Separable Gating Mechanisms in a Mammalian Pacemaker Channel*

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Despite permeability to both K\(^+\) and Na\(^+\), hyperpolarization-activated cyclic nucleotide-gated (HCN) pacemaker channels contain the K\(^+\) channel signature sequence, GYG, within the selectivity filter of the pore. Here, we show that this region is involved in regulating gating in a mouse isoform of the pacemaker channel (mHCN2). A mutation in the GYG sequence of the selectivity filter (G404S) had different effects on the two components of the wild-type current; it eliminated the slowly activating current (I\(_{\text{f}}\)) but, surprisingly, did not affect the instantaneous current (I\(_{\text{inst}}\)). Confocal imaging and immunocytochemistry showed G404S protein on the periphery of the cells, consistent with the presence of channels on the plasma membrane. Experiments with the wild-type channel showed that the rate of I\(_{\text{f}}\) deactivation and I\(_{\text{f}}\) amplitude had a parallel dependence on the ratio of K\(^+\)/Na\(^+\) driving forces. In the amplitude of fully activated I\(_{\text{f}}\), unlike I\(_{\text{inst}}\), was not well predicted by equal and independent flow of K\(^+\) and Na\(^+\). The data are consistent with two separable gating mechanisms associated with pacemaker channels: one (I\(_{\text{f}}\)) that is sensitive to voltage, to a mutation in the selectivity filter, and to driving forces for permeating cations and another (I\(_{\text{inst}}\)) that is insensitive to these influences.

Pacemaker channels (also known as Hyperpolarization-activated Cyclic Nucleotide-gated or HCN\(^1\) channels) are expressed by many excitatory cells of the central nervous system and heart where they produce a slowly activating current known as I\(_{\text{f}}\), I\(_{\text{h}}\), or I\(_{\text{q}}\) (1–7). I\(_{\text{f}}\) is involved in regulating membrane potential and spontaneous activity in a variety of excitable cells (8, 9). I\(_{\text{f}}\) has a clear role in the sino-atrial node, where its activation upon hyperpolarization, deactivation upon depolarization, and permeability to both Na\(^+\) and K\(^+\) are important for regulating the rate of diastolic depolarization (9). I\(_{\text{f}}\) is also important in thalamocortical neurons where its deactivation produces a slowly decaying after-depolarization that determines the length of refractory periods separating episodes of synchronized oscillations (8, 10).

We have recently shown that HCN2 channels produce an instantaneous and Cs\(^+\)-insensitive current component (I\(_{\text{inst}}\)) in addition to the hyperpolarization-activated and Cs\(^+\)-sensitive I\(_{\text{f}}\) component (11). I\(_{\text{inst}}\) was not affected by a mutation in the S4 transmembrane segment (S306Q) whereas I\(_{\text{f}}\) was greatly reduced. Thus, our data support a role for the S4 segment in voltage-dependent gating (I\(_{\text{f}}\)), as suggested previously for HCN channels (12, 13) but not in voltage-independent gating (I\(_{\text{inst}}\)).

Despite permeability to both K\(^+\) and Na\(^+\), the HCN pore contains a conserved GYG sequence that is found in many potassium-selective channels (14, 15). Studies examining the effects of mutations in the selectivity filter of K\(^+\) channels have shown that conformational changes in this region contribute directly to channel gating (16, 17). Permeant and blocking ions such as Rb\(^+\), K\(^+\), and Cs\(^+\) also affect the closing of K\(^+\) channels, implicating the selectivity filter in voltage-dependent gating (18–21). Finally, the KcsA K\(^+\) channel selectivity filter and activation gate have recently been shown to have different conformations in conditions of low K\(^+\) and high K\(^+\), which could explain the effects of permeant ions on gating (22). Based on the similarity of the pore structure in HCN and K\(^+\) channels, it seems likely that the selectivity filter and the voltage-dependent gate of HCN channels are linked structurally such that changes in one influence the function of the other. However, this has not been directly demonstrated.

