An External Determinant in the S5-P Linker of the Pacemaker (HCN) Channel Identified by Sulfonyl Modification*

Received for publication, May 18, 2002, and in revised form, September 24, 2002
Published, JBC Papers in Press, September 25, 2002, DOI 10.1074/jbc.M204915200

Tian Xue and Ronald A. Li‡
From the Institute of Molecular Cardiobiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels underlie spontaneous rhythmic activities in the heart and brain. Sulfonyl modification of ion channels is a proven approach for studying their structure-function relationships; here we examined the effects of the hydrophilic sulfonyl-modifying agents methanethiosulfonate ethylammonium (MTSEA*) and methanethiosulfonate ethylsulfonate (MTSES) on wild-type (WT) and engineered HCN1 channels. External application of MTSEA* to WT channels irreversibly reduced whole-cell currents (I_{MTSEA}/I_{Control} = 42 ± 2%), slowed activation and deactivation kinetics (–6- and –3-fold at –140 and –20 mV, respectively), and produced hyperpolarizing shifts of steady-state activation (V_{1/2(MTSEA)} = –125.8 ± 9.0 mV versus V_{1/2(Control)} = –76.4 ± 1.7 mV). Sequence inspection revealed the presence of five endogenous cysteines in the transmembrane domains of HCN1: three are putatively close to the extracellular milieu (Cys303, Cys318, and Cys347 in the S5, S5-P, and P segments, respectively), whereas the remaining two are likely to be cytoplasmic or buried. To identify the molecular constituent(s) responsible for the effects of MTSEA*, we mutated the three “external” cysteines individually to serine. C303S did not yield measurable currents. Whereas C347S channels remained sensitive to MTSEA*, C318S was not modified (I_{MTSEA}/I_{Control} = 101 ± 2%), V_{1/2(MTSEA)} = –78.4 ± 1.1 mV, and V_{1/2(Control)} = –79.8 ± 2.3 mV). Likewise, WT (but not C318S) channels were sensitive to MTSES*. Despite their opposite charges, MTSES* produced changes directionally similar to those effected by MTSEA* (I_{MTSEA}/I_{Control} = 22 ± 1.6% and V_{1/2(MTSEA)} = –145.9 ± 4.9 mV). We conclude that S5-P Cys318 of HCN1 is externally accessible and that the external pore vestibule and activation gating of HCN channels are allosterically coupled.

The hyperpolarization-activated, cyclic nucleotide-gated (HCN1–4) or the so-called pacemaker channel gene family encode the pacemaking current I_{r} or I_{h} that underlies the spontaneous periodic activities found in parts of the heart and brain (1–3). Like voltage-gated K⁺ channels, HCN channels are tetramers (4) made up of monomeric subunits consisting of six transmembrane segments (S1–6) with a pore-forming P-loop between S5 and S6 (1–3). Different HCN isoforms can co-assemble with each other to form heteromultimeric complexes, greatly increasing the diversity of the molecular identity of native I_{h} (4–6). Despite the presumed structural similarity to K⁺ channels, direct evidence regarding the topology of HCN channels is relatively sparse.

Inspection of the primary sequences of HCN channels (1–3) reveals the presence of a total of five endogenous cysteines in the transmembrane domains: three appear to be close to the extracellular side (Cys303, Cys318, and Cys347 in the S5, S5-P, and P segments, respectively; HCN1 numbering), whereas the remaining two are likely to be cytoplasmic or buried (S5 Cys298 and S6 Cys374) (Fig. 1). It is known that the cysteine sulfonyl, in its reduced form, may readily undergo chemical reactions (such as alkylation, acylation, and arylation) or even cross-link with a second nearby cysteine to form a disulfide bridge (7, 8). These properties render free cysteinyl the most chemically reactive amino acid side chain (9). When exposed to the aqueous phase, cysteinyls can be selectively and covalently modified by hydrophilic sulfonyl-reactive agents (10). Indeed, engineering cysteines into proteins, followed by the assessment of their accessibility pattern using a variety of sulfonyl-reactive probes with different physical and chemical properties (i.e. cysteine scanning mutagenesis), has been a proven approach to study the structure-function relationships of numerous ion channels, receptors, and proteins (7, 8, 10–17).

