segment determines the difference in activation speeds

The amino acid at position 272 in the S1 segment of HCN4 is responsible for the difference in the inherent activation kinetics of HCN2 and HCN4. Exchanging this residue between the two channels transfers the activation kinetics to the respective other channel. Especially, HCN4 with a phenylalanine – the respective residue in HCN2 - instead of a leucine at position 272 is activated almost exactly as fast as HCN2. The responsiveness to cAMP in this mutant channel is comparable to HCN2. Thus, we conclude that this single residue determines the distinct cAMP-independent activation time constant of HCN2 and HCN4.

Replacement of leucine 272 by tryptophan, an aromatic and hydrophobic amino acid like phenylalanine, had a similar effect on the activation time of HCN4: The speed of activation was significantly increased and the response to cAMP was maintained. However, this mutant
channel did not exactly result in an HCN2-like channel since the voltage dependence of activation was shifted towards more positive activation potentials indicating that tryptophan is not sufficient to preserve proper gating in HCN4. Although alanine at position 272 had no effect on the activation kinetic it affected as tryptophan the voltage dependence of activation supporting the notion that gating of HCN4 is rather sensitive to the nature of the amino acid at position 272.

Methionine, the corresponding amino acid of the HCN1 channel, had no effect on the activation kinetics of HCN4. In agreement with Ishii and coworkers (19), we had to replace the whole S1-S2 segment plus parts of the S6-C-terminal region to change HCN4 into an HCN1-like channel with regard to activation speed (data not shown). The need for this large replacement is not too surprising, if we consider that the effect of cAMP on the activation kinetic of HCN 1 is rather modest compared with its effect on HCN4. Ishii and coworkers (19) already concluded that the functional and structural differences between HCN4 and HCN1 are pronounced and that exchanging single residues in the S1-S2 regions of HCN1 and HCN4 did not markedly affect activation kinetics. The inability of alanine or methionine to change the speed of activation indicates that other parts of the HCN4 channel must contribute to its slow activation time constant. HCN3, an HCN channel which activates faster than HCN4 but considerably slower than HCN2 (10) has a leucine at the position corresponding to 272. SPIH, an HCN channel from sea urchin testis (25), also has a leucine at the corresponding position but is activated faster than the HCN4 channel. These considerations support the notion that additional elements are needed to affect fast or slow opening gating.

*Single residues in the S2-segment and in the S1-S2 linker influence cAMP modulation*

Replacing the S1-S2 linker and the S2 segment in HCN4 with the respective parts from HCN2 together renders the mutant channel almost as rapidly activated as HCN2
(H4291/293/308H2, Fig. 5C). However, activation is only weakly accelerated by cAMP and $\tau_{act}$ of the mutated channel is never faster than $\tau_{act}$ for the cAMP-modulated HCN4 channel.

Replacing those three amino acids individually reveals the same principle: The activation time constants of the mutant channels are always within the HCN4 range and the responsiveness to cAMP is limited and even abolished as in the case of the I308M mutation. Most likely, I308 interacts with L272 since the negative effect of the single mutation I308M on cAMP decreased activation time is surmounted if the mutation L272F is combined with I308M.

Interaction between these two amino acids are apparently also necessary to increase the activation speed by removal of the C-terminus. The presented results show that mutation of the three amino acids in the S1-S2-linker and S2-segment do not contribute to the inherent $\tau_{act}$ difference between HCN2 and HCN4. Rather, the mutant channels are HCN4-like channels which are arrested in some rigid state that does not relax if the conformation of the inhibitory C-terminal region is modulated by cAMP.

In conclusion, the basic, cAMP-independent and -dependent activation kinetics of HCN2 and HCN4 channels reside in the S1-S2 transmembrane region. Residue 272 of the S1-segment determines the difference in activation kinetics between HCN2 and HCN4 whereas residues 291 and 293 of S1-2-linker and 308 of the S2-segment are important for the proper activation gating of these HCN channels. Disrupting the structure in any of the described positions by inserting unsuitable residues results in functional but incorrectly gated channels.

The relative ease with which the basic kinetic properties of HCN2 can be transferred to HCN4, and considering that these basic kinetic properties determines the function of the respective channel in its native environment, suggests that mutations occurring in the described segments of the HCN-channels could be associated with diseases based on
improper pacemaker activities such as certain cardiac arrhythmias (14), brainstem-based respiratory impairments (21) or epilepsies (14, 23, 24).

