

# Reversal of HCN Channel Voltage Dependence via Bridging of the S4–S5 Linker and Post-S6

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Voltage-gated ion channels possess charged domains that move in response to changes in transmembrane voltage. How this movement is transduced into gating of the channel pore is largely unknown. Here we show directly that two functionally important regions of the spHCN1 pacemaker channel, the S4–S5 linker and the C-linker, come into close proximity during gating. Cross-linking these regions with high-affinity metal bridges or disulfide bridges dramatically alters channel gating in the absence of cAMP; after modification the polarity of voltage dependence is reversed. Instead of being closed at positive voltage and activating with hyperpolarization, modified channels are closed at negative voltage and activate with depolarization. Mechanistically, this reversal of voltage dependence occurs as a result of selectively eliminating channel deactivation, while retaining an existing inactivation process. Bridging also alters channel activation by cAMP, showing that interaction of these two regions can also affect the efficacy of physiological ligands.

## INTRODUCTION

Many ion channels show voltage-dependent changes in conductance. Sensing of membrane voltage is achieved via movement of charged domains within the channel (termed voltage sensors), primarily the positively charged S4 segment in channels that contain this domain. The exact extent of such voltage sensor movements remains controversial and may differ between channels (Ruta et al., 2005; Tombola et al., 2005). However, movement of S4 apparently occurs in the same general direction (at least in the plane perpendicular to the membrane) for both depolarization and hyperpolarization-activated channels, i.e., hyperpolarization drives the positively charged S4 segments inward for both channel types (Larsson et al., 1996; Mannikko et al., 2002; although see Bell et al., 2004, for evidence that apparent movements of S4 may occur via alteration of the membrane field).

Depolarization- and hyperpolarization-activated channels have also both been shown to possess intracellular gates that are located in roughly the same regions of their S6 transmembrane segments. These gates open and close in response to changes in transmembrane voltage and have been proposed to limit the flow of ions in the closed state, based on their ability to limit access of drugs and small ions to the channel pore (Liu et al., 1997; Shin et al., 2001; Rothberg et al., 2002).

A currently unresolved question is therefore the following: if the S4 voltage sensors move in the same direction and the gates show a broadly common location, how is opposite polarity of gating achieved in

hyperpolarization-versus depolarization-activated channels? It seems possible that differences in the transduction mechanism linking voltage sensing to movement of the gate might underlie their opposite polarity. It has been proposed that an interaction of the S4–S5 linker with the post-S6 region might be important for this transduction in various ion channels (Lu et al., 2002; Decher et al., 2004; Long et al., 2005; Ferrer et al., 2006). However, how this potential transduction mechanism might differ between depolarization-activated and hyperpolarization-activated channels, and whether such differences can account for the opposite polarity of these channels, is currently unknown.

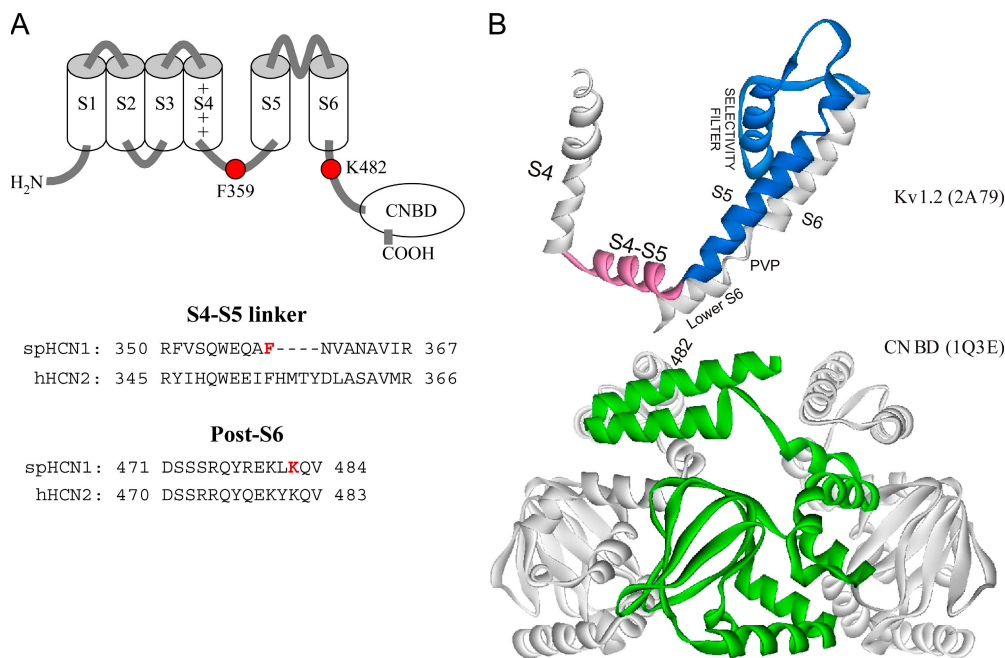
We sought more direct evidence that interactions between the S4–S5 linker and post-S6 regions occur in the spHCN1 channel: a hyperpolarization-activated, cyclic nucleotide-sensitive, nonselective cation (HCN) channel (Gauss et al., 1998). We chose to study potential interactions of the S4–S5 linker with a region of the post-S6 termed the C-linker. This region is of additional interest as it is conserved in many channels sensitive to cyclic nucleotides and is thought to facilitate transduction of cyclic nucleotide binding into channel gating (Wang et al., 2001; Zagotta et al., 2003). This region seemed a potential candidate for interaction with the S4–S5 linker, as cyclic nucleotide binding alters the voltage dependence of spHCN1 gating (Gauss et al., 1998; Shin et al., 2001).

We introduced cysteines into the S4–S5 and C-linker regions of the spHCN1 channel and attempted to form

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The online version of this article includes supplemental material.

Abbreviations used in this paper: DTDP, 2,2'-dithiodipyridine; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol.