The presence of endogenous cysteines in HCN channels that are putatively exposed to the extracellular milieu raises the possibility that wild-type (WT) channels are susceptible to modifications by sulfonyl-reactive compounds, which in turn provide an excellent opportunity for localizing structurally and functionally important channel domains. In this report, we examined the effects of the hydrophilic covalent sulfonyl modifiers methanethiosulfonate ethylammonium (MTSEA) and methanethiosulfonate ethylsulfonate (MTSES), which are positively and negatively charged, respectively, on the functions of HCN1 channels. We found that WT HCN1 channels were indeed modified by these agents. Using site-directed mutagenesis, we identified the native cysteine in the S5-P linker (i.e. Cys318) as the residue responsible for the post-translational changes caused by these agents. Novel insights into the structure-function relationships of HCN channels from these results are discussed.

EXPERIMENTAL PROCEDURES

Molecular Biology and Heterologous Expression—Murine HCN1 (kindly provided by Drs. Siegelbaum and Santoro) was subcloned into R01 HL-52768 and a research career development award from the Cardiac Arrhythmias Research and Education Foundation (to R. A. L.).

* This work was supported by National Institutes of Health Grant 1R01 HL-52768 and a research career development award from the Cardiac Arrhythmias Research and Education Foundation (to R. A. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Inst. of Molecular Cardiobiology, The Johns Hopkins University School of Medicine, 720 Rutland Ave., Ross 844, Baltimore, MD 21205. Tel.: 410-614-0035; Fax: 410-955-7953; E-mail: ronald@jhmi.edu.

The abbreviations used are: WT, wild-type; MTS, methanethiosulfonate; MTSEA, methanethiosulfonate ethylammonium; MTSES, methanethiosulfonate ethylsulfonate; DTT, dithiothreitol.

This paper is available online at http://www.jbc.org
FIG. 1. Putative transmembrane topology of HCN channels. The six transmembrane segments (S1–6) of a monomeric subunit of HCN1 channels are shown. A total of five endogenous cysteines are present in the transmembrane domains of all four isoforms of HCN channels. Their approximate locations, as well as the GYG signature motif, are highlighted as shown. Three of these cysteines (Cys318, Cys324, and Cys347) are putatively close to the extracellular side, whereas the remaining two (Cys365 and Cys374) are likely to be cytoplasmic or buried. The cyclic nucleotide-binding domain (CNBD) is in the C-terminal region, whereas the remaining cysteines can be found; an extra cysteine can be found in the N-terminal region.

Electrophysiology—Two-electrode voltage-clamp recordings were performed at room temperature using a Warner OC-725B amplifier. Agarose-plugged electrodes (TW120-6, World Precision Instruments, Inc., Sarasota, FL) were pulled using a Narishige PP-83 vertical puller, with final tip resistances of 2–4 megohms when filled with 3 M KCl.

FIG. 2. MTSEA-induced current reduction of WT HCN1 channels. A, representative traces of whole-cell currents through WT HCN1 channels before and after modification by MTSEA. Currents were elicited by stepping to a family of 3-s electrical pulses ranging from 0 to -150 mV in 10-mV increments from a holding potential of -30 mV. B, time course of the development of block upon addition of 2.5 mM MTSEA (arrow) with a stimulation frequency of 0.1 Hz (■) or 0.03 Hz (○). HCN1 currents did not run down under control conditions (i.e. without MTSEA (□)) over the same recording period. Co-application of MTSEA and the reducing agent DTT (10 mM) prevented channels from becoming modified (△). C, sustained I-V relationships (at 3 s) of WT channels before and after MTSEA modification. All data shown are means ± S.E. Data are presented as means ± S.E. Statistical significance was determined using an unpaired Student’s t test.

RESULTS

Modification of WT HCN1 Channels by MTSEA—To study whether any of the endogenous cysteines present in HCN channels are accessible from the extracellular aqueous environment, we first examined the effects of external application of

the pGHE expression vector (3). Mutations were created using PCR with overlapping mutagenic primers. The desired mutations were confirmed by DNA sequencing. cRNA was transcribed from Xhel-linearized DNA using T7 RNA polymerase (Promega, Madison, WI). HCN1 channel constructs were heterologously expressed and studied in Xenopus oocytes. Stage IV–VI oocytes were surgically removed from female frogs anesthetized by immersion in 0.3% 3-aminobenzoic acid ethyl ester, followed by digestion with 1 mg/ml collagenase (type IIA) in OR-2 solution containing 88 mM NaCl, 2 mM KCl, 1 mM MgCl2, and 5 mM HEPES (pH 7.6) for 30–60 min. Isolated oocytes were injected with cRNA (50 ng/cell) and stored in 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES (pH 7.6) supplemented with 50 mg/ml gentamycin, 5 mM pyruvate, and 0.5 mM theophylline for 1–2 days before experiments.