In this study, we carried out experiments to determine whether the selectivity filter is coupled to gating in HCN channels using mouse HCN2 subunits expressed in Chinese hamster ovary (CHO) cells. We found that I\(_{\text{f}}\) was sensitive to a mutation in the selectivity filter and to different concentrations of permeating cations, whereas I\(_{\text{inst}}\) was insensitive to these influences. The results may be explained by changes in pore conformation upon hyperpolarization, and/or by the presence of a second pore that is: 1) found within the same channel, 2) formed by a second population of the same channel subunits, or 3) associated with HCN channels in the form of up-regulated endogenous channels.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Expression**—The G404S mutant was constructed by overlapping PCR mutagenesis from a mouse HCN2 template as previously described (11). The amplified mutagenic product and wild-type mHCN2, in the mammalian expression vector pcDNA3 (6), were subsequently digested using NheI and BglII. The fragment digested out of wild-type mHCN2 was replaced by the complementary fragment carrying the mutation. The mutation was confirmed by restriction analysis and automated sequencing (Biotechnology Laboratory, University of British Columbia, Vancouver Canada).

**Tissue Culture**—CHO-K1 cells were obtained from ATCC (Manassas, VA), maintained in Hams F-12 media supplemented with antibiotics and 10% fetal bovine serum (Invitrogen) and incubated at 37 °C with

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1 The abbreviations used are: HCN, hyperpolarization-activated cyclic nucleotide-gated channel; I\(_{\text{f}}\), I\(_{\text{h}}\), or I\(_{\text{q}}\) hyperpolarization-activated current; I\(_{\text{inst(HCN2)}}\), instantaneous current in CHO cells expressing HCN2; G404S, HCN2 selectivity filter mutant; I\(_{\text{inst(G404S)}}\), instantaneous current in CHO cells expressing G404S; GFP, green fluorescent protein; I\(_{\text{inst(G404S)}}\), instantaneous current in CHO cells expressing GFP; CHO, Chinese hamster ovary cells; PBS, phosphate-buffered saline.

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% CO₂. Cells were plated onto glass coverslips and 1 day after splitting were transiently co-transfected with mammalian expression vectors encoding wild-type and/or mutant mHCN2 channels (4 µg per 35-mm dish) along with a green fluorescent protein (GFP) reporter plasmid (0.3 µg per dish) using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals).

Electrophysiology—Cells expressing GFP were chosen for whole-cell recordings 24–48 h after transfection or co-transfection with mHCN2. The pipette solution contained (in mM): 130, KCl; 10, NaCl; 0.5, MgCl₂; 1, EGTA; 5, HEPES; pH adjusted to 7.4 with KOH. The extracellular solution contained (in mM) 140, XCl (X = sum of K⁺ and Na⁺); 1.8, CaCl₂; 5, MgCl₂; 10, HEPES; pH adjusted to 7.4 with NaOH. For solution changes, a 200-µl bath was completely exchanged and perfused (0.5–1 ml/min) for at least 1 min prior to collecting data. Whole-cell patch clamp currents were recorded using an Axopatch 200B amplifier and Clampex software (Axon Instruments, Union City, CA) at room temperature (20–22 °C). Currents were not leak-subtracted and capacitance compensation was not used. Patch clamp pipettes were pulled from borosilicate glass and were fire polished before use (pipette resistance is 2.5–4.5 MΩ). Data were filtered at 2 kHz and were analyzed using Clampfit (Axon Instruments), Origin (Microcal, Northampton, MA) and Excel (Microsoft, Seattle, WA) software. The current densities plotted in Fig. 2, A and B were determined by dividing measured currents by the capacitance, which was estimated by the ClampEx software from the time constant of the current elicited by a 2 nA test pulse at the beginning of whole-cell recording.

Time constants to assess rates of activation and deactivation were generated using a single exponential fitting procedure. An initial delay occurred prior to Iᵢ deactivation that was not well described by a single exponential function, as described previously (5, 25) and therefore was not used in our fits. Fig. 4 uses values of Iᵢ, corrected for the presence of endogenous instantaneous currents. Previously, we found that the amplitude of Iᵢ was 41 and 48% larger than the instantaneous current determined in cells transfected with only GFP or with the pHOOK membrane protein (a single chain antibody, fused to the C terminus of the transmembrane domain from the platelet-derived growth factor receptor, that allows for the antibody to be anchored and displayed on the extracellular side of the CHO cell plasma membrane), at −140 mV and −65 mV, respectively (11). These proportions were used to estimate the amplitudes of instantaneous current due only to HCN2, plotted in Fig. 4, C and D (Iᵢ,corrected).