**Figure 1.** Location of spHCN1 mutations involved in reversed voltage dependence. (A) Topology diagram of spHCN1, showing the location of the S4–S5 linker and post-S6 C-linker regions. Also shown are the S1–S6 transmembrane segments including the S4 voltage sensor, and the cyclic nucleotide-binding domain (CNBD). Locations of the residues F359 and K482 discussed in the text are indicated by circles. Also shown are alignments of sequences in these regions for spHCN1 and hHCN2, with F359 and K482 of spHCN1 shown in red. (B) Clues to the positions of the mutations from related crystal structures of the Kv1.2 pore-forming domain (top) and the HCN2 cyclic nucleotide binding domain (bottom). The S4–S6

regions of one subunit of Kv1.2 (Long et al., 2005) are shown (aa288–416); the S5 and S6 are roughly in the plane of the paper with the N-terminal end of S4–S5 running  $\sim 35^\circ$  into the paper. The alignment of HCN with Kv channels is difficult in the linker and S5 regions, making it hard to speculate on the location of individual residues. Three subunits of the HCN2 CNBD (Zagotta et al. 2003) are shown, with the position corresponding to spHCN1-482 indicated for the green subunit in the foreground. The relative position of the pore-forming and CNB domains is not known. The Kv1.2 structure is apparently an open state; the CNBD structure has four molecules of cAMP bound, but the C-linker conformation may correspond more to a closed-preferring form (Craven and Zagotta, 2004).

high-affinity metal bridges or disulfide cross-links between the introduced cysteines, hoping that such modifications might result in a measurable functional effect. We describe here channels containing a pair of cysteines: F359C in the S4–S5 linker and K482C in the C-linker. These channels exhibited profound changes in gating when cross-linking agents were applied. These effects included an altered response of channels to cAMP, demonstrating the potential for interaction of these regions to alter modulation of channels by physiological ligands. Most dramatically, application of cross-linking agents led to a reversal in the effective voltage dependence of gating in the absence of cAMP, demonstrating that interaction of the S4–S5 linker with the post-S6 can in principle dictate the polarity of voltage-dependent gating in ion channels.

## MATERIALS AND METHODS

### Molecular Biology

Point mutations of the spHCN1 channel subunit (GenBank/EMBL/DBJ accession no. Y16880) were made as described previously (Rothberg et al., 2002). All subunits contained the M349I and H462Y mutations (Rothberg et al., 2003), except constructs containing F459L, where H462 was not mutated. All mutations were confirmed by sequencing.

### Electrophysiology and Solutions

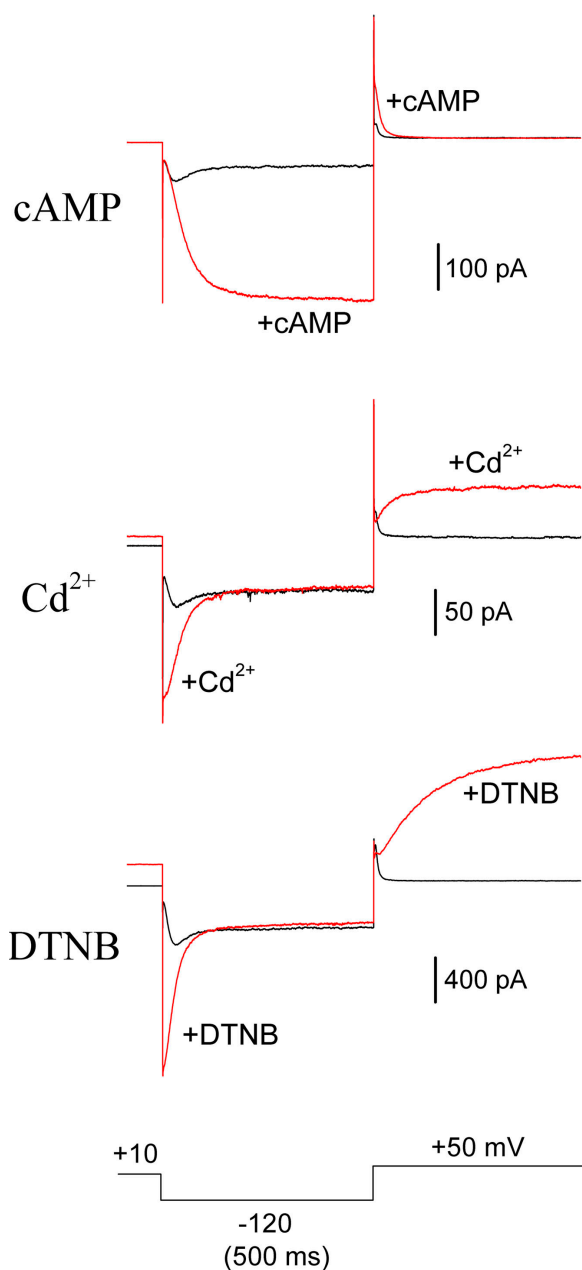
Experiments were performed with excised inside-out patches from HEK-293 cells transfected as described previously (Rothberg

et al., 2002). Cells were pretreated with dithiothreitol (DTT; 5 mM) for 20 min before recording. Methods for rapid perfusion switches and electrophysiological recordings were as described previously (Liu et al., 1997). Currents were not leak subtracted. Both the intrapipette and control bathing solutions consisted of (in mM) 160 KCl, 10 HEPES, 0.5 MgCl<sub>2</sub>, and 0.2 EGTA (or 0.05 for experiments involving Cd<sup>2+</sup>), pH 7.4 with KOH. For bathing solutions containing Cd<sup>2+</sup>, EGTA was omitted. Experimental concentrations of Cd<sup>2+</sup> are quoted as the free Cd<sup>2+</sup> concentration, calculated using reported equilibrium constants (Martell and Smith, 1998).

### Cysteine Cross-linking

For attempting cross-linking of cysteines, we avoided the use of common oxidizing agents such as copper-phenanthroline, as these reagents have been shown to dramatically inhibit wild-type spHCN1 (Roncaglia et al., 2002). Instead, we used Cd<sup>2+</sup> ions at submicromolar concentrations (i.e., much lower than those reported to block spHCN1 by Roncaglia et al., 2002). In addition, we used reagents that we anticipated would be pore impermeant, such as the established cross-linking agents 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB; Means and Feeney, 1971; Lehrer, 1975; Zilberberg et al., 2001) or 2,2'-dithiodipyridine (DTDP). Independent modification of cysteines by DTNB or DTDP is difficult to rule out completely, and almost certainly occurs to some degree. However, for reasons discussed in the text, it seems likely that cross-linking dominates the main functional effects seen in this study. DTNB-modified currents are shown after washout of reagent (used at 100  $\mu$ M). DTT was made as a 1 M stock solution in distilled water, kept on ice, and applied at the required concentration within 6 h. Values stated are mean  $\pm$  SEM.

