The modulation of ion channel activity by extracellular ions plays a central role in the control of heart function. Here, we show that the sinoatrial pacemaker current \( I_f \) is strongly affected by the extracellular Cl\(^-\) concentration. We investigated the molecular basis of the Cl\(^-\) dependence in heterologously expressed hyperpolarization-activated cyclic nucleotide-gated (HCN) channels that represent the molecular correlate of \( I_f \). Currents carried by the two cardiac HCN channel isoforms (HCN2 and HCN4) showed the same strong Cl\(^-\) dependence as the sinoatrial \( I_f \) and decreased to about 10% in the absence of external Cl\(^-\). In contrast, the neuronal HCN1 current was reduced to only 50% under the same conditions. Depletion of Cl\(^-\) did not affect the voltage dependence of activation or the ion selectivity of the channels, indicating that the reduction of \( I_f \) was caused by a decrease of channel conductance. A series of chimeras between HCN1 and HCN2 was constructed to identify the structural determinants underlying the different Cl\(^-\) dependence of HCN1 and HCN2. Exchange of the ion-conducting pore region was sufficient to switch the Cl\(^-\) dependence from HCN1- to HCN2-type and vice versa. Replacement of a single alanine residue in the pore of HCN1 (Ala-352) by an arginine residue present in HCN2 at equivalent position (Arg-405) induced HCN2-type chloride sensitivity in HCN1. Our data indicate that Arg-405 is a key component of a domain that allosterically couples Cl\(^-\) binding with channel activation.

The slow diastolic depolarization in sinoatrial node (SAN)\(^1\) cells is the motor of cellular automaticity that drives the spontaneous beating of the heart (1–3). In addition, the slow diastolic depolarization is in the center of the autonomous control of heart rate because its kinetics is speeded up or slowed down by the release of norepinephrine and acetylcholine from sympathetic and parasympathetic nerve terminals, respectively. Several ionic currents have been proposed to control the time course and slope of slow diastolic depolarizations (e.g. T-type and L-type Ca\(^{2+}\) currents and the sustained inward current \( I_{Na} \)), some of which are directly regulated by the autonomous nervous system (4). Among these currents, the hyperpolarization-activated (“pacemaker”) current \( I_f \) plays an outstanding role. As the only current in SAN node, \( I_f \) is dually gated by both hyperpolarized voltages and binding of cAMP, a second messenger, the concentration of which is directly controlled by the activity of \( \beta \)-adrenergic and muscarinic receptors. Owing to its dual gating behavior, \( I_f \) contributes both to basal heart rate and to the autonomous regulation of rhythmicity. \( I_f \) is carried by a family of four hyperpolarization activated- and cyclic nucleotide-gated channels (HCN1–4) (1, 5). Genetic studies in human patients suffering from sick sinus syndrome (6, 7) and the analysis of HCN4-deficient mice (8) indicated that the HCN4 channel is required for normal sympathetic stimulation of pacemaker activity. In contrast, HCN2 is not involved in autonomous heart rate control but probably provides a safety mechanism in SAN cells that prevents the diastolic membrane potential from becoming too negative in the presence of excessive hyperpolarizations (e.g. by increased K\(^+\)-channel activity) (9). In most species, HCN1 and HCN3 are expressed only in minor amounts in the SAN (1). The relevance of these subunits for heart function has not been determined so far. Given their key role for cardiac pacemaking and heart pathology, it is important to understand how the fine-tuning of HCN channels is achieved. Several mechanisms including the formation of heteromers (10–15), the coassembly with auxiliary subunits (16–18), the interaction with cellular scaffolding proteins (19, 20) and protons (21, 22), and the phosphorylation by protein kinases (23–29), have been implicated to regulate HCN channel activity in the heart. In the clinical setting, modulation of \( I_f \) by serum electrolytes could be pivotal for cardiac pacemaker function. Indeed, there is experimental evidence that \( I_f \) activity is sensitive to changes of extracellular Cl\(^-\) concentration (30). It has been convincingly demonstrated that \( I_f \) is a purely catonic current and that Cl\(^-\) does not pass HCN channels (5, 30). Nevertheless, Frace et al. (31) showed that the amplitude of rabbit sinoatrial \( I_f \) decreased when extracellular Cl\(^-\) was replaced by larger anions such as aspartate (31). This finding could have considerable clinical relevance since in hypochloremia, plasma Cl\(^-\) concentration can drop to values as low as 60 mM (32–34). The inhibition of \( I_f \) at low Cl\(^-\) may well lead to the impairment of pacemaker function and, hence, to the induction of dysrhythmia.