Immunocytchemistry and Confocal Microscopy—At 24–48 h post-transfection, cells on coverslips were washed with PBS and fixed in 2% paraformaldehyde in PBS for 5 min. The cells were then washed with PBS (3 × 5 min) using 0.2% Triton X-100, and blocked with 10% normal goat serum (NGS). After one wash with PBS containing 1% NGS, the cells were incubated with a rabbit polyclonal antibody to NGS (Alomone Labs, Jerusalem, Israel) at a dilution of 1:200 in PBS with 1% anti-rabbit antibody tagged with cyanine 3 (Cy3) (Jackson Laboratories, West Grove, Pennsylvania) at a dilution of 1:400 in PBS with 1% Triton X-100. After washing with PBS, the coverslips with cells were permeabilized using 0.2% Triton X-100, and blocked with 10% NGS, the cells were incubated with a rabbit polyclonal antibody to HCN2 (Alomone Labs, Jerusalem, Israel) at a dilution of 1:400 in PBS, and then washed with PBS. Cells were then incubated with a donkey anti-rabbit antibody tagged with cyanine 3 (Cy3) (Jackson Laboratories). The antibody was removed, and cells were washed in PBS, again washed with PBS. Cells were then incubated with a mouse monoclonal antibody (clone HC2.1, 1:125 in PBS with 1% NGS) that recognizes the N-terminal of the CHO cell plasma membrane, at −85°C in the dark. The antibody was removed, and cells were washed in PBS, and the coverslips with cells were mounted on slides using Permount (Fisher Scientific). Cells were examined using wide-field and confocal microscopy with an Olympus BX 51 microscope, equipped with epifluorescence and appropriate filters, and an inverted Zeiss TurboPascal confocal microscope, respectively. For confocal microscopy, serial sections were taken in 0.8–1.0 µm steps using a ×63 oil immersion objective lens and an excitation wavelength of 543 nm.

RESULTS

A Pore Mutation (G404S) Reduces If but Does Not Affect Iᵢ, corrected—Our initial strategy to examine the involvement of the selectivity filter in gating was to introduce a mutation into the GYG sequence of mHCN2 (G404S) and determine its effects on expressed current. Fig. 1 A and B show the region of the channel encompassing the selectivity filter has significant homology among different classes of K⁺ channels and of HCN channels. We mutated the glycine at position 404 to a serine (Fig. 1, asterisk). Serine was chosen because the corresponding mutation (G629S) in the human ether-a-go-go-related channel (HERG), which is related to HCN channels, eliminated slowly activating current but not trafficking to the plasma membrane (23). Cells expressing G404S produced little If, even in a high K⁺ (135 mM) extracellular solution (Fig. 2A). We determined If and Iᵢ, corrected densities (see “Experimental Procedures”) in cells expressing wild-type HCN2 and G404S in order to control for variability in cell surface area. Cells expressing G404S produced significantly less If than did cells expressing wild-type HCN2 (Fig. 2B). The instantaneous current associated with G404S (Iᵢ,corrected(G404S)) and wild-type HCN2 (Iᵢ,corrected(HCN2)) were significantly larger than the instantaneous current observed in cells expressing only GFP (Iᵢ,corrected(GFP)) but were not significantly different from each other (Fig. 2, A and C). The reversal potentials of Iᵢ,corrected(G404S) and Iᵢ,corrected(HCN2) were significantly more positive than the reversal potential of Iᵢ,corrected(GFP) but not significantly different from each other (Fig. 2D). This indicates that the expression of either wild-type HCN2 or G404S added a conductance with a reversal potential positive to that of the endogenous channels found in CHO cells expressing only GFP. The similar reversal potentials for Iᵢ,corrected(G404S) and Iᵢ,corrected(HCN2) suggests similar selectivities for the wild-type HCN2 and G404S channels. Like Iᵢ,corrected(G404S), Iᵢ,corrected(HCN2) was significantly (p < 0.05) and reversibly reduced by cAMP elevation with perfusion of 100 µM IBMX (3-isobutyl-1-methylxanthine), an inhibitor of phosphodiesterase that inhibits the breakdown of cAMP, and 100 µM forskolin, an activator of adenyl cyclase that increases cAMP formation (~931.2 ± 139.8 pA, n = 17, −858.8 ± 127.5 pA, n = 17, −1048.0 ± 174.1 pA, n = 15; before IBMX and forskolin, after 1 min in IBMX and forskolin, and after a 1-min wash, respectively). Finally, we found that Iᵢ,corrected(G404S) was unaffected by 2 mM Cs⁺, like Iᵢ,corrected(HCN2), (11) but unlike If, which is blocked by 2 mM Cs⁺ (26).

