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# Intracellular cAMP-modulated Gate in Hyperpolarization Activated Cation Channels

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**Abstract:** Hyperpolarization-activated nonselective cation channels (HCNs) play a pivotal role in producing rhythmic electrical activity in the heart and the nerve cells. In our previous experiments, voltage-dependent $Cd^{2+}$ access to one of the substituted cysteines in S6, T464C, supports the existence of an intracellular voltage-dependent activation gate. Direct binding of intracellular cAMP to HCN channels also modulates gating. Here we attempted to locate the cAMP-modulated structure that can modify the gating of HCN channels. SpHCN channels, a sea urchin homologue of the HCN family, became inactivated rapidly and intracellular cAMP removed this inactivation, resulting in about eight-fold increase of steady-state current level. T464C was probed with $Cd^{2+}$ applied to the intracellular side of the channel. We found that access of $Cd^{2+}$ to T464C was strongly gated by cAMP as well as voltage. Release of bound $Cd^{2+}$ by DMPS was also gated in a cAMP-dependent manner. Our results suggest the existence of an intracellular cAMP-modulated gate in the lower S6 region of spHCN channels.

**Key words:** HCN channels, cAMP, gating, $Cd^{2+}$

Hyperpolarization-activated cation currents (or pacemaker currents, termed $I_f$, $I_h$ or $I_q$) play an important role in the generation of spontaneous rhythmic activity in the heart and the brain (Brown et al., 1979; Brown and DiFrancesco, 1980; Pape and McCormick, 1989; for review see DiFrancesco, 1993; Pape, 1996). Cloning of the HCN gene family revealed the molecular entities of the pacemaker currents (Gauss et al., 1998; Ludwig et al., 1998; Santoro et al., 1998; Ishii et al., 1999; Ludwig et al., 1999). These channels are related to depolarization-activated $K^+$ channels ($K_V$ channels), with six transmembrane domains. Despite this structural similarity to $K_V$ channels, which open at depolarized voltages, heterologously expressed cloned HCN channels are activated upon hyperpolarization.

Our previous efforts to elucidate the molecular basis for the voltage-dependent gating of HCN channels provided evidence for the existence of the intracellular activation gate in HCN channels. Specific HCN channel blocker ZD7288 applied to the intracellular side blocks HCN channels only when the channels are open. Also, ZD7288 can be trapped in the channels by holding the blocked channels at a depolarized voltage (Shin et al., 2001). We also demonstrated that T464C in lower S6 region forms an irreversible binding site for the metal cation $Cd^{2+}$. $Cd^{2+}$ applied to the intracellular side of the channels irreversibly blocks currents through T464C channels and the apparent blocking rate can be slowed by holding the channel closed at depolarized voltages, which is consistent with the voltage-dependent intracellular gate that can prevent $Cd^{2+}$ access to 464C (Rothberg et al, 2002).

Along with voltage-dependent gating, HCN channels are also modulated by direct binding of cAMP to a cyclic nucleotide binding domain (CNBD) motif in the C-terminal region, similar to that of CNG channels (DiFrancesco and Tortora, 1991). The binding of cAMP causes a depolarizing shift of the activation curve, allowing the channels to open more easily. cAMP modulation of HCN current in the heart allows the cardiac diastolic depolarization rate to be finely tuned by β-adrenergic and cholinergic stimuli (Pape and McCormick, 1989; DiFrancesco, 1993).

One HCN homologue cloned from sea urchin, named spHCN, shows unique activation properties among HCN channels (Gauss et al., 1998). In the presence of cAMP, the spHCN channel mediates a slowly activating current similar to that produce by the mammalian HCN channels. However, in the absence of cAMP, spHCN channel is inactivated, resulting in approximately 8-fold decrease of steady state current. In our previous experiments, we found that rate of channel blockade by ZD7288 in the absence of cAMP was much slower then in the presence of cAMP. In

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addition, ZD7288 could be trapped inside the channel in the absence of cAMP at a hyperpolarized voltage where channel is inactivated. These observations provided the evidence for a cAMP-modulated structure at the intracellular entrance to the pore (Shin et al., 2004).

In the present study, for further insights into the structural basis of cAMP-modulated inactivation gating in spHCN channel, we examined gated access of $Cd^{2+}$ to its T464C residue in the presence or absence of cAMP using excised inside-out patches. The apparent blocking rate of T464C by $Cd^{2+}$ was much slower in the absence of cAMP, and the $Cd^{2+}$ escape rate by DMPS was also slower without cAMP, consistent with the notion that a cAMP-modulated inactivation gate is present, located intracellular to the 464 residue.

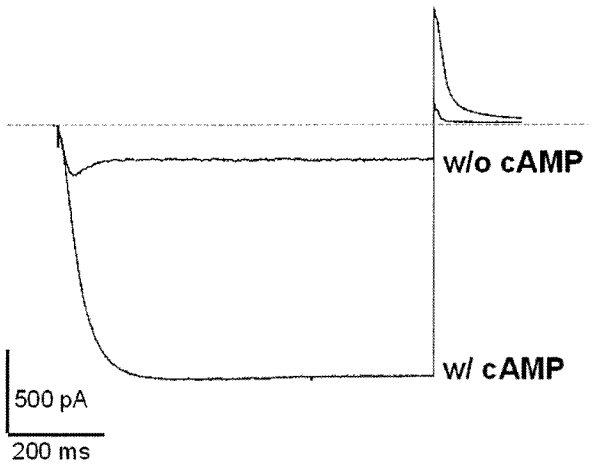


**Fig. 1.** Effect of cAMP on spHCN channel gating. Currents were measured in response to a hyperpolarized voltage from +10 to −120 mV with excised inside-out patch. cAMP (0.1 mM) was applied to the intracellular side of the channels. cAMP removed the inactivation of the channels and increased the steady state current level by ~8-fold.

## MATERIALS AND METHOD

### Expression of recombinant $I_h$ channels and site-directed mutagenesis

For channel expression we used spHCN channel (SPIH, Gauss et al., 1998). We found that a point mutation in S4 region of spHCN (M349I) increased the expression as previously described (Shin et al., 2001). This mutation did not change qualitative features of wild-type inactivation in the absence of cAMP as well as voltage-dependent activation. Therefore, we used this modified spHCN as a wild-type channel. Human embryonic kidney 293 cells (HEK 293; Amer. Type Culture Collection, Rockville, MD, USA) were transiently transfected with expression plasmid containing spHCN cDNA (40 μg in 200 μl cell suspension) using electroporation. The channel expression plasmid was cotransfected with πH3-CD8 plasmid (Seed and Aruffo, 1987) that expresses the α subunit of the human CD8 lymphocyte antigen. Cells expressing the CD8 antigen were identified visually by decoration with antibody-coated beads (Jurman et al., 1994). Point mutations were introduced by PCR (Ausubel et al., 1996). The nucleotide sequences of the mutants were verified by sequencing.

### Solutions and electrophysiological recordings

All experiments were done with excised inside-out patches (Hamill et al., 1981) from transfected and identified cells 1-2 days after transfection. The methods for electrophysiological recordings and rapid perfusion switches have been described previously (Liu et al., 1997). Both internal and external solutions contained 160 mM KCl, 0.5 mM $MgCl_2$, 1 mM EGTA and 10 mM HEPES, pH 7.4. ZD7288 is known as a specific blocker for hyperpolarization-activated cation currents (BoSmith et al., 1993). ZD7288 (Tocris, Ballwin, MO, USA) was dissolved in distilled water to make a 20 mM stock solution, which was stored at −20°C. An aliquot was diluted into the internal solution to obtain the desired final concentration. Solution with $Cd^{2+}$ contained no EGTA. For experiments using 2,3-dimercapto-1-propanesulfonate (DMPS, Aldrich), the reagents was dissolved in internal solution within a few minutes before use.

## RESULTS AND DISCUSSION

In response to membrane hyperpolarization, the spHCN channel opens a gate at the intracellular entrance to the pore (Shin et al., 2001; Rothberg et al., 2002). In addition to the voltage-dependent gating, cAMP also modulates spHCN channel gating. Direct binding of cAMP to the CNBD in SpHCN channel removes inactivation, resulting in an 8-fold increase in steady state current level as shown in Fig. 1. In the present study, we attempted to gain insights into the molecular mechanism by which cAMP regulates the channel gating in spHCN channels.

We have previously shown evidence that the voltage-controlled intracellular gate is able to control $Cd^{2+}$ access between the intracellular solution and introduced cysteines in the pore, at position 464 (Rothberg et al., 2002). We found that access of $Cd^{2+}$ to 464C was also highly cAMP-dependent. As shown in Fig. 2A, $Cd^{2+}$ applied to intracellular side of the channel slowly inhibited the T464C mutant in the absence of cAMP, while $Cd^{2+}$ acted rapidly in the presence of cAMP. The $Cd^{2+}$ inhibition rate in the presence of cAMP was ~70 times higher than that in the absence of cAMP (Fig. 2B). Considering ~9.4-fold increase in the cAMP-induced conductance of M464C channels, the cAMP-induced difference in $Cd^{2+}$ inhibition rates appeared to be much higher than expected. Irreversible $Cd^{2+}$ binding to T464C appears to require more than 3 cysteines (Rothberg