In the last couple of years, genetic mouse models have opened novel perspectives to investigate the (patho)physiological role of ion channels in heart. Since it was not known so far whether or not murine \( I_f \) is regulated by Cl\(^-\), we set out in this study to characterize in mouse SAN the modulatory effect of this anion. We then determined structural determinants underlying the Cl\(^-\) dependence of \( I_f \) in cloned cardiac HCN channels. To this end, we made use of our finding that HCN2 and HCN4 channels profoundly differ from the HCN1 isoform in terms of their sensitivity to Cl\(^-\). This diversity allowed us to use chimeras constructed between HCN1 and HCN2 to pinpoint individual amino acid residues underlying the Cl\(^-\) effect.
Experimental Procedures—Cl− Sensitivity in Pacemaker Channels

Molecular Biology—mHCN1/mHCN2 chimeric channel mutants and site-directed mutations were constructed in the pcDNA3 mammalian expression vector (Invitrogen). Briefly, the 389-bp Accl-Blap fragment of the wild-type mHCN2 expression plasmid (35) or the 625-bp BspHl-PstI fragment of mHCN1 (36) was ligated into DNA fragments self-catalyzed in several overlapping PCR steps, which contained the desired sequence and mutations. All constructs were confirmed by restriction enzyme analysis and sequencing. In Ch1, amino acids Ile 326–Leu 419 of mHCN2 were replaced by Ile 273–Leu 366 of mHCN1. In Ch2, amino acids Asn 376–Ser 410 of mHCN2 were replaced by Asn 323–Ser 357 of mHCN1. In Ch3, amino acids Ile 273–Ser 388 of mHCN1 were replaced by Ile 326–Ser 411 of mHCN4. Amino acids Asn 376–Ser 357 of mHCN1 were replaced by Asn 376–Ser 411 of mHCN2. All other chimeric and mutant HCN1/HCN2 channels were as indicated in Fig. 5.

Cell Culture and Heterologous Expression—HEK293 cells (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin and incubated at 37 °C with 10% CO2. For microcopy and electrophysiological analysis, glass coverslips were coated in 24-well dishes with poly-L-lysine (Sigma), and 30,000 HEK293 cells/well were seeded. After 24 h, the cells were transiently transfected with expression plasmids using FuGENE 6 transfection reagent (Roche Diagnostics).

Electrophysiology—Currents were measured at room temperature 2–3 days after transfection using whole cell patch clamp technique. The following extracellular solutions were used. The standard extracellular chloride-solution was composed of (in mM): 110 sodium aspartate, 0.5 magnesium gluconate; 1.8 calcium gluconate; 5 HEPES; 30 potassium gluconate, pH 7.4. The extracellular solutions were exchanged by a monoexponential function fitting the current evoked during hyperpolarizing voltage pulses to −140 mV. As has been described earlier, the initial tail currents were recorded immediately after stepping to a family of test voltages ranging from −70 to 0 mV preceded by a 2.5-s prepulse to either −140 or −20 mV. The difference of tail currents resulting from the two prepulse potentials was plotted against the test potentials, and I0mV was determined from the intersection of the I/V curve with the voltage axis (35). Changes of liquid junction potentials were either calculated using the JPcal software (Dr. P. Barry, University of South Wales, Sydney, Australia (37)) or directly measured by a free-flowing KCl electrode. Liquid junction potentials were −7.4 mV for solution containing 130 mM aspartate, 3.1 mV for solution containing 145 mM Cl−, and 2.9 mV for solution containing 140 mM I−. These values were used for a posteriori correction (36). For all experiments, agar bridges were used as reference electrode to minimize electrode offsets.