For confirmation of the presence of G404S on the plasma membrane, we examined subcellular localization in CHO cells of wild-type or mutant channels and labeled with an anti-HERG primary antibody and a Cy3-tagged secondary antibody. As shown by optical sections of cells expressing either wild-type HCN2 or G404S, there was a very strong pattern of fluorescence along the periphery of the cells suggesting the presence of HCN2 protein on the plasma membrane (Fig. 2E). Bright patches of fluorescence were observed in the interior of the cells, which probably represented channel protein present in discrete intracellular compartments such as the endoplasmic reticulum.

FIG. 1. A comparison of the selectivity filter from channels structurally related to HCN channels. Black shaded columns represent amino acid identity and gray shaded columns represent conserved amino acids. Asterisks represent the residue mutated in this study. Mouse HCN-gated 1, 2 channels (mHCN1 (NP_034538) and mHCN2 (NP_032250)) and human HCN4 (NP_005468) channel; sea urchin sperm channel (spHCN, CAA76493), a member of the HCN channel family; Arabidopsis, KAT1 (BABI11079), hyperpolarization-activated potassium channel found in plants; Drosophila melanogaster Shaker potassium channel (S00479); human ether-a-go-go (HERG, I38465) potassium channel; mouse inward rectifier potassium channel (Kir2.1, P35561); Streptomyces lividans, KcsA potassium channel (S86172); rat olfactory cyclic nucleotide-gated subtype a channel (olf-CNGa, AAD14173); human voltage-gated potassium channel (Kv 2.1, Q14721).
reticulum, Golgi apparatus, vesicles mediating transport to or from the plasma membrane, or degradatory compartments. There was relatively little fluorescence in non-transfected cells present in the image field of Fig. 2E, or in mock-transfected cells (not shown). We have shown previously that the wild-type channel and the S306Q mutant demonstrated a similar pattern of localization, whereas a mutant lacking the cyclic-nucleotide binding domain (CNBD) and distal C terminus did not express currents and demonstrated a very different pattern of localization that did not include fluorescence on the periphery of CHO cells (11). The imaging data and the significant increase in instantaneous current support the presence of G404S on the plasma membrane and the selective disruption of hyperpolarization-activated gating (I_{h}) by the G404S mutation.

Increasing the Ratio of K\(^{+}\)/Na\(^{+}\) Driving Forces Increases Fully Activated I_{h} and the Rate of I_{h} Deactivation—To further examine the role of the selectivity filter in voltage-dependent gating of HCN channels, we determined the effects of permeating cations on I_{h} kinetics. Whole-cell current traces from cells expressing HCN2 are shown in Fig. 3A. We compared rates of deactivation and amplitudes of fully activated I_{h} at the same potential but at different ratios of extracellular K\(^{+}\)/Na\(^{+}\) because both cations permeate HCN channels (24). We used a protocol consisting of a prepulse to −140 mV followed by a test pulse to −65 mV. These voltages were chosen for several reasons. First, the channels are close to fully activated at −140 mV and fully closed at −65 mV in CHO cells (11). Second, fully activated I_{h} amplitude and the rate of I_{h} deactivation were easily determined at −65 mV over the range of extracellular K\(^{+}\) and Na\(^{+}\) concentrations studied here. Finally, −65 mV is...
within the ranges of potentials observed normally in many excitable cells expressing $I_f$.

Increasing the $K^+/Na^+$ ratio produced reversible increases in the amplitude of $I_f$ at $-140$ mV and of the tail current at $-65$ mV (Fig. 3, A–C). Current activation for 2-s pulses at $-140$ mV and deactivation at $-65$ mV were both well fit with a single exponential function (Fig. 3A, inset) from which time constants were determined. Increasing the $K^+/Na^+$ ratio did not significantly increase the rate of $I_f$ activation at $-140$ mV but substantially increased the rate of $I_f$ deactivation at $-65$ mV (Fig. 3, A, D, and E). Deactivation also included a delay (Fig. 3A) that has been attributed to complex changes in conformation during channel closing in both native and cloned HCN channels (25, 26). This delay is clearly smaller in the higher $K^+/Na^+$ ratio as compared with the medium $K^+/Na^+$ ratio and cannot be distinguished in the lowest $K^+/Na^+$ ratio. Together, the increased deactivation rate and reduction in delay suggest that permeant cations affect $I_f$ deactivation preferentially.