Experimental Protocols and Data Analysis—The steady-state current-voltage (I-V) relationship was determined by plotting the HCN1 currents measured at the end of a 3-s pulse ranging from -150 to 0 mV from a holding potential of -30 mV against the test potentials. The voltage dependence of HCN channel activation was assessed by plotting tail currents measured immediately after pulsing to -140 mV as a function of the preceding 3-s test pulse normalized to the maximum tail current recorded. Data were fit to the Boltzmann functions using the Marquardt-Levenberg algorithm in a nonlinear least-squares procedure: 

\[ m = \frac{1}{(1 + \exp((V - V_{1/2})/k))} \]

where \( V_{1/2} \) is the half-point of the relationship, \( k = R T z / F \) is the slope factor, and \( R, T, z, \) and \( F \) have their usual meanings.

The time course of MTSEA modification was fit with the following single-exponential equation, \( F = (1 - S) \times \exp(-t/\tau_{MTS}) + S \), where \( F \) is the fraction of remaining current measured at -140 mV in the presence of MTSEA, \( t \) is the cumulative exposure time, \( \tau_{MTS} \) is the time constant for MTSEA modification, and \( S \) is the steady-state plateau.

For tail I-V relationships, currents were recorded immediately after stepping to a family of test voltages ranging from -100 to -40 mV, preceded by a 3-s prepulse to either -140 or -20 mV. The difference in tail currents resulting from the two prepulse potentials was plotted against the test potentials and fitted with linear regression to obtain the reversal potential (\( E_{rev} \)). Assuming that \( pF \) was not changed under our experimental conditions and that N-methyl-d-glucamine was non-permeant, the permeability ratio (\( pD/pF \) for a given cation (X) was calculated from the corresponding reversal potentials recorded under the ionic conditions as noted using the Goldman-Hodgkin-Katz equation (18). For current kinetics, the time constants for activation (\( \tau_{act} \)) and deactivation (\( \tau_{deact} \)) were estimated by fitting macroscopic and tail currents with bi- and monoeXponential functions, respectively.

Data are presented as means ± S.E. Statistical significance was determined using an unpaired Student’s t test.
the positively charged sulfhydryl modifier MTSEA on WT HCN1 channels. Fig. 2A shows representative records of a family of WT HCN1 currents before and after MTSEA treatment. With a stimulation frequency of 0.1 Hz, WT currents elicited at \(-140 \text{ mV}\) reduced in amplitude progressively upon addition of 2.5 mM MTSEA (Fig. 2B). (HCN1 currents were

**Fig. 3. MTSEA-induced functional changes of WT HCN1 channels.** A, activation curves of WT HCN1 before (■) and after (○) MTSEA modification. B, tail I-V relationships. Current amplitudes were reduced by MTSEA, but the reversal potential was not changed. C, normalized activation and deactivation current tracings at \(-140\) and \(-30 \text{ mV}\), respectively, before and after MTSEA modification as indicated. D, summary of the fast and slow components for activation (\(\tau_{\text{act (fast)}}\), left panel), the relative amplitude of \(\tau_{\text{act (slow)}}\) (middle panel), and the time constants for deactivation (\(\tau_{\text{deact (slow)}}\), right panel) in the absence and presence of MTSEA. *, \(p < 0.05\).
unchanged over the same period under control MTSEA-free conditions.) The modification time constant (\( \tau_{\text{MTSEA}} \)) was 51 ± 11 s (n = 6) under these conditions; steady-state block was achieved ~5 min after MTSEA application (\( I_{\text{MTSEA/Control}} = 42 ± 2\% \), n = 6). Neither \( \tau_{\text{MTSEA}} \) (56 ± 10 s, n = 8) nor \( I_{\text{MTSEA/Control}} \) (41 ± 2\%, n = 8) was altered when the stimulation frequency was slowed to 0.03 Hz (Fig. 2B). The I-V relationships of the sustained currents measured at 3 s after hyperpolarization before and after MTSEA modification are compared in Fig. 2C.