Isolation of Murine Sinoatrial Node Cells—Isolated SAN cells were prepared from adult C57BL/6J mice of either sex, aged 8–12 weeks as described (9, 39). The animals were treated in accordance to German legislation on the protection of animals. Single SAN cells were plated onto glass coverslips. For whole cell patch clamp recordings, 5 mM BaCl2 was added to the recording solution; in experiments with 0 mM Cl−, 1 mM BaOH2 was added. Higher BaOH concentrations were not compatible with a pH of 7.4.

Statistics—All values are given as mean ± S.E. n = number of experiments. An unpaired student’s t test was performed for the comparison between two groups. Significance was also tested by analysis of variance if multiple comparisons were made. Values of p < 0.05 were considered significant.

Results—I− of Murine SAN Cells Is Highly Sensitive to Changes in Extracellular Cl− Concentration—Spindle-shaped pacemaker cells were isolated from the SAN node region of adult mice. Murine heart muscle and were analyzed using the whole cell patch clamp method (Fig. 1A). In extracellular solution containing a physiological concentration of Cl− (105 mM), nearly every cell investigated showed a prominent I− upon hyperpolarizing steps to −140 mV (Fig. 1B). The current was non-inactivating and reached steady-state values within 3 s. Increasing extracellular Cl− to 125 mM did not significantly affect the maximal current (I−max), whereas reduction to 85, 65, and 55 mM decreased I−max by 9.9 ± 1.9% (n = 23), 21 ± 3.8%, (n = 4), and 54 ± 4.2% (n = 6), respectively (Fig. 1C). On complete substitution of extracellular Cl− by aspartate, the remaining current (I−rem) was only 12.6 ± 2.1% (n = 14) of the current evoked at 105 mM extracellular Cl− (Fig. 1, B and C).

HCN2- and HCN4-mediated Currents Are More Affected by Changes in Extracellular Cl− than HCN1 Currents—To investigate whether or not sensitivity to extracellular Cl− was also a feature of cloned HCN channels, we expressed the two cardiac HCN channel isoforms, HCN2 and HCN4, as well as the neuronal HCN1, in HEK293 cells. Fig. 2A shows representative current traces of the three channels obtained in 145 mM Cl− and in the absence of Cl−. Like the native cardiac I−, HCN2 and HCN4 currents were almost completely abolished (about 90% reduction) when Cl− was replaced by aspartate. In contrast, the maximal HCN1 current was suppressed by only about 50% in the complete absence of Cl−. Replacement of extracellular Cl− by aspartate or gluconate in inside-out patch measurements had no influence on HCN channel currents (data not shown). To exclude that the inhibition of I−max was caused by a block induced by aspartate rather than reflecting a true Cl−-depletion effect, HCN1 and HCN2 currents were also measured in the presence of gluconate, an anion that has a comparable size as aspartate but is structurally unrelated to it. The reduction of I−max measured under these ionic conditions was virtually identical to that seen in aspartate solution. (HCN1, I−rem = 48.6 ± 1.5%, n = 7; HCN2, I−rem = 8.3 ± 1.9%, n = 4). There was no difference between the absolute magnitude of HCN1 and HCN2 currents recorded in either gluconate or aspartate solution (not shown). Taken together, these data indicated that I− is indeed controlled by extracellular Cl−.

The failure of the large anions aspartate and gluconate to...
In the following, we set out to determine the structural dependence of the Cl– sensitivity of HCN currents. Reduction of Cl– concentration decreased the slope conductance of the fully activated I/V curves (Fig. 3, E and F), whereas replacement of Cl– by I– increased it (not shown).