**Fig. 3.** Increasing extracellular $K^+/Na^+$ ratio increases the fully activated current and speeds deactivation. A, current traces (bottom) recorded from a representative cell in response to a voltage protocol (top) in solutions containing the indicated concentrations of $K^+$ and $Na^+$ (a, b, c). Note the faster deactivation and shorter delay in deactivation in the high $K^+/Na^+$ solution. Inset, tail currents (dotted lines) fitted with a single exponential function (solid lines), and tau values at each $K^+/Na^+$ ratio; residuals (i.e. observed, fitted) of each fit are shown as solid lines above each tail current trace while the dashed lines represent the zero current level. Current amplitudes elicited by voltage steps to $-140$ mV ($B$) or $-65$ mV ($C$) at the three different ratios of $K^+/Na^+$. Bar graphs of time constants ($\tau$) of activation at $-140$ mV ($D$) and deactivation at $-65$ mV ($E$) at three different ratios of $K^+/Na^+$ concentrations. Values represent absolute means ± S.E.M., and the numbers in parentheses represent the total number of cells at each ratio.
The Dependence of \( I_{\text{tot}} \) and \( I_{\text{tot}} \) Amplitudes on \( K^+ \) and \( Na^+ \) Driving Forces—We next compared how \( I_{\text{tot}} \) and \( I_{\text{tot}} \) amplitudes depended on the changes in the \( K^+ \) and \( Na^+ \) driving forces, and how these compared with theoretical current values generated using the total current calculated in Equation 1,

\[
I_{\text{tot}} = I_{Na} + I_{K} = g_{Na,\text{max}}(E_{test} - E_{Na}) + g_{K,\text{max}}(E_{test} - E_{K}) \quad (\text{Eq. 1})
\]

where \( I_{\text{tot}} \), \( I_{Na} \), and \( I_{K} \) are the predicted current values for \( I_{\text{tot}} \), the \( Na^+ \) component, and the \( K^+ \) component; \( (E_{test} - E_{Na}) \) and \( (E_{test} - E_{K}) \) are the driving forces for \( Na^+ \) and \( K^+ \); and \( g_{Na,\text{max}} \) and \( g_{K,\text{max}} \) are the maximum values of conductance for \( Na^+ \) and \( K^+ \). We assumed that: (i) each ion moved through the pore independently and (ii) \( g_{Na,\text{max}} = 1 \) ns, a value that was obtained using the above equation and the measured \( I_{\text{tot}} \) value at a ratio of 0 (when only \( Na^+ \) flows through the channel).

Fig. 4A (filled boxes) shows the fully activated \( I_{\text{tot}} \) as a function of the ratio of \( K^+ \) and \( Na^+ \) driving forces \( [(E_{test} - E_{K})/(E_{test} - E_{Na})] \) at \(-65 \) mV. An additional set of data at \( 10 \) mM \( K^+ \) and \( 130 \) mM \( Na^+ \) was added to determine more accurately the range over which the changes occurred. The greatest change in measured \( I_{\text{tot}} \) occurred between \( 5.4 \) mM \( K^+/135 \) mM \( Na^+ \) and \( 10 \) mM \( K^+/130 \) mM \( Na^+ \) where a slope of \( 677 \) pA/unit ratio of driving forces was determined (solid line, negative ratios). A smaller change occurred between \( 10 \) mM \( K^+/130 \) mM \( Na^+ \) and \( 135 \) mM \( K^+/5.4 \) mM \( Na^+ \) where a slope of \( 124 \) pA/unit ratio of driving forces was determined (dotted vertical line represents a ratio of zero).

Using \( g_{Na,\text{max}} \) equal to \( g_{K,\text{max}} \), we found that the measured \( I_{\text{tot}} \) lay on \( I_{\text{tot}} \) only at ratios where the \( K^+ \) driving force was relatively small (Fig. 4A, lower dashed curve). Extrapolation of the measured \( I_{\text{tot}} \) to 0 pA (x-intercept) yielded a ratio of
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Parallel Dependence of Fully Activated If and If Deactivation on Driving Forces for K+/Na+ in the Physiological Range—We next re-examined I\textsubscript{f} deactivation at −65 mV to determine whether this was modified by permeating cations in the same range of driving forces that modify I\textsubscript{f} amplitude. Here, we included the set of data points at 10 mM K+/130 mM Na\textsuperscript{+} for both I\textsubscript{f} amplitude and I\textsubscript{f} deactivation (Fig. 5). We found that the increase in rate of I\textsubscript{f} deactivation (filled squares) paralleled the increase in fully activated I\textsubscript{f} (filled diamonds) at increasing K+/Na\textsuperscript{+} driving forces. Both sets of values changed most dramatically in the range of driving forces normally found in cells and where K\textsuperscript{+} moves in the outward direction. As for the fully activated I\textsubscript{f} at −65 mV, deactivation time constants were joined with two straight lines on either side of the K\textsuperscript{+} reversal concentration. The most dramatic change occurred between 5.4 mM K+/135 mM Na\textsuperscript{+} and 10 mM K+/130 mM Na\textsuperscript{+} where a straight line of slope = −4647 ms/unit ratio of driving forces joined these values. A less dramatic change occurred between 5.4 mM K+/135 mM Na\textsuperscript{+} and 136.7 mM Na\textsuperscript{+} where permeating cations affected If deactivation.