## 359C-482C



**Figure 2.** Cross-linking S4–S5 linker and post-S6 regions reverses voltage dependence of spHCN1. Effect of cAMP (100  $\mu$ M), Cd<sup>2+</sup> (130 nM), or DTNB treatment, on 359C-482C channel currents from independent patches. Fold increases in peak time-dependent current at  $-120$  mV in response to application were as follows: cAMP ( $8.0 \pm 1.2$ ,  $n = 9$ ), Cd<sup>2+</sup> ( $3.5 \pm 1.5$ ,  $n = 9$ ), and DTNB ( $4.7 \pm 1.7$ ,  $n = 9$ ), i.e., cAMP induced a greater increase of peak current than either Cd<sup>2+</sup> or DTNB. DTNB results were obtained after washout of the reagent (see Materials and methods).

#### Online Supplemental Material

See the supplemental material (<http://www.jgp.org/cgi/content/full/jgp.200609590/DC1>) for control experiments on the single cysteine mutants (359C or 482C) with Cd<sup>2+</sup> and DTNB in the absence (Fig. S1) or presence (Fig. S2) of cAMP.

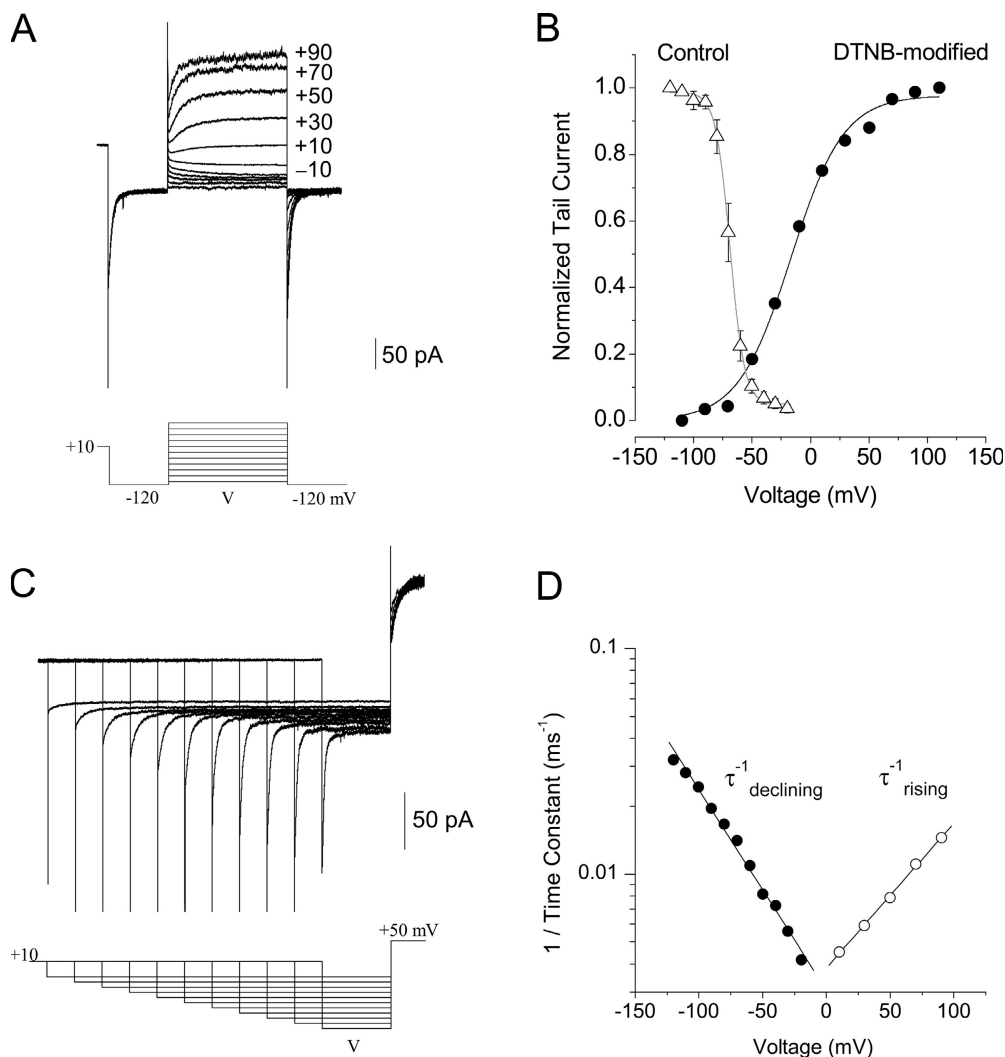
## RESULTS

### Cross-linking S4–S5 and Post-S6 Regions Reverses the Voltage Dependence of spHCN1

Fig. 1 A shows a putative topology diagram of spHCN1, indicating the position of the S4–S5 linker and the post-S6 C-linker region studied. The location of these regions in the context of related crystal structures is shown in Fig. 1 B. To probe the effects of S4–S5 linker interaction with the C-linker on gating properties of spHCN1, we introduced cysteines into the S4–S5 linker and C-linker, either singly or simultaneously. We then applied submicromolar concentrations of Cd<sup>2+</sup> to the intracellular face of excised patches and attempted to form high-affinity metal bridges between the introduced cysteines. In addition, we used cysteine cross-linking agents such as DTNB (see Materials and methods) to study the effects of disulfide formation between these respective cysteines.