Exchange of the Pore Region Is Sufficient to Switch between HCN1- and HCN2-like Cl– Dependence—The finding that Cl– and I– affected the slope of the fully activated I/V curves indicated that small anions control the channel conductance of HCN1 and HCN2. We assumed that the amino acid residues conferring Cl– dependence were localized in the outer pore region of the channel, where they were involved in the control of ion permeation. To test for this hypothesis, we constructed four chimeric channels in which the region spanning from transmembrane domain S5 to S6 (including the pore) or only the pore region was exchanged between HCN1 and HCN2 (Fig. 4). Chimera (Chi) 1 and Chi2 containing the S5-S6 sequence of HCN1 inserted into the backbone of HCN2, respectively, showed virtually the same sensitivity to depletion of Cl– as HCN2. The remaining current fraction in the absence of external Cl– was 7.8 ± 1.5% (n = 11) and 6.4 ± 1.4% (n = 8), which is not significantly different from the value of 6.5% determined for HCN2 (Fig. 2). Similarly, HCN2 acquired a HCN1-like Cl– sensitivity when its S5-S6 sequence (Chi3, Irem 46.6 ± 1.7 (n = 16)) or pore (Chi4 Irem 39.2 ± 1.7 (n = 11)) was exchanged by the corresponding portions of HCN1.

Identification of Amino Acid Residues in the Pore Contributing to the Sensitivity to External Cl–—The sequence that was sufficient in Chi2 and Chi4 to switch the Cl– dependence from HCN1-type to HCN2-type and vice versa consists of 35 amino acid residues from which all but eight residues are identical between HCN1 and HCN2 (Fig. 5A). To determine the individual contribution of the eight residues for the Cl– dependence, we constructed a number of chimeras and channel mutants based on Chi4. Our goal was to define the minimal sequence
that conferred HCN1-like Cl⁻ dependence in the context of a HCN2 backbone. In the chimeras Chi5 and Chi6, the first five and the last three amino acids of the eight different residues were exchanged by the corresponding segments of HCN1 (Fig. 5B). The $I_{\text{rem}}$ was 18.7 ± 2.5% ($n = 10$) for Chi5 and 37.6 ± 1.6% ($n = 23$) for Chi6. The $I_{\text{rem}}$ of Chi6 was almost identical to the $I_{\text{rem}}$ of Chi4 (39%) and was very close to that of wild-type HCN1 (47%), indicating that the three HCN1-derived residues present in this chimera were key determinants of the HCN1-type Cl⁻ dependence. Conversely, the relatively low $I_{\text{rem}}$ of Chi5, which was close to that of HCN2 (10%), indicated that the five HCN1-derived residues present in this chimera were less influential in inducing HCN1-like behavior. In support of this notion, chimeras in which only subsets of the five residues were exchanged revealed a $I_{\text{rem}}$ that was even closer to that of wild-type HCN2 (data not shown).