DISCUSSION

Separable Gating Mechanisms in Pacemaker Channels—Our present findings show that in the HCN2 G404S mutant, a reduction in external K\textsuperscript{+} or application of Cs\textsuperscript{+} reduce or eliminate I\textsubscript{f} without blocking I\textsubscript{inst}. Together with our previous work demonstrating the co-existence of a slow Cs\textsuperscript{+}-sensitive current (I\textsubscript{S}) and an instantaneous Cs\textsuperscript{+}-insensitive current (I\textsubscript{inst}) (11), these data suggest that two gating mechanisms, which can be separated functionally and structurally, are associated with HCN channels.

Is it possible for two currents with different properties and structural requirements to travel through the same pore? The inhibition of I\textsubscript{f} by Cs\textsuperscript{+} and G404S may be analogous to the production of distinct subconductance states in single channel currents of K\textsubscript{v} 2.1 by the permeant cation Ti\textsuperscript{4+} and by mutations of the glycine corresponding to G404 in HCN2 (16, 18). Certain closed-time states in this inward rectifier were inde-
dependent of membrane voltage and were not produced by open-pore blockade by external or internal cations, suggesting the co-existence of a separate gating mechanism (18, 30). Voltage-independent closed to open fluctuations and subconductance levels have also been observed in Shaker K+ channels (31, 32). By analogy, Ifnat may thus represent voltage-independent subconductance states and/or closed to open transitions, which are not inhibited by Cs+, G4048, or a decrease in external potassium. In this model, hyperpolarization would then produce additional subconductance states that are sensitive to Cs+ and to driving forces for permeating cations. Measurement of single channel currents will be required to examine this hypothesis in HCN channels, although this may be challenging given the small (~1 pS) single channel conductance determined in studies of native HCN channels (33).

Other voltage-gated K+ channels, such as the cloned plant K+ channels, AKT2 and AKT3 (34, 35), exhibit combinations of instantaneous leak and slowly-activating inward rectification. Single channel and whole-cell current analysis suggested that the AKT2 channels exist in two modes whose relative amounts may change over time (36). The co-existence of instantaneous, voltage-independent leak with voltage-dependent, slowly activating rectification has also been described in the KCNK2 K+ channel (37). The SLO-2 calcium-activated K+ channel has both a time-dependent outward current component and a rapidly activating current component (38). Finally, Liu and Joho (39) have proposed the co-existence of slow voltage-dependent and rapid voltage-independent gating mechanisms in Kv2.1. These authors suggested that there are two gates within the pore of Kv2.1 that are close, but physically separated. Given the conserved nature of the pore structure, voltage-independent leak through a separate gating mechanism may be an inherent property of channels in these related families. However, it is also possible that the co-existence of instantaneous, slowly activating currents is due to a second pore that is found within the same channel or formed by a second population of the same channel subunits or associated with the channels in the form of up-regulated endogenous channels.

**Coupling between the Selectivity Filter and I_{\alpha}-Deactivation—**

Permeation and gating were originally thought to be independent in ion channels but more recent evidence indicates that permeant ions affect gating and hence that these properties are coupled. Previous studies of I_{\alpha} in sensory neurons, apical dendrites of hippocampal CA1 pyramidal neurons, and myocytes from the sinoatrial node have shown that deactivation was affected by relatively large changes in external cations (28, 40, 41). Despite concurrent changes in I\alpha amplitude that reflected interactions of Na+ and K+ in the pore, a separate mechanism was proposed for the effects of cations on I_{\alpha} deactivation that involve an external binding site (28). The primary argument in favor of an external binding site was that only K+ could be present in the pore during measurements of outward tail currents because Na+ was not present in the intracellular solution and thus would have left the pore almost instantly upon depolarization. Our data suggest the effects of permeating cations do involve interactions in the pore because the rate of I_{\alpha} deactivation and I\alpha amplitude depended on K+/Na+ driving forces in a parallel manner. Our findings also suggest that the changes in I_{\alpha} deactivation and I\alpha amplitude follow from hyperpolarization-induced changes in pore conformation that occur slowly during I_{\alpha} activation. These changes are related to the ability of K+ to permeate the channel more readily than Na+ after hyperpolarization as well as to the direction of K+ flow. Thus, we suggest that the selectivity filter is an important structural element of voltage-dependent gating and is coupled to cation selectivity in HCN channels, as it is in related potassium channels (42).