*Acknowledgements* – The research was supported by grants from Deutsche Forschungsgemeinschaft und Fonds der Chemie.
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Figure Legends

Figure 1

**Basic properties of the human HCN2 and HCN4 channels.** A, current traces from HCN2 and HCN4-expressing HEK-cells, recorded on hyperpolarizing steps from a holding potential of -40 mV to the range of -140 mV to -30 mV. The pulse protocols are displayed below the current traces. Note the different time scales. B, voltage dependent activation curves for HCN2 and HCN4, in the absence ("-cAMP", filled circles, n=24 and filled squares, n=31, respectively) and presence ("+cAMP", open circles, n=17 and open squares, n=16, respectively) of 100 µM cAMP. Values are in mean ± s.e.m. \( V_{\text{max}1/2} \) do not differ between HCN2 and HCN4. cAMP shifted \( V_{\text{max}1/2} \) by +13 mV for both channels. C, voltage dependence of activation time constant \( \tau_{\text{act}} \) for HCN2 and HCN4 in the absence and presence of cAMP. Activation of both HCN channels is accelerated by cAMP over the whole range of potentials. Meaning of symbols and n-numbers are the same as in B, values are mean ± s.e.m.
**Figure 2**

**Effect of N- and C-termini on the activation kinetics of HCN4 and HCN2.** A, left panel: Schematic representation of wild type and mutant channels. Thick lines and black parts indicate HCN4, thin lines and white parts indicate HCN2. A, right panel: Voltage dependence of $\tau_{\text{act}}$ for HCN4, $H4\text{NCH2}$ and $H4\Delta\text{C}$. Shown is mean ± s.e.m., in the absence (-cAMP) and presence (+cAMP) of 100 µM cAMP. Numbers of experiments (n) are given in the graphics. The C- and N-terminal regions do not change the speed of activation or reaction to cAMP of the "core"-channel, represented by the S1-S6 segments. Deleting the C-terminus accelerates the channel activation and abolishes sensitivity to cAMP. B, activation kinetics of the chimera reciprocal to the one displayed in A: The $H2\text{NCH4}$-chimera does likewise not show a difference to the wild type HCN2 with respect to $\tau_{\text{act}}$ or modulation by cAMP.
Figure 3

**Activation time constants of HCN4/HCN2 chimeras.** Left panel: Schematic representation of HCN4 (black), HCN2 (white) and the HCN4/HCN2 chimeras. HCN4-derived parts are black, HCN2-derived parts are white; parts that have been exchanged are labeled in addition, with "S..(number)" referring to the transmembrane segments 1-6 as indicated above the drawing. Right panel: $\tau_{\text{act}}$ at $-140$ mV in the presence (open circles) and absence (closed circles) of 100 µM cAMP for each channel. Values are mean ± s.e.m. with the number of experiments (n) shown at the right side.
Figure 4

Current traces for S1-S2 segment chimeras. A, example current traces of HCN4, H41-2H2, HCN2 and H21-2H4 channels. B, Voltage dependence of $\tau_{act}$ for the channels shown in A. C, voltage dependent activation curves for the channels shown in A. $V_{max1/2}$ was $-97.4 \pm 3$ mV for all channels. Values in B and C are mean $\pm$ s.e.m. for 12 to 31 experiments.
Figure 5

**Effect of single amino acid replacement.** A, sequence alignment of the S1-S2 region of HCN4 and HCN2. Differing amino acids are highlighted in black. B, schematic model of HCN4 with the approximate position of the differing amino acids in white. C, left panel: schematic representation of the S1-S2-region with the respective mutations. Black bars/circles refer to HCN4, white bars/circles refer to HCN2. The letter in the circles refers to the amino acid inserted at this point in exchange for the wild type amino acid. ΔCT stands for the mutant with a deleted C-terminal region. Right panel: \( \tau_{\text{act}} \) at –140 mV for each channel in the absence (filled symbols) and presence (open symbols) of 100 µM cAMP with the number of the experiments at the right side.
Figure 6

**Current traces of L272 and I308 chimeras.** A, left panel: Current traces of H4272H2 and H2221H4. Note identical time scale for each chimera. A, right panel: voltage dependence of $\tau_{\text{act}}$ (mean ± s.e.m for 15 to 31 experiments) for wild type and mutant channels in the absence of cAMP. B, left panel: Current traces for the H4308H2 and the wild type HCN4 channel in the presence and absence of cAMP. B, right panel: voltage dependence of $\tau_{\text{act}}$ for wild type and mutant channels in the absence (filled symbol) and presence (open symbols) of cAMP. Values are mean ± s.e.m. for 8 to 31 experiments.
Figure 7

**Variation of amino acid at position 272 of HCN4.** A, scheme of HCN4 with the amino acids introduced at position 272. B, $\tau_{\text{act}}$ at $-140$ mV for wild type and mutant channels in the presence (open circles) and absence (filled circles) of 100 $\mu$M cAMP with the number of experiments at the right side. C, voltage dependence of $\tau_{\text{act}}$ for wild type and mutant channels. Values are mean ± s.e.m. for 8 to 31 experiments. D, voltage dependent activation curves for wild type and mutant channels. Values are mean ± s.e.m. for 8 to 31 experiments. Please note, that $H4_{272}$F and $H4_{272}$M don't differ in their $V_{\text{max}1/2}$ from the wild types whereas $V_{\text{max}1/2}$ of $H4_{272}$W and $H4_{272}$A are slightly shifted to more positive potentials.
Supplement Table 1