We sought to define the relative importance of the HCN1-derived residues present in Chi6 (F389Y, R405A, and E409V) by analyzing another set of chimeras (Chi7–12; Fig. 5B). First, we replaced Phe-389 in the pore helix of HCN2 by the analogous tyrosine of HCN1 (HCN2F389Y; Chi7). The resulting $I_{\text{rem}}$ was 29.6 ± 1.8% ($n = 29$), which is in between the respective values of HCN1 and HCN2. This finding suggested that Phe-389 is influential in determining high Cl⁻ sensitivity in HCN2. In support of this notion, a chimera containing all HCN1-derived amino acids present in Chi4 with the exception of the F389Y mutation (Chi8) showed a substantial decrease in $I_{\text{rem}}$ with respect to Chi4 ($I_{\text{rem}}$ of Chi8 = 25.3 ± 1.2% ($n = 11$)). We next explored the role of position 409. A HCN2 channel containing F389Y and R405A mutations (Chi9) did not yield $I_{\text{rem}}$ currents, indicating that the residue present at position 409 (the only residue in which Chi9 differs from Chi6) is critical for principal channel activity. However, the finding that Chi10 (HCN2F389Y, E409Y) carrying a HCN1-type valine at position 409 had a somewhat lower $I_{\text{rem}}$ than Chi7 (HCN2F389Y) carrying a HCN2-type glutamate argues against a major role of position 409 in determining HCN1- and HCN2-type Cl⁻ dependence. Finally, we replaced Arg-405 by the corresponding alanine present in HCN1 (HCN2A405A; Chi11). Arg-405 is an attractive candidate for controlling Cl⁻ dependence because it is adjacent to the ion selectivity filter, the narrowest part of the pore, and because its positively charged side chain might well interfere with the flux of cations. Unfortunately, Chi11 did not yield functional channels. Similarly, a chimera in which both Arg-405 and Glu-409 were mutated (HCN2A405A, E409V; Chi12) failed to express currents. However, when Ala-405 in Chi6 was back-mutated to arginine (HCN2F389Y, E409Y; Chi10), $I_{\text{rem}}$ decreased from 37.6 ± 1.6% (Chi6) to 17.9 ± 3.5% ($n = 7$). This result strongly indicated that Arg-405 is an important residue in conferring strong (HCN2-type) Cl⁻ dependence. Interestingly, Arg-405 is also conserved in the pore of the highly Cl⁻ sensitive HCN4 channel, whereas this channel carries HCN1-type tyrosine and valine residues at positions equivalent to residues 389 and 409 of HCN2 (Fig. 6A). To evaluate the role of Arg-405 directly, we introduced this residue into the backbone of HCN1 (HCN1A352R). Unlike the complementary HCN2 mutant (Chi11), this channel mutant expressed well and gave robust currents (Fig. 6A). Importantly, the current revealed the same Cl⁻ sensitivity as wild-type HCN2 ($I_{\text{rem}}$ of HCN1A352R; 6.3 ± 1.5%, $n = 12$). In contrast, the mutation had no influence on the voltage dependence of activation (Fig. 6B; $V_{0.5, \text{act}} = -74.2 ± 5.5$ mV ($n = 6$)), nor did it alter the opening kinetics with respect to wild-type HCN1 ($\tau_{\text{on}} = 65.4 ± 13.9$ ms ($n = 12$)). Thus, in the backbone of HCN1, the exchange of Ala-352 by Arg was sufficient to achieve a complete switch of the Cl⁻ sensitivity from HCN1 to HCN2-type behavior.

**DISCUSSION**

In this study, we provide evidence that replacement of extracellular Cl⁻ by large anions induces a profound reduction of $I_{\text{max}}$ and a moderate deceleration of opening kinetics of both native and cloned pacemaker channels. In principal, this effect could result from the blockage of the channels by large anions as has been proposed previously (40, 41). However, such a model, which requires that large anions bind to the channel pore, and because its positively charged side chain might well interfere with the flux of cations. Unfortunately, Chi11 did not yield functional channels. Similarly, a chimera in which both Arg-405 and Glu-409 were mutated (HCN2A405A, E409V; Chi12) failed to express currents. However, when Ala-405 in Chi6 was back-mutated to arginine (HCN2F389Y, E409Y; Chi10), $I_{\text{rem}}$ decreased from 37.6 ± 1.6% (Chi6) to 17.9 ± 3.5% ($n = 7$). This result strongly indicated that Arg-405 is an important residue in conferring strong (HCN2-type) Cl⁻ dependence. Interestingly, Arg-405 is also conserved in the pore of the highly Cl⁻ sensitive HCN4 channel, whereas this channel carries HCN1-type tyrosine and valine residues at positions equivalent to residues 389 and 409 of HCN2 (Fig. 6A). To evaluate the role of Arg-405 directly, we introduced this residue into the backbone of HCN1 (HCN1A352R). Unlike the complementary HCN2 mutant (Chi11), this channel mutant expressed well and gave robust currents (Fig. 6A). Importantly, the current revealed the same Cl⁻ sensitivity as wild-type HCN2 ($I_{\text{rem}}$ of HCN1A352R; 6.3 ± 1.5%, $n = 12$). In contrast, the mutation had no influence on the voltage dependence of activation (Fig. 6B; $V_{0.5, \text{act}} = -74.2 ± 5.5$ mV ($n = 6$)), nor did it alter the opening kinetics with respect to wild-type HCN1 ($\tau_{\text{on}} = 65.4 ± 13.9$ ms ($n = 12$)). Thus, in the backbone of HCN1, the exchange of Ala-352 by Arg was sufficient to achieve a complete switch of the Cl⁻ sensitivity from HCN1 to HCN2-type behavior.
region as the crucial determinant controlling HCN1- or HCN2-type Cl\(^{-}\) dependence. Within the identified region, HCN1 and HCN2 differ from each other only in eight amino residues. Among these residues, the residue directly adjacent to the selectivity filter sequence was identified as the key determinant of Cl\(^{-}\) sensitivity. The two channels with high Cl\(^{-}\) sensitivity (HCN2 and HCN4) carry an arginine at this position, arguing against a general role of this domain in determining subtype specificity of Cl\(^{-}\) dependence.