Reduction of whole-cell HCN1 currents by MTSEA could result from changes in the permeation pathway, gating properties, or a combination of both. To distinguish among these possibilities, we studied channel activation before and after MTSEA modification. Fig. 3A shows that MTSEA modification led to a significant hyperpolarizing shift of the activation curve (\( V_{1/2,\text{MTSEA}} = -125.8 ± 9.0 \text{ mV (n = 5) versus } V_{1/2,\text{Control}} = -76.4 ± 1.6 \text{ mV (n = 10)} \)), which accounts for >80% of the MTSEA-induced reduction in current amplitude. The rest can plausibly be attributed to partial blockade of the permeation pathway as a result of the physical size of MTSEA (66 Å).

Furthermore, MTSEA significantly slowed the kinetics of both activation gating (by increasing the magnitudes of both the fast and slow time components (\( \tau_{\text{act(fast)}} \) and \( \tau_{\text{act(slow)}} \) respectively) as well as the relative amplitude of \( \tau_{\text{act(slow)}} \) (i.e. \( A_{\text{slow}} \)) when assessed at ~140 mV) and deactivation gating (by 3-fold at ~30 mV (Fig. 3, C and D; and Table I)). Despite these changes in gating, MTSEA did not, however, noticeably alter the reversal potential as gauged by the tail I-V relationships (Fig. 3B). Taken collectively, modification of WT HCN1 channels by MTSEA appeared to more prominently modify gating than permeation properties. All MTSEA-induced changes were irreversible even after >15 min of washout (data not shown), suggesting that the actions of MTSEA involve covalent modification of one or more of the endogenous cysteines. Consistent with this notion, co-application of MTSEA and the reducing agent dithiothreitol (DTT; 10 mM) to WT HCN1 channels did not lead to functional changes in channel properties (Fig. 2B). However, WT channels became susceptible to MTSEA again upon removal of DTT (data not shown). Furthermore, external application of 200 or 500 µM MTSEA irreversibly induced similar changes in HCN1 gating properties over time (i.e. at steady state), although the time course of modification was much slower compared with that of 2.5 mM as anticipated from the classical collision theory for chemical reactions. These results (summarized in Table I) strongly suggest that the changes observed were chemical and covalent in nature rather than due to nonspecific (dose-dependent) effects resulting simply from the physical presence of MTSEA in the bath solution.

MTS Sensitivities of Cysteine-to-Serine HCN1 Mutants—To identify which particular cysteine(s) is (are) responsible for the effects of MTSEA modification, we mutated all three putative “external” cysteines in HCN1 (i.e. Cys303, Cys318, and Cys347) individually to serine and studied their susceptibilities to MTSEA. The results are summarized in Figs. 4 and 5. All cysteine-to-serine constructs except C303S yielded measurable currents. The C318S and C347S mutations did not alter ionic selectivity remarkably, as gauged by their effects on the permeability ratios for K⁺, Na⁺, and Li⁺ (Tables II and III); the selectivity of WT, C318S, and C347S channels calculated under these conditions follows the sequence K⁺ > Na⁺ > Li⁺, comparable with those reported previously for cloned HCN channels and native I\(_{\text{h}}\) (19–21). Similar to WT channels, addition of 2.5 mM MTSEA externally to the C347S construct reduced whole-cell currents (\( I_{\text{MTSEA/Control}} = 23.5 ± 3.1\% \), n = 5), slowed activation and deactivation kinetics (Table IV), and shifted channel activation in the hyperpolarizing direction (\( V_{1/2,\text{MTSEA}} = -147.0 ± 7.3 \text{ mV (n = 6) versus } V_{1/2,\text{Control}} = -82.2 ± 0.8 \text{ mV (n = 7)} \)). In fact, C347S channels were more susceptible to MTSEA block than WT channels (\( p < 0.05 \)) (cf. Fig. 4B) although the modification rate was not different (\( V_{1/2,\text{MTSEA}} = 60 ± 7 \text{ s, n = 5}; p > 0.05 \)). As in WT channels, the \( E_{\text{rev}} \) of C347S channels was also not altered by MTSEA (Fig. 5B). In contrast to WT and C347S channels, the S5-P mutation C318S rendered HCN1 channels completely insensitive to MTSEA block (\( I_{\text{MTSEA/Control}} = 101 ± 2\% \), n = 7) (Fig. 4). Their activation curves (\( V_{1/2,\text{MTSEA}} = -78.5 ± 1.1 \text{ mV (n = 8) versus } V_{1/2,\text{Control}} = -79.8 ± 2.3 \text{ mV (n = 5)} \)) (Fig. 5), gating kinetics (Table IV), and \( E_{\text{rev}} \) were not altered by MTSEA, indicating that the native cysteine at position 318 is responsible for the functional changes associated with MTSEA modification of WT channels.