Fig. 2 shows the effects of applying cAMP, Cd<sup>2+</sup>, or DTNB on currents mediated by spHCN1 channels containing cysteines simultaneously introduced into the S4–S5 linker (F359C) and C-linker (K482C) (subsequently termed “359C-482C”). These mutant channels showed properties similar to those of wild-type spHCN1 under similar conditions (e.g., Shin et al., 2004). In the absence of cAMP, currents were of small amplitude, activating upon hyperpolarization and showing a subsequent time-dependent decline referred to as inactivation (Gauss et al., 1998; Shin et al., 2004). Addition of cAMP increased current amplitude and eliminated time-dependent inactivation (top). Remarkably, modification with Cd<sup>2+</sup> (middle) or DTNB (bottom) caused dramatic changes to channel gating in the absence of cAMP. After modification, currents showed a time-dependent decline in response to hyperpolarization and a time-dependent increase in response to depolarization, i.e., the voltage dependence of these channels was reversed with respect to unmodified channels. Channels containing only an introduced cysteine in the S4–S5 linker (359C) or C-linker (482C) did not show this effect and showed only very mild effects of modification with Cd<sup>2+</sup> or DTNB (see Fig. S1, available at <http://www.jgp.org/cgi/content/full/jgp.200609590/DC1>). This shows that both cysteines are required for the effects of Cd<sup>2+</sup> and DTNB, suggesting either that modifications at these positions interact, or that cross-linking of these two cysteines occurs.

Cd<sup>2+</sup> is a well-established reagent for binding multiple, but not single, cysteines with high affinity. The dramatic effects of Cd<sup>2+</sup> on 359C-482C channels, but not on the respective single mutants 359C and 482C, suggests that a high-affinity metal bridge can be formed between these two introduced cysteine residues. The similarity of DTNB effects to those of Cd<sup>2+</sup> suggests that DTNB induces formation of disulfide bridges between the introduced cysteine residues in 359C-482C channels. Further evidence for disulfide formation was provided by the



**Figure 3.** Quantitative assessment of reversed voltage dependence. Properties of 359C-482C currents after modification with DTNB. (A) Depolarization-activated currents elicited by steps from  $-120$  mV (500-ms pulse) to test potentials ranging from  $-110$  mV to  $+90$  mV (1 s duration), in  $+20$ -mV steps. Pulse voltage is indicated alongside the corresponding current. (B) Normalized conductance–voltage relationship for modified channels (filled circles, from tail current relaxation amplitudes at  $-120$  mV in A after 500-ms depolarizing prepulses), or unmodified channels (open triangles, from tails at  $+50$  mV after hyperpolarizing prepulses; measured in the presence of cAMP [ $100 \mu\text{M}$ ] for experimental ease, as parameters of G-V fits are not markedly different  $\pm$  cAMP; e.g. Shin et al., 2004). Values for modified and unmodified channels were independently normalized. Mean parameters from individual fits to four patches were as follows: modified channels,  $V_{1/2} = -4.1 \pm 8$  mV and slope =  $25.8 \pm 1.3$  mV; unmodified channels,  $V_{1/2} = -69 \pm 2$  mV and slope =  $5.7 \pm 0.2$  mV (similar to wild-type spHCN1 under these conditions; e.g., Shin et al., 2004).

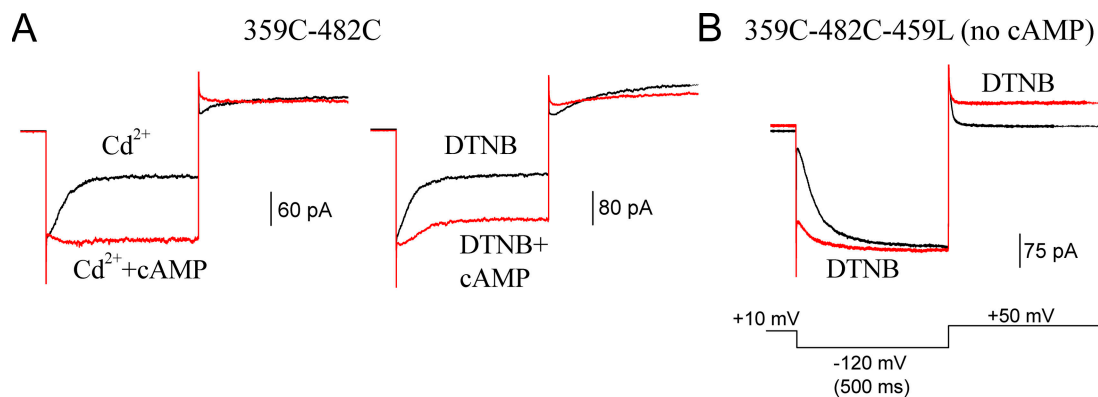
Note that normalized tail current relaxation amplitudes are shown, and hence residual steady-state current at  $-120$  mV is not represented; we did not routinely measure ZD7288-blockable currents in these patches and therefore cannot reliably estimate the fraction of channel current contributing to leak. (C) Current declines elicited by steps from  $+10$  mV to test potentials ranging from  $-20$  to  $-120$  mV, in  $-10$  mV steps (along with a 400-ms decrement in duration, to give shorter durations at hyperpolarized potentials, for increased patch stability). (D) Time constants for time-dependent relaxations: rising depolarization-induced relaxations in A ( $\tau_{\text{rising}}$ ), and declining hyperpolarization-induced relaxations in C ( $\tau_{\text{declining}}$ ). Mean parameters from individual fits were as follows: rising, slope =  $+0.0080 \pm 6.4 \times 10^{-4} \text{ mV}^{-1}$  ( $n = 4$ ; i.e.,  $e$ -fold in  $\sim 54$  mV); declining, slope =  $-0.0091 \pm 2.4 \times 10^{-4} \text{ mV}^{-1}$  ( $n = 3$ ; i.e.,  $e$ -fold in  $\sim 48$  mV).

observation that these dramatic effects were reproduced by other cross-linking agents such as DTDP, but not by the structurally similar thiol-reactive, non-cross-linking reagent benzyltoluenethiosulfonate (BTTS; unpublished data). Effects of modification with DTNB could be reversed with DTT, but only slowly ( $\tau \sim 6$  min with 5 mM DTT; unpublished data). This slow reversal by DTT also suggests the presence of a cysteine–cysteine disulfide bridge, since cysteines independently modified with DTNB would be expected to exhibit much faster reversal with DTT, as thionitrobenzoic acid is a good leaving group (although this is only circumstantial evidence, as poor solvent accessibility could also contribute to slow reversal by DTT).