**Physiological Relevance of Two Gating Mechanisms and Modulation by Permeating Ions in Pacemaker Channels**—A physiologically important aspect of our experiments is that the changes in the concentration of K+ and Na+ that influence both I_{\alpha} deactivation and I\alpha amplitude are in a range that exist under certain physiological and pathophysiological conditions and thus could provide a mechanism for controlling cellular excitability in vivo. In the heart, changes in external K+ between 3 and 10 mM may occur in situations such as exercise and can produce bradycardia mediated through I_{\alpha} (43–45). In the central nervous system, extracellular K+ may vary from 3 to 12 mM, and extracellular Na+ may decrease by up to 7 mM during repetitive activity (46, 47). These changes in cation concentration could significantly affect the spontaneous activity of neurons that contain HCN channels, such as thalamic relay neurons, in which I_{\alpha} deactivation and amplitude play critical roles in determining the period of time separating periods of sustained activity (10).

The ability to balance I_{\alpha} nat versus I_{\alpha} would greatly expand the role of HCN channels in modulating the firing characteristics of neurons and cardiomyocytes. A dramatic reduction of I_{\alpha} compared with I_{\alpha} nat may explain why some neurons express HCN mRNA but do not express I_{\alpha} (4). In the sino-atrial node of the heart, I_{\alpha} nat may contribute to the Na+ - sensitive background current (I_{\alpha} Na) with which it shares several properties (48). The relative effects of I_{\alpha} Na and I_{\alpha} are important for regulating the pacemaking activity of the sino-atrial node (49), and a similar balance of these current components may be important in neurons.

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**REFERENCES**

1. Shi, W., Wymore, R., Yu, H., Wu, J., Wymore, R. T., Pan, Z., Robinson, B. B., Dixon, J. E., McKinnon, D., and Cohen, I. S. (1999) Circ. Res. 85, e1–6
2. Mosemann, S., BieI, M., Hofmann, F., and Ludwig, A. (1999) J. Biol. Chem. 380, 975–980
3. Montegia, L. M., Schi, A. J., Tang, M. D., Czatkow, L. K., and Nestler, E. J. (2000) Brain Res. Mol. Brain Res. 81, 129–139
4. Santoro, B., Liu, D. T., Yao, H., Bartsch, D., Kandel, E. R., Siegelbaum, S. A., and Tibbs, G. B. (1998) Cell 95, 717–729
5. Santoro, B., Chen, S., Luthi, A., Pavlidis, P., Shumakovsky, G. P., Tibbs, G. R., and Siegelbaum, S. A. (2000) J. Neurosci. 20, 5264–5275
6. Ludvig, A., Zang, X., Jeglitsch, M., Hofmann, F., and BieI, M. (1998) Nature 393, 587–591
7. Stevens, D. R., Seifert, R., Bufe, B., Muller, F., Kremmer, E., Gauss, R., Meyerhof, W., Kaupp, U. B., and Lindemann, B. (2001) Nature 413, 631–635
8. Pape, H. C. (1996) Annu. Rev. Physiol. 58, 299–327
9. DiFrancesco, D. (1993) Annu. Rev. Physiol. 55, 455–472
10. Bal, T., and McCormick, D. (1997) J. Neurophysiol. 77, 3145–3156
11. Prenza, C., Angoli, D., Agrawalc, E., Macci, V., and Accieli A. E. (2002) J. Biol. Chem. 277, 5101–5109
12. Chen, J., Mitzechos, J. S., Lin, M., and Sanguinetti, M. C. (2000) J. Biol. Chem. 275, 36465–36471
13. Chen, J., Mitzechos, J. S., Tristani-Firouzi, M., Lin, M., and Sanguinetti, M. C. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11277–11282
14. Lipkind, G. M., Hanck, D. A., and Fozzard, H. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9215–9219
15. Heginbotham, L., Lu, Z., Abramson, T., and MacKinnon, R. (1994) Biophys. J. 66, 1061–1067
16. Lu, T., Wu, L., Xiao, J., and Yang, J. (2001) J. Gen. Physiol. 118, 509–522
17. Su, L., Ashmore, I., Davies, N. W., Sutcliffe, M. J., and Stanfield, P. R. (2001) J. Physiol. 531, 37–50
18. Lu, T., Ting, A. Y., Mainland, J., Jan, L. Y., Schultz, P. G., and Yang, J. (2001) Nat. Neurosci. 4, 239–246
19. Wang, Z., Zhang, X., and Fedida, D. (1999) J. Physiol. 515, 331–339
20. Baukrowitz, T., and Yellen, G. (1995) Neuron 15, 951–960
21. Armstrong, C. M., Bezanilla, F., and Rojas, E. (1973) J. Gen. Physiol. 62, 375–391
22. Zhou, Y., Morais-Cabral, J. H., Kaufman, A., and MacKinnon, R. (2001) Nature 414, 43–48
23. Zhou, Z., Gong, Q., Epstein, M. L., and Janeway, C. T. (1998) J. Biol. Chem. 273, 17653–17656