MTSES Exerts Effects Similar to Those of MTSEA Despite Their Opposite Charges—To obtain further insights into how modification of Cys318 led to the observed changes in channel functions, we next investigated the effects of negatively charged MTSES. Interestingly, despite their opposite charges, 2.5 mM MTSES produced functional consequences similar to those caused by MTSEA, i.e. current reduction (\( I_{\text{MTSEA/Control}} = 22 ± 1.6\% \), n = 9), leftward shifts of the activation curve (\( V_{1/2,\text{MTSEA}} = -145.9 ± 4.9 \text{ mV, n = 6} \)), decelerated gating kinetics (Table IV), and no effect on \( E_{\text{rev}} \) (Figs. 6 and 7). The time constant for MTSES modification (\( \tau_{\text{MTSES}} \)) was 109.7 ± 9.6 s (n = 7). Like MTSEA, MTSES-induced changes were irreversible after extensive washout. However, these functional effects could be reversed, at least partially, by applying the reducing agent DTT (5 mM) to MTSES-modified channels (Fig. 6B, inset). As anticipated from their lack of MTSEA sensitivity, MTSES had no effect on C318S channels (Figs. 6 and 7 and Table IV). Taken together, the above observations are consistent with the notion that the action of MTSEA involves covalent modification of Cys318 within the S5-P linker.

DISCUSSION

Mechanistic Interpretations—In this study, we demonstrate that covalent modification of an endogenous cysteine (viz. Cys318) in the S5-P linker of WT HCN1 by MTSEA and MTSES altered channel properties. Alterations by MTS reagents depend on a combination of factors, including the bulk and charge of the modifier and the location and accessibility of the cysteine
FIG. 4. C318S (but not C347S) channels are insensitive to MTSEA modification. A, representative traces of whole-cell currents through C318S and C347S constructs before and after modification by MTSEA. Similar to WT channels, external application of 2.5 mM MTSEA to C347S channels led to current reduction and slowed gating kinetics. In contrast, C318S channels were completely insensitive. B, time course of MTSEA block of the same constructs in A. Stimulation frequency was 0.03 Hz. C, sustained I-V relationships of C318S (left panel) and C347S (right panel) before (■) and after (○) MTS modification.
in question (which may be state-dependent) (10, 22). The lack of frequency dependence of MTSEA modification suggests that Cys318 is always accessible from the external milieu regardless of the channel state. This finding was consistent with the outermost external location of Cys318 predicted from the putative HCN transmembrane topology. Despite the opposite charges of MTSEA and MTSES, these agents produced similar functional changes, suggesting that the microenvironments surrounding this native S5-P cysteine, at least under our experimental conditions, are non-electrostatic. The slower modification rate of MTSES relative to MTSEA might be due to steric hindrance resulting from the larger physical size of MTSES (90 Å³). Nonetheless, the observation that MTS modification almost exclusively altered activation properties suggests that Cys318 and its surrounding regions are involved in channel gating. Perhaps, the HCN outer pore undergoes gating-induced dynamic rearrangements such that it is constricted and dilated when depolarized and hyperpolarized, respectively (Fig. 8). Attachment of an MTS moiety at position 318 may hinder these conformational changes of the HCN pore, thereby slowing the transition of channels from closed to open states (which is also accompanied by a stabilization of the closed state under resting conditions) and vice versa. This proposed gating feature of HCN channels accounts for the MTS-induced decelerated gating kinetics and negative shifts of channel activation. Indeed, it is analogous to slow and C-type inactivation of Na⁺/Hlopen and K⁺/Hlopen channels, respectively (18, 23, 24), except that HCN channels do not exhibit the “transient” depolarization-activated open state because they are activated by hyperpolarization instead (cf. Fig. 8). This pore-to-gate theory is also in accord with previous experiments demonstrating that the plant hyperpolarization-activated KAT1 channels do not inactivate (25). Furthermore, pore motions of HCN channels and others that slow or C-type inactivate appear to have similar overall voltage dependence (i.e. constriction when “sufficiently” depolarized). Alternatively, it is probable that the attached MTS bulk allosterically influences the movements of the S4 voltage sensor. However, direct electrostatic interactions between the positively charged S4 and the ethylammonium or ethylsulfonate moiety anchored at position 318 are unlikely, due to the lack of charge dependence of their reactions.