Overall, these results suggest that regions within the S4–S5 linker and C-linker come into close proximity during gating and can likely be bridged or cross-linked by a variety of reagents, producing dramatic changes to channel gating.

#### Voltage Dependence of Reversed Gating

Fig. 3 shows the voltage-dependent characteristics of reversed gating seen after modification of 359C-482C with DTNB. Depolarizing pulses after a prepulse to  $-120$  mV elicited rising currents that showed substantial voltage dependence (Fig. 3 A). The conductance–voltage relationship of reversed gating in a representative patch, constructed from tail current relaxation amplitudes at



**Figure 4.** Reversed gating is sensitive to cAMP and to a mutation in the pore. (A) Effect of cAMP (100  $\mu$ M) on 359C-482C currents modified with  $\text{Cd}^{2+}$  (130 nM, left) or DTNB (right). (B) Effect of DTNB on 359C-482C-459L currents, in the absence of cAMP.

$-120$  mV, is shown in Fig. 3 B. For comparison, the conductance–voltage relationship of unmodified channels is also shown. Unmodified channels showed similar properties to those of wild-type spHCN1 (Shin et al., 2004): currents activated with hyperpolarization and exhibited a relatively steep voltage dependence of steady-state activation. In contrast, modified 359C-482C channel currents activated with depolarization and showed a more shallow voltage dependence (approximately one quarter that of unmodified channels). Hyperpolarizing pulses from a holding potential of  $+10$  mV elicited declining currents that again showed substantial voltage dependence (Fig. 3 C). The voltage-dependent kinetics of both rising (Fig. 3 A) and declining (Fig. 3 C) phases of reversed gating in a representative patch are shown in Fig. 3 D.

#### Reversed Gating Is Sensitive to cAMP and to a Mutation in the Pore

What is the physical basis for the reversed gating seen in modified channels, and how might this gating process be modulated? We first examined the effect of cAMP, a physiological ligand of HCN channels, on the reversed gating. Addition of cAMP to  $\text{Cd}^{2+}$ -modified 359C-482C currents practically eliminated the time-dependent relaxations (Fig. 4 A, left). Similarly, relaxations were largely eliminated in DTNB-modified currents, although some residual time-dependent gating processes were still evident (Fig. 4 A, right). These residual relaxations were not affected by increasing the concentration of cAMP (to 1 mM; unpublished data), demonstrating that this effect is not due to a DTNB-induced reduction of channel affinity for cAMP. The sensitivity to cAMP suggested that the reversed gating might represent some form of inactivation process, since inactivation of wild-type spHCN1 is also removed by cAMP (Gauss et al., 1998). Another manipulation that can remove inactivation is the mutation F459L within the pore (Shin et al., 2004). We therefore tested whether this mutation could also eliminate the reversed gating relaxations

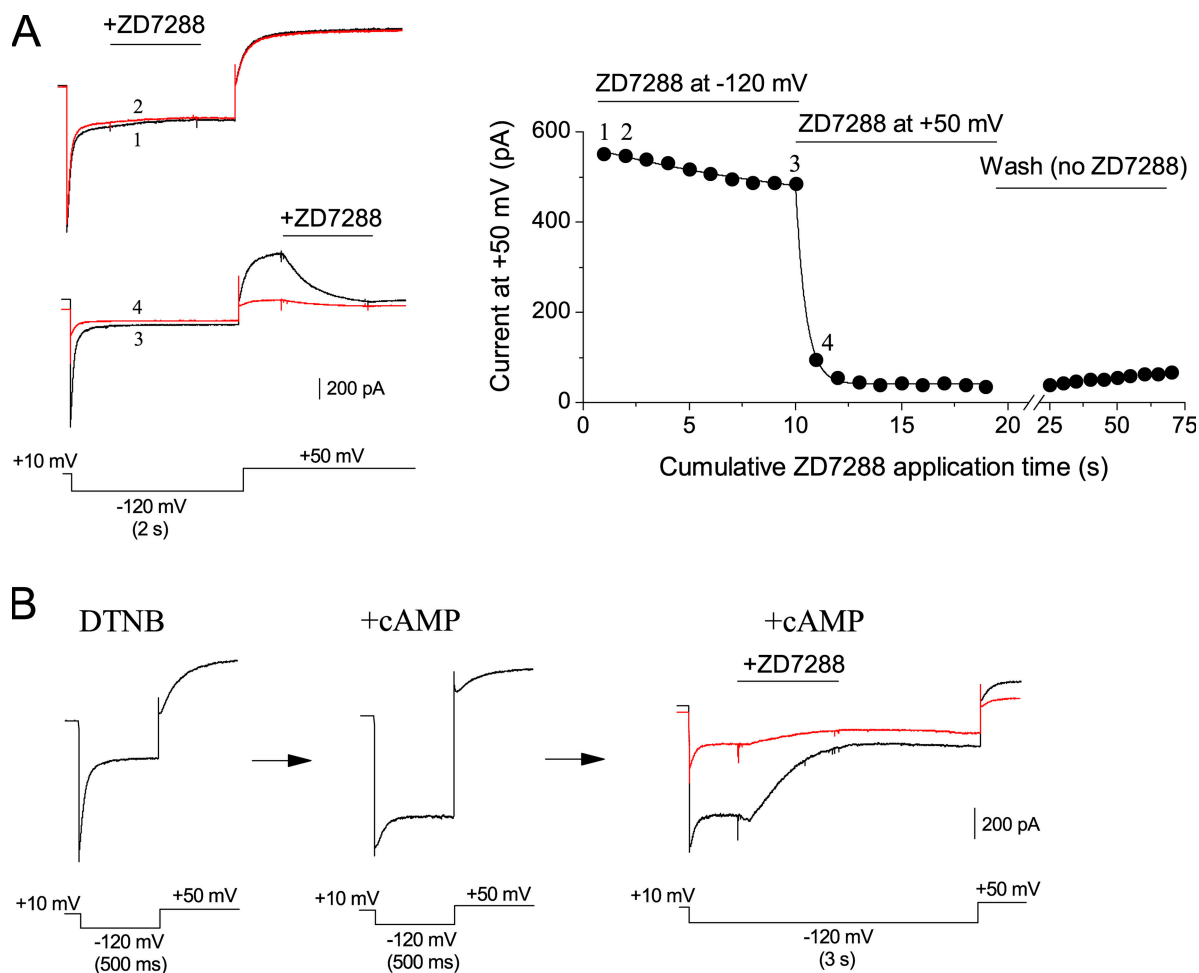
seen in cross-linked channels. Fig. 4 B shows the effect of DTNB on 359C-482C-459L currents in the absence of cAMP. Modification with DTNB resulted in a pronounced lock-open effect, with no evidence of reversed gating relaxations.