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24. Moroni, A., Barbati, A., Altomare, C., Viscomi, C., Morgan, J., Baruscotti, M., and DiFrancesco, D. (2000) Pflugers Arch. 439, 618–626
25. Altomare, C., Bucchi, A., Camatini, E., Baruscotti, M., Viscomi, C., Moroni, A., and DiFrancesco, D. (2001) J. Gen. Physiol. 117, 519–532
26. DiFrancesco, D. (1984) J. Physiol. 348, 341–367
27. Wellnath, L. P. (1995) Pflugers Arch. 430, 34–43
28. Marouka, F., Nakashima, Y., Takano, M., Ono, K., and Noma, A. (1994) J. Physiol. 477, 423–435
29. DiFrancesco, D. (1982) J. Physiol. 329, 485–507
30. Choe, H., Palmer, L. G., and Sackin, H. (1999) Biophys. J. 76, 1988–2003
31. Zheng, J., and Sipworth, F. J. (1998) J. Gen. Physiol. 112, 457–474
32. Hashi, T., Zagotta, W. N., and Aldrich, R. W. (1994) J. Gen. Physiol. 103, 249–278
33. DiFrancesco, D. (1986) Nature 324, 470–473
34. Lacombe, B., Plot, G., Michard, E., Gaymard, F., Sentenac, H., and Thibaud, J. B. (2000) Plant Cell 12, 837–851
35. Marten, I., Hoth, S., Deeken, R., Ache, P., Ketchum, K. A., Hoshi, T., and Hedrich, R. (1999) Proc. Natl. Acad. U. S. A. 96, 7581–7586
36. Dreyer, I., Michard, E., Lacombe, B., and Thibaud, J. B. (2001) FEBS Lett. 505, 233–239
37. Bockenhauer, D., Zilberberg, N., and Goldstein, S. A. (2001) Nat. Neurosci. 4, 486–491
38. Yuan, A., Dourado, M., Butler, A., Walton, N., Wei, A., and Salkoff, L. (2000) Nat. Neurosci. 3, 771–779
39. Liu, Y., and Joho, R. H. (1998) Pflugers Arch. 435, 654–661
40. Mayer, M. L., and Westbrook, G. L. (1983) J. Physiol. 340, 19–45
41. Magee, J. C. (1998) J. Neurosci. 18, 7613–7624
42. Kiss, L., LoTurco, J., and Korn, S. J. (1999) Biophys. J. 76, 253–263
43. Paterson, D. J. (1996) J. Appl. Physiol. 80, 1853–1862
44. Kleber, A. G. (1983) Circ. Res. 52, 442–450
45. Choate, J. R., Nandhabalan, M., and Paterson, D. J. (2001) Exp. Physiol. 86, 19–25
46. Dietzel, I., Heinemann, U., Hofmeier, G., and Lux, H. D. (1982) Exp. Br. Res. 46, 73–84
47. Sykova, E. (1983) Prog. Biophys. Mol. Biol. 42, 135–169
48. Hagiwara, N., Irisawa, H., Kasanuki, H., and Hosoda, S. (1992) J. Physiol. 448, 53–72
49. Noble, D., Denyer, J. C., Brown, H. F., and DiFrancesco, D. (1992) Proc. R. Soc. Lond. B. Biol. Sci. 250, 199–267
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