According to the above HCN pore-to-gate model, like K⁺/Hlopen and Na⁺/Hlopen channels, pacemaker channel gating should also be mod-

### Table II

| Channel | 32 K⁺/64 NMG⁺  | 32 K⁺/64 Na⁺  | 32 K⁺/64 Li⁺  |
|---------|----------------|---------------|---------------|
| WT      | -28.2 ± 1.1 (5)| -23.2 ± 1.3 (5)| -26.6 ± 1.3 (6)|
| C318S   | -19.2 ± 0.8 (5)| -16.5 ± 1.3 (5)| -18.3 ± 1.1 (6)|
| C347S   | -27.4 ± 0.5 (6)| -23.8 ± 1.5 (8)| -25.8 ± 0.9 (5)|

### Table III

| Channel | $P_{K}P_{Na}$ | $P_{Na}/P_{K}$ | $P_{Li}/P_{K}$ |
|---------|---------------|----------------|---------------|
| WT      | 1             | 0.11           | 0.03          |
| C318S   | 1             | 0.06           | 0.02          |
| C347S   | 1             | 0.08           | 0.03          |

Fig. 5. **Functional effects of MTSEA on C318S and C347S channels.** Shown are channel activation curves (A) and tail I-V relationships (B) of C318S (left panels) and C347S (right panels) channels before (●) and after (○) MTSEA modification.
ulated by external permeants presumably via a “foot-in-the-door” mechanism (18, 24, 26). In support of this notion, our preliminary observations also indicate that this is indeed the case: activation kinetics of WT HCN1 channels are substantially accelerated when external K\(^{+}\) increases (27), consistent with a destabilizing effect on the closed state by the permeant ion. Therefore, it is becoming increasingly apparent that HCN and K\(^{+}\) channels share a number of key structural and functional features from evolution; only a few subtle differences in design (albeit unidentified) may suffice to explain their vastly different gating and permeation behaviors (37).

Structural Inferences in the HCN Outer Pore—Covalent attachment of a bulky moiety to an inserted cysteine located in a narrow pore region can block permeation simply by steric hindrance regardless of its charge, an effect previously observed in similar studies performed with the Na\(^{+}\) channel pore (11, 12, 28–30). However, because the hyperpolarizing activation shifts induced by MTSEA and MTSES modifications were already sufficient to account for most of the current reduction observed with these agents (cf. the corresponding activation curves and the whole-cell current reduction at −140 mV), these findings suggest that physical blockade of the permeation pathway by these agents, if present, was not substantial. By analogy with the KcsA K\(^{+}\) channels (31), Cys318 corresponds to a wide open pore region with a diameter of \(\sim 35\) Å. This diameter also does not place the native cysteines close enough to cross-link with each other. Taken collectively, our observations support the notion that Cys318 is located in a relatively wide region of the external pore mouth.

Because HCN channels are tetramers (4), it is possible that up to all four S5-P cysteines were modified by the MTS agents to produce the observed functional changes. However, the modification of one cysteine might in turn influence the accessibility and susceptibility of the remaining ones for reaction, de-
pending on the side chain orientation of Cys\(^{318}\) and the local geometry. Our present results did not reveal how many Cys\(^{318}\) copies were modified. Experiments using tandem constructs to place one, two, or three cysteines in a channel combined with single-channel recordings may answer this question and are currently underway. Regardless of the outcome, it is clear that residue 318 is located in a region where allosteric interactions critical for the gating properties and global structure of HCN.
channels are present. In addition, the S5-P linker, a highly nonconserved region even among channels of the same selectivity, has been reported to regulate many isoform- and channel-specific properties such as toxin sensitivities and permeation (32–34). Further studies of this extrapore linker may reveal additional determinants underlying the specific structural and functional profiles of HCN and other ion channels. Our finding that the S5-P linker is externally accessible also does not support the hypothesis that HCN channels are incorporated into the bilayer membrane in an orientation opposite from that of voltage-gated \( K^+ \) channels.