How may Cl\(^{-}\) control the activity of HCN channels? The crystal structure of the HCN channel pore has not been determined so far. However, based on sequence alignments, one can assume that the pore is structurally related to that of the KscA potassium channel (42, 43) (Figs. 5 and 7). On the basis of this structure, all eight amino acids that are different between HCN1 and HCN2 are localized within or close to the external portion of the pore and are probably localized outside of the electrical field. This is in line with the finding that Cl\(^{-}\) does not affect the voltage dependence of channel activation. Two models could explain the effect of Cl\(^{-}\). In the first model, Cl\(^{-}\) promotes channel opening by binding to an activatory domain that tonically blocks channel closure and thereby sensitizes the channel to voltage-gating. In the second model, Cl\(^{-}\) binding could increase channel activity by relieving the influence of an inhibitory domain that tonically blocks channel closure. Alternatively, Cl\(^{-}\) binding could increase channel activity by relieving the impact of an inhibitory domain that tonically blocks channel conductance in the absence of small anions.

The question arises as to which role the identified residues fulfill in the allosteric regulation of HCN channel activity. The \(K_v\) values and Hill coefficients deduced from the dose-response relation curves of the Cl\(^{-}\) dependence were virtually identical for both HCN1 and HCN2. Thus, the binding site for Cl\(^{-}\) is most likely conserved between both channels and is probably formed by amino residues of the pore that are identical in both channels. Although unlikely, it cannot be excluded that the binding site of Cl\(^{-}\) is situated even outside of the pore.

### Table I

| Channel | Anion | \(V_{0.5,act}\) | \(h_{act}\) | \(V_{rev}\) | \(\tau_{act}\) |
|---------|-------|----------------|-------------|-------------|-------------|
| HCN1    | 145 Cl\(^{-}\) | −75.6 ± 0.9 | 6.7 ± 0.3 | −24.2 ± 0.4 | 87.7 ± 2.9 |
| HCN1    | 10 Cl\(^{-}\)  | −76.6 ± 0.9 | 7.0 ± 0.3 | −24.0 ± 0.4 | 128.9 ± 6.3*|
| HCN1    | 145 I\(^{-}\)  | ND           | ND         | −24.5 ± 0.7 | ND         |
| HCN2    | 145 Cl\(^{-}\) | −97.3 ± 0.9 | 5.2 ± 0.2 | −22.9 ± 1.5 | 196.6 ± 9.4 |
| HCN2    | 10 Cl\(^{-}\)  | −97.4 ± 1.1 | 5.0 ± 0.2 | −22.3 ± 1.1 | 380.7 ± 17.4*|
| HCN2    | 145 I\(^{-}\)  | ND           | ND         | −24.2 ± 1.0 | ND         |

*Statistical differences are given in \(p < 0.05\). ND, not determined.
over, since Hill coefficients had values of about 1.5, more than one, Cl\/H<sub>11002</sub> may bind to the tetrameric HCN channel complex.