These results suggest that 359C and 482C come into close proximity in an open state of the channel and that cross-linking prevents normal channel closure, while still allowing a time-dependent gating process with reversed voltage-dependent polarity to occur.

#### Gated Access to the Pore during Reversed Gating

Based on the sensitivity of the reversed gating processes to cAMP and to the pore mutation F459L, we reasoned that these relaxations might share a similar structural origin to the inactivation relaxations shown by wild-type spHCN1 (Shin et al., 2004). We therefore tested whether an HCN channel blocker (ZD7288) showed state-dependent accessibility to the channel pore, as previously reported for inactivation of spHCN1 (Shin et al., 2004). Fig. 5 shows the effects of ZD7288 on DTNB-modified 359C-482C channels. ZD7288 was applied at different voltages using a rapid-perfusion system. In Fig. 5 A, ZD7288 was applied at either  $-120$  mV or at  $+50$  mV, after the gating relaxations at these respective voltages had occurred. At  $-120$  mV, block by ZD7288 occurred only very slowly, whereas at  $+50$  mV, block occurred much more quickly ( $\sim 30$ -fold increase in rate).

To exclude the possibility that this difference in rates arose by virtue of any intrinsic voltage dependence of block, we used cAMP instead of voltage to open the channels at a constant voltage of  $-120$  mV (Fig. 5 B). In the presence of cAMP, the rate of ZD7288 block was increased  $\sim 30$ -fold compared with block in the absence of cAMP. The rate observed in cAMP at  $-120$  mV was similar to that observed at  $+50$  mV in the absence of cAMP, indicating a lack of intrinsic voltage dependence of ZD7288 block over this 170-mV voltage range. This is consistent with the lack of intrinsic voltage dependence



**Figure 5.** State-dependent block by ZD7288 during reversed gating. (A) 359C-482C currents after modification with DTNB. ZD7288 (100  $\mu$ M) was applied during the periods indicated. The sequential first and second current traces during which ZD7288 was applied are shown. Block at +50 mV occurred with  $\tau = 300 \pm 12$  ms ( $n = 3$  patches). The graph shows cumulative block of currents by ZD7288 (100  $\mu$ M) applied for 1 s during each pulse at the voltage indicated. Pulses were applied every 5 s. Block was virtually irreversible over the time course studied, shown by the absence of recovery upon washout. Cumulative block occurred slowly when ZD7288 was applied at -120 mV ( $\tau \sim 13$  s), but much more rapidly when applied at +50 mV ( $\tau \sim 460$  ms). Note ZD7288-sensitive residual steady-state current was evident at -120 mV, comprising  $\sim 8\%$  of maximum ZD7288-blockable inward current in this patch. (B) 359C-482C current traces after modification with DTNB (left) and after subsequent application of cAMP (100  $\mu$ M). ZD7288 was then applied for 1 s during pulses to -120 mV in the presence of cAMP (right). Sequential first and second traces during which ZD7288 was applied are shown. Block occurred with  $\tau \sim 460$  ms.

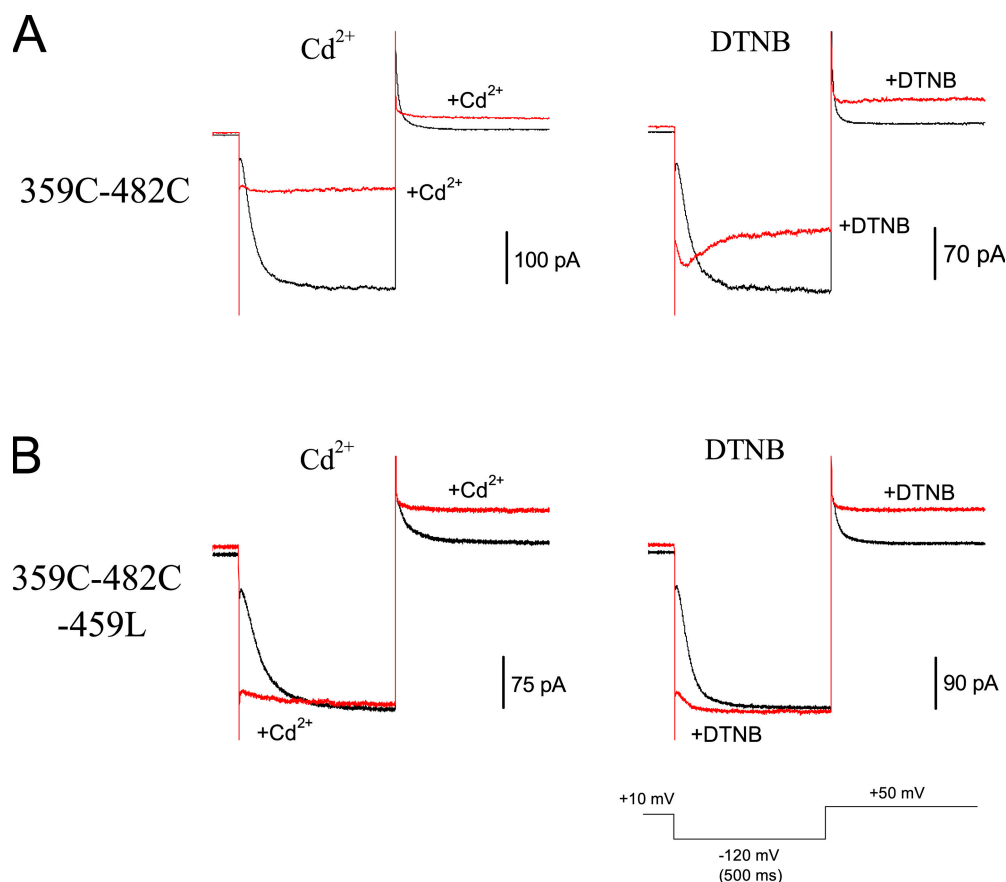
observed in a previous study (Shin et al., 2001). Fig. 5 A also shows that modified currents display a residual steady-state current at -120 mV that is blocked by ZD7288. The slow rate at which current is blocked at -120 mV has implications for the structural etiology of this residual steady-state current. It suggests that this current reflects either channels open with low probability (whose drug entry rate is proportional to open probability), or the presence of an inherently “leaky” (but only slowly blockable) inactivated state. A small separate population of high open-probability channels would be expected to be blocked at a faster rate (e.g., Proenza and Yellen, 2006).