Permeation Insights—Although HCN and \( K^+ \) channels share substantial homology, one distinguishing feature that discriminates the pacemaker channels from their \( K^+ \) counterparts, other than their obvious gating differences (i.e., activation by hyperpolarization rather than depolarization), is that HCN channels permeate both \( K^+ \) and \( Na^+ \). The molecular basis of this nonspecific nature of HCN channels is unknown. Although we have recently shown that the glycine-tyrosine-glycine (GYG) motif of HCN channels is prerequisite for ion conduction (4), its conservation in \( K^+ \) channels does not explain why HCN channels are only weakly selective for \( K^+ \) over \( Na^+ \). It is possible that variant pore residues in or near the pore region render HCN channels nonspecific by altering the pore diameter and/or influencing the selectivity filter. Sequence comparison reveals that although Cys\(^{347} \), located two residues N-terminal to the GYG signature sequence (4, 31), is conserved among all HCN isoforms, the equivalent residue in \( K^+ \)-selective channels is a conserved threonine. Indeed, it has been hypothesized that this cysteine variant in the pore of HCN channels underlies their nonselectivity (1–3, 19, 35, 36). However, our results indicate that the permeability ratios of C347S channels were not different from those of WT channels, suggesting that this pore variant is unlikely to contribute to ionic selectivity. Clearly, further experiments studying additional variant sites are needed to address this question.

Conclusion—In summary, sulphydryl modification of Cys\(^{318} \) in the S5-P linker of HCN1 channels primarily modified activation gating. These effects were abolished by the mutation C318S. Our results form the basis for future cysteine scanning experiments of HCN channels. We conclude that the side chain of the S5-P native cysteine is externally accessible and that the outermost pore vestibule and activation gating of HCN channels are allosterically coupled.

Acknowledgments—We are indebted to Dr. Eduardo Marbán for guidance, encouragement, and generous support and for critical reading of the manuscript. We also thank Peihong Dong for constructing the channel mutants.

REFERENCES

1. Gauss, R., Seifert, R., and Kaupp, U. B. (1998) Nature 393, 583–587
2. Ludwig, A., Zong, X., Jeglitsch, M., Hofmann, F., and Biel, M. (1998) Nature 393, 587–591
3. Santoro, B., Liu, D. T., Yao, H., Bartsch, D., Kandel, E. R., Siegelbaum, S. A., and Tsien, R. Y. (1998) Cell 93, 717–729
4. Xie, T., Marbán, E., and Li, B. A. (2002) Circ. Res. 90, 1267–1273
5. Chen, S., Wang, J., and Siegelbaum, S. A. (2001) J. Gen. Physiol. 117, 491–504
6. Ulena, C., and Tytsga, J. (2001) J. Biol. Chem. 276, 6069–6072

### TABLE IV

Summary of activation and deactivation kinetics of WT and mutant HCN1 channels before and after MTSEA or MTSES modification

| Channel          | \( \tau_{\text{act(slow)}} \) | \( \tau_{\text{act(fast)}} \) | \( \lambda_{\text{slow}} \) | \( \% \) |
|------------------|-----------------|-----------------|-----------------|-------------|
| WT               | 27.1 ± 1.9 (8)  | 259.1 ± 51.5 (8)| 20.5 ± 2.1 (8)  | 71.5 ± 3.0 (9)|
| WT + MTSEA      | 98.0 ± 10.5 (6) | 1800 ± 269 (6)  | 78.0 ± 1.8 (6)  | 200.7 ± 9.2 (4) |
| WT + MTSES      | 73.8 ± 5.2 (7)  | 1790 ± 104 (7)  | 80.3 ± 2.5 (7)  | 246.4 ± 8.5 (10) |
| C318S           | 29.1 ± 2.3 (7)  | 240.6 ± 61.2 (7)| 19.8 ± 2.2 (7)  | 67.7 ± 2.9 (10)|
| C318S + MTSEA   | 42.7 ± 5.0 (6)  | 350.5 ± 521.6 (6)| 26.3 ± 2.1 (6)  | 65.0 ± 4.1 (4) |
| C318S + MTSES   | 32.8 ± 1.8 (6)  | 269.6 ± 36.3 (6)| 18.7 ± 1.7 (6)  | 70.5 ± 4.0 (10)|
| C347S           | 26.9 ± 1.8 (7)  | 188.0 ± 31.1 (7)| 14.2 ± 0.9 (7)  | 40.7 ± 1.7 (6) |
| C347S + MTSEA   | 59.1 ± 5.2 (7)  | 2459 ± 393 (7)  | 81.9 ± 3.5 (7)  | 228.4 ± 15.3 (3) |

* Statistically different (\( p < 0.01 \)) from the corresponding channels recorded under control MT-free conditions.