Our data suggest that Arg-405 is part of a regulatory domain that, in addition to the sensors for voltage (S4 segment) and cAMP (the cyclic nucleotide-binding domain), controls HCN channel gating. Since Arg-405 of HCN2 is localized close to the narrow opening of the pore, it may prevent a substantial flux of cations due to its positive charge (Fig. 7). Binding of Cl\/H<sub>11002</sub> may promote cation flux by inducing a conformational change that alters the relative position of Arg-405. Phe-389, and to minor extent, other residues in the proximal pore region, are probably involved in the coupling between Cl\/H<sub>11002</sub> binding and pore opening.

In support of this model, HCN4, which, like HCN2, is highly Cl\/H<sub>11002</sub>-dependent, also contains an arginine at the position equivalent to Arg-405. By contrast, HCN1 carries an alanine instead of arginine at a position equivalent to Arg-405. The smaller size and the uncharged nature of the alanine may explain why HCN1 exhibits a substantial ion flux even in the absence of Cl\-. 

FIG. 5. Contribution of distinct portions of the pore loop to the determination of [Cl\^-] sensitivity. A, sequence alignment of the pore regions from HCN1, HCN2, HCN4, and the KcsA potassium channel (42, 43). The localization of the S5- and S6-transmembrane helices, the pore helix, and the selectivity filter are indicated on top of the alignment. The amino acid residues different between the three HCN channel types are highlighted. B, schematic representation of Chi4–12. Only the eight amino acid residues in the pore that differ between HCN1 and HCN2 are depicted. Residues are highlighted in black if derived from HCN1 or boxed if derived from HCN2. The I<sub>rest</sub> measured at 0 mM [Cl\^-] is given beside each chimera. Values are mean ± S.E. with the numbers of experiments (n) shown at the right. n.c., no current upon six independent transfections.

FIG. 6. Functional properties of HCN1<sub>A352R</sub> mutant. A, whole cell current traces of HCN1<sub>A352R</sub> obtained in extracellular solutions containing 145 mM Cl\^- (closed circle) and 0 mM Cl\^- (open circle). B, steady-state activation curves of wild-type HCN1 (open circles) and HCN1<sub>A352R</sub> at 145 mM (n = 6).

FIG. 7. Structure model of the KcsA channel. For clarity, only two out of four subunits are shown. The inner and outer transmembrane helices corresponding to S6 and S5 of HCN channels are blue. The pore helices are shown in orange. The residues of KcsA corresponding to residues different between HCN2 and HCN1 are highlighted green or pink. Residues 64 and 80 correspond to Phe-389 and Arg-405 in HCN2.
A potential role of the Arg-405 in channel gating is also supported by a recent study of Azene et al. (44), showing that this particular residue affects the modulation of HCN channel currents by external K⁺. Unlike Azene et al. (44), we did not observe a hyperpolarizing shift of V_{0.5} and a slow down in activation kinetics in the HCN1_{R452R} mutant. The reason for this discrepancy is not known but may be due to use of different expression systems (Xenopus oocytes in Azene et al. (44) and HEK293 cells in the present study).

With respect to its strong Cl⁻ dependence, the sinoatrial Iₚ closely resembles the HCN2- and HCN4-mediated currents. This finding corroborates previous studies, indicating that, at least in mouse and human, HCN2 and HCN4 represent the major molecular correlate of cardiac I_{f}(45). The strong dependence of the sinoatrial Iₚ on the extracellular Cl⁻ could have significant clinical implications. We observed an about 10 and 20% reduction when Cl⁻ decreased from its physiological concentration of 105 mM to pathological concentrations of 85 and 65 mM, respectively. In genetic mouse models, it has been shown that a reduction of total Iₚ current amplitude of 20% is sufficient to induce sinus dysrhythmia (9, 45). Moreover, such a mechanism could well contribute to arrhythmia occurring during hypochloremia associated with metabolic alkalosis (46, 47). Finally, HCN channels are widely expressed in the central nervous system, where they fulfill various functions (2, 48). Thus, a decrease in the activity of HCN channels could also be involved in the pathomechanism of important neurological disorders including absence seizures and ataxia (9, 49).

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