**Numbering and crossover-points of the chimeric HCN2/4-channels.** A, numbering of wild type HCN4 and HCN2, as well as HCN4- and 2-C-terminal deletion mutants (ΔC). "N-terminus" is the sequence from the start-methionine to the amino acid prior to the first amino acid of the S1-segment, C-terminus is the sequence from the amino acid following the last amino acid of the S6-segment to the last amino acid of the protein. "Total length" therefore refers to the number of amino acids from the start-methionine to the last amino acid of the protein. B, crossover-points/assembled regions for chimeras with exchanged N- and C-termini. The sequence of the chimeric channel can be obtained by assembling the respective N-, S1-6 and C-terminal regions. C, numbering of transmembrane segments S1 to S6 and linkers between segments. Note that there is no difference in the length of the individual segments and linkers between HCN4 and HCN2; hence, the chimeras in Supplement Table 1D are always as long as the backbone channel (in the name of construct: first wild type in *italics*). D, crossover-points/assembled regions for chimeras with exchanged transmembrane segments. All chimeras displayed here contain the full-length N-and C-terminus of the respective backbone wild type HCN-channel.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
A

Wildtype HCN4 and 2 and C-terminal deletion mutants:

| Name of construct | N-terminus aa # | S1-6-region aa # | C-terminus aa # | Total length aa |
|-------------------|-----------------|-----------------|----------------|----------------|
| HCN4 (=H4)        | H4, 1-257       | H4, 258-520     | H4, 521-1203   | 1203           |
| HCN2 (=H2)        | H2, 1-206       | H2, 207-469     | H2, 470-889    | 889            |
| H4ΔC              | H4, 1-257       | H4, 258-520     | H4, 521-534    | 534            |
| H2ΔC              | H2, 1-206       | H2, 207-469     | H2, 470-483    | 483            |

B

Chimeras with exchanged N- and C-termini:

| Name of construct | N-terminus aa # | S1-6-region aa # | C-terminus aa # | Total length aa |
|-------------------|-----------------|-----------------|----------------|----------------|
| H4NH2             | H2, 1-206       | H4, 258-520     | H4, 521-1203   | 1152           |
| H4CH2             | H4, 1-257       | H4, 258-520     | H2, 470-889    | 940            |
| H4NCH2            | H2, 1-206       | H4, 258-520     | H2, 470-889    | 889            |
| H2NH4             | H4, 1-257       | H2, 207-469     | H2, 470-889    | 940            |
| H2CH4             | H2, 1-206       | H2, 207-469     | H4, 521-1203   | 1152           |
| H2NC              | H4, 1-257       | H2, 207-469     | H4, 521-1203   | 1203           |

C

Transmembrane segments S1-S6 and linkers between segments:

| Segment/linker   | HCN4 aa # | HCN2 aa # |
|------------------|-----------|-----------|
| S1-6             | 258-520   | 207-469   |
| S1               | 258-280   | 207-229   |
| S1-2-linker      | 281-294   | 230-243   |
| S2               | 295-316   | 244-265   |
| S2-3-linker      | 317-340   | 266-289   |
| S3               | 341-359   | 290-308   |
| S3-4-linker      | 360-367   | 309-316   |
| S4               | 368-399   | 317-348   |
| S4-5-linker      | 400-417   | 349-366   |
| S5               | 418-442   | 367-391   |
| S5-pore-S6       | 443-491   | 392-440   |
| S6               | 492-520   | 441-469   |

D

Chimeras with exchanged transmembrane segments:

| Name of construct | HCN4-region | HCN2-region | exchanged region |
|-------------------|-------------|-------------|-----------------|
| H41-6H2           | ---         | 207-469     | S1 to S6        |
| H41-4H2           | 400-520     | 207-348     | S1 to S4        |
| H45-6H2           | 258-399     | 349-469     | S4-5-linker, S5 to S6 |
| H43-4H2           | 258-316, 400-520 | 266-348 | S2-3-linker, S3 to S4 |
| H43-6H2           | 258-316     | 266-469     | S2-3-linker, S3 to S6 |
| H41-2H2           | 317-520     | 207-265     | S1 to S2        |
| H42H2             | 258-280, 317-520 | 230-265 | S1-2-linker, S2 |
| H41H2             | 281-520     | 207-229     | S1               |
| H21-2H4           | 258-316     | 266-469     | S1-2-linker, S2 |

Supplement Table 1
Molecular basis for the different activation kinetics of the pacemaker channels HCN2 and HCN4

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*J. Biol. Chem.* published online June 17, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M305318200

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