Fig. 8. Schematic diagram demonstrating the dynamic rearrangements of the pore mouth of depolarization-activated (A) and HCN (B) channels during gating. The S5-P cysteines of HCN are exposed to the extracellular milieu at all times.
An Externally Accessible Pore Cysteine of HCN Channels

7. Benitah, J. P., Tomaselli, G. F., and Marbán, E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7392–7396
8. Tsushima, R. G., Li, R. A., and Backx, P. H. (1997) J. Gen. Physiol. 110, 59–72
9. Creighton, T. (1997) Protein: Structures and Molecular Properties, 5th Ed., W. H. Freeman & Co., New York
10. Karlin, A., and Akabas, M. H. (1998) Methods Enzymol. 293, 123–145
11. Li, R. A., Velez, P., Chiamvimonvat, N., Tomaselli, G. F., and Marbán, E. (2000) J. Gen. Physiol. 115, 81–92
12. Perez-Garcia, M. T., Chiamvimonvat, N., Marbán, E., and Tomaselli, G. F. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 300–304
13. Lu, Q., and Miller, C. (1995) Science 268, 304–307
14. Akabas, M. H., Stauffer, D. A., Xu, M., and Karlin, A. (1992) Science 258, 307–310
15. Akabas, M. H., and Karlin, A. (1995) Biochemistry 34, 12496–12500
16. Akabas, M. H., Kaufmann, C., Archdeacon, P., and Karlin, A. (1994) Neuron 13, 919–927
17. Sun, Z. P., Akabas, M. H., Goulding, E. H., Karlin, A., and Siegelbaum, S. A. (1996) Neuron 16, 1037–1047
18. Hille, B. (2001) Ion Channels of Excitable Membranes, 3rd Ed., Sinauer Associates, Inc., Sunderland, MA
19. Santoro, B., and Tibbs, G. B. (1999) Annu. N. Y. Acad. Sci. 868, 741–764
20. Zagotta, W. N., and Siegelbaum, S. A. (1996) Annu. Rev. Neurosci. 19, 235–263
21. Ludwig, A., Zong, X., Hofmann, F., and Biel, M. (1999) Cell. Physiol. Biochem. 9, 179–186
22. Horn, R. (1998) Methods Enzymol. 293, 145–155
23. Balser, J. R., Nuss, H. B., Chiamvimonvat, N., Perez-Garcia, M. T., Marbán, E., and Tomaselli, G. F. (1996) J. Physiol. (Lond.) 491, 69–77
24. Yellen, G. (1998) Q. Rev. Biophys. 31, 239–295
25. Marten, L., and Hoshi, T. (1996) Biophys. J. 74, 2953–2962
26. Townsend, C., and Horn, R. (1997) J. Gen. Physiol. 110, 23–33
27. Azene, E., Xue, T., Marbán, E., and Li, R. A. (2002) Circulation, in press
28. Chiamvimonvat, N., Perez-Garcia, M. T., Ranjan, R., Marbán, E., and Tomaselli, G. F. (1996) Neuron 16, 1037–1047
29. Tsushima, R. G., Li, R. A., and Backx, P. H. (1997) J. Gen. Physiol. 109, 463–475
30. Chiamvimonvat, N., Perez-Garcia, M. T., Tomaselli, G. F., and Marbán, E. (1996) J. Physiol. (Lond.) 491, 51–59
31. Doyle, D. A., Morais-Cabral, J., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) Science 280, 69–77
32. Li, R. A., Ennis, E., Tomaselli, G., and Marbán, E. (2001) Circulation 104, 230–236
33. Dun, W., Jiang, M., and Tseng, G. N. (2002) Pflügers Arch. 439, 141–149
34. Pardo-Lopez, L., Zhang, M., Liu, J., Jiang, M., Pussani, L. D., and Tseng, G. N. (2002) J. Biol. Chem. 277, 16403–16411
35. Kaupp, U. B., and Sfedert, R. (2001) Annu. Rev. Physiol. 63, 235–257
36. Clapham, D. E. (1998) Neuron 21, 5–7
37. Henrikson, C. A., Azene, E., Dong, P., Marbán, E., and Li, R. A. (2002) Circulation, in press
An External Determinant in the S5-P Linker of the Pacemaker (HCN) Channel
Identified by Sulfhydryl Modification
Tian Xue and Ronald A. Li

J. Biol. Chem. 2002, 277:46233-46242.
doi: 10.1074/jbc.M204915200 originally published online September 25, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204915200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 33 references, 13 of which can be accessed free at
http://www.jbc.org/content/277/48/46233.full.html#ref-list-1