These results indicate that during the gating process that underlies reversed voltage dependence of modified

channels, there is a concomitant change in the accessibility of ZD7288 to the channel pore.

#### Cross-linking S4–S5 and Post-S6 Alters Channel Regulation by cAMP

When performed in the presence of cAMP, modification of 359C-482C channels with cross-linking agents also had dramatic effects on gating. Fig. 6 shows the effect of  $\text{Cd}^{2+}$  or DTNB on 359C-482C currents in the constant presence of cAMP.  $\text{Cd}^{2+}$  increased the steady-state current at +50 mV, indicating a lock-open effect, but also caused a marked reduction in total current amplitude (Fig. 6 A, left). These two effects could not be separated by using a lower concentration of  $\text{Cd}^{2+}$  (10 nM; unpublished data), suggesting that they may



**Figure 6.** Cross-linking S4–S5 and post-S6 produces tonic inactivation that can no longer be completely removed by cAMP. (A) Effects of  $\text{Cd}^{2+}$  (130 nM, left) or DTNB (right) on 359C-482C currents in the constant presence of cAMP. (B) Effects of  $\text{Cd}^{2+}$  (130 nM, left) or DTNB (right) on 359C-482C-459L currents in the constant presence of cAMP.

arise through a single common cross-linking process. Modification with DTNB led to a dramatic lock-open effect, with some residual time-dependent current that exhibited reversed voltage dependence (Fig. 6 A, right). These dramatic effects of modification were not seen for channels in which cysteines were introduced only at position 359 or 482 (see Fig. S2, available at <http://www.jgp.org/cgi/content/full/jgp.200609590/DC1>). Interestingly, at first glance, results shown in Fig. 6 A and Fig. 4 A seem somewhat contradictory. In Fig. 4 A, cAMP removes a proposed time-dependent inactivation process, whereas in Fig. 6 A, currents were inhibited, suggesting a disruption of cAMP effects. However, the overall inhibitions seen on modification with  $\text{Cd}^{2+}$  or DTNB in the presence of cAMP (Fig. 6 A) are consistent with the observation that modification with these reagents in the absence of cAMP produced smaller mean increases in current than cAMP itself (legend to Fig. 2). Taken together, these results suggest that after modification with  $\text{Cd}^{2+}$  or DTNB, cAMP can largely remove the time-dependent processes in Fig. 4 A, but can no longer increase current to the full original extent possible before modification (see Fig. 2). We hypothesized that inhibition seen after modification of 359C-482C channels with either  $\text{Cd}^{2+}$  or DTNB in the presence of cAMP (Fig. 6 A) resulted from an inability of cAMP to fully remove inactivation from cross-linked channels.

We therefore tested whether the pore mutation F459L could abolish this inhibition (Fig. 6 B). When channels containing the 359C-482C-459L mutations were modified with  $\text{Cd}^{2+}$  (left) or DTNB (right), currents were locked open, but there was no concomitant inhibition. This suggests that cross-linking the S4–S5 linker and C-linker regions does impair the ability of cAMP to remove inactivation from these channels.

## DISCUSSION

### Interaction of S4–S5 and Post-S6

The results presented here show that the S4–S5 linker and C-linker regions of spHCN1 channels come into close proximity and can interact during gating. The profound effects of  $\text{Cd}^{2+}$  and DTNB on 359C-482C channels seem likely to result from cross-linking of these two residues, via a metal bridge or disulfide bridge, respectively, although we cannot completely exclude the possibility that independent modifications at both sites interact to induce the observed effects. Close proximity of the S4–S5 linker and C-linker shown here for spHCN1 is consistent with proposals for the related HCN2 channel, based on extensive mutagenesis (Decher et al., 2004). The interactions described for HCN2 were inferred from apparent interaction of mutant effects at

TABLE I

Comparison of Depolarization- and Hyperpolarization-induced Closure in spHCN1 Channels

“Activation gating”	“Inactivation gating”
Closed at positive V	Closed at negative V
cAMP opens (shift in g-V in F459L) <sup>a</sup>	cAMP opens (removal of inactivation)
359C-482C bridge locks open <sup>b</sup>	F459L removes inactivation <sup>a</sup>
Common features	
When closed, both prevent ZD7288 entry from inside <sup>a,b,c</sup>	
Both locked open by 462C-Cd <sup>2+</sup> -466C bridge <sup>a,d</sup>	

<sup>a</sup>Shin et al., 2004.

<sup>b</sup>This study.

<sup>c</sup>Shin et al., 2001.

<sup>d</sup>Rothberg et al., 2003.

two sites. These sites were located some distance away (approximately three helical turns from the positions described in the present study) within both the S4–S5 linker and the post-S6 and were reported to stabilize the closed state (Decher et al., 2004). This is in contrast to the results presented in this study, which showed stabilization of open (and possibly inactivated) states upon cross-linking. This suggests that highly specific interactions between the S4–S5 linker and post-S6 may be involved in channel opening, inactivation, and closure, respectively. Such interactions are apparently not critical for gating to occur, as HCN2 channels in which the C-linker is partially deleted still show voltage-dependent gating (Decher et al., 2004). However, the potential clearly exists for interaction of these two domains to dramatically modulate gating under appropriate conditions, and our results indicate that there is likely to be relative motion between the two domains during gating. Interaction of the S4–S5 linker with the post-S6 has also been proposed for the depolarization-activated channels Kv1.2 (Long et al., 2005) and hERG (Tristani-Firouzi et al., 2002; Ferrer et al., 2006), although it is not yet clear how interactions of the S4–S5 linker and post-S6 may differ between these respective channels in different gating states.

#### Origin of Voltage Dependence and Gating in Cross-linked Channels

Forming a constitutive interaction between the S4–S5 and C-linker residues studied here essentially reverses the net voltage dependence of gating (in the absence of cAMP), or leads to largely time-independent currents (in the presence of cAMP). It appears that the bridging interaction prevents normal deactivation at positive voltages but allows the inactivation that is normally seen in the absence of cAMP at negative voltages to persist. The result is reversed voltage dependence; like *Shaker* potassium channels, the modified channels are mostly closed at negative voltage and open at positive voltage.

The closure at negative voltages bears several specific similarities to the inactivation process seen for wild-type spHCN1 (Shin et al., 2004): it is sensitive to cAMP; it is prevented by the mutation F459L; and it regulates access of ZD7288 to the pore (see Table I for a summary of experimental manipulations in relation to HCN channel gating).

The residual voltage dependence of modified 359C-482C channels is about one quarter that of unmodified channels, as judged from the steepness of the Boltzmann curves (Fig. 3 B). This remaining voltage dependence might result from S4 movement (perhaps limited by the modification), or it might be due to charge movement elsewhere in the protein. S4-related movements in *Shaker* can apparently occur in multiple steps, some of which occur during the final concerted opening transition (Pathak et al., 2005). Non-S4-related voltage dependence is clearly seen for KcsA channels (which lack the S4 voltage sensor domain entirely); for these channels, charged residues in the pore loop influence a voltage-dependent gating transition of the selectivity filter (Cordero-Morales et al., 2006).

Gating of modified channels at negative voltages can limit access of ZD7288 to the channel pore, as previously seen for both activation and inactivation gating of spHCN1 (Shin et al., 2001, 2004). This suggests involvement of the lower S6 in the gating process, though the actual point at which ion flow is prevented could either be at the S6 bundle crossing (as in *Shaker* channels; del Camino and Yellen, 2001), or at an S6-coupled selectivity filter gate (Flynn and Zagotta, 2001; Cordero-Morales et al., 2006).

How might bridging between 359C in the S4–S5 linker and 482C in the C-linker produce the alteration in gating? One possibility is that bridging produces partial or complete immobilization of the main S4 voltage sensor, and that this primary effect prevents deactivation but permits a rather separate inactivation process, with its own voltage dependence, to dominate. Another possibility is that bridging prevents a relative motion of the S4–S5 linker and C-linker that is crucial to deactivation but not to inactivation.

These same gating processes may be affected by native salt bridges between these two regions. It also seems possible that physiologically relevant ions or molecules (e.g., divalent ions, PIP<sub>2</sub>) might intercalate between these two regions (both of which contain several charged residues), to either facilitate or inhibit their interaction. In addition, alteration of the exact disposition and interactions of S4, S4–S5, and C-linker regions may underlie some of the more complex effects of cross-linking, such as the inhibition observed in the presence of cAMP.

#### Inhibition of cAMP Efficacy by Cross-linking

Cross-linking of the S4–S5 segment and C-linker made it impossible to achieve full activation of channels by cAMP



at negative voltages. This was not due to reduced affinity for cAMP and could be overcome by a pore mutation (F459L) that abolishes inactivation. This suggests that cross-linking S4–S5 to the C-linker impairs the ability of cAMP binding to be transduced into an alteration of gating. This makes intuitive sense given recent proposals that the C-linker of HCN2 undergoes movements in response to cAMP binding (Craven and Zagotta, 2004) and that this region is important for transducing the effects of cyclic nucleotide binding (Wang et al., 2001; Zagotta et al., 2003). Cross-linking the C-linker to the S4–S5 linker would presumably inhibit movement of the C-linker to some degree and therefore impair downstream effects of cAMP binding. An alternative explanation might be that cross-linking prevents normal cAMP-dependent interactions of the C-linker with the S4–S5 linker. It is interesting that cAMP is still capable of largely eliminating the reversed gating of cross-linked channels (Fig. 4), despite being unable to remove inactivation completely. This apparent contradiction suggests that multiple inactivation mechanisms may exist in cross-linked 359C-482C channels: both a time-dependent process that is still sensitive to cAMP, and a second process that, due to cross-linking, is relatively insensitive to cAMP. It seems possible that both of these processes occur in wild-type unmodified spHCN1, where they are both sensitive to cAMP. Whether the gates limiting ion flow during these two processes share a common structural origin is currently unclear.

#### Implications for Other Voltage-gated Channels

The reversed net voltage dependence of gating we describe here seems to be made possible by the presence of multiple gating processes with opposing polarity in unmodified spHCN1 channels. The balance between these two processes can apparently dictate the overall polarity of voltage dependence. Removal of one process (deactivation) is therefore able to expose a second process (inactivation) and lead to reversal of voltage dependence. Interestingly, a similar principle is exploited physiologically by hERG potassium channels (Smith et al., 1996), and the phenomenon can also be produced by mutation in *Shaker* potassium channels (Miller and Aldrich, 1996). Similarly, many other voltage-gated channels show multiple distinct voltage-dependent gating processes, which are often of opposite polarity. By analogy with the results presented here, biasing voltage sensor movement of these channels in favor of activation (e.g., by glycosylation, phosphorylation, toxin binding, or interaction of appropriate regions) might be expected to lead to a reversed voltage dependence in these channels also. It therefore seems possible that other voltage-gated channels might undergo physiologically regulated or evolutionary changes to their net voltage-dependent polarity in this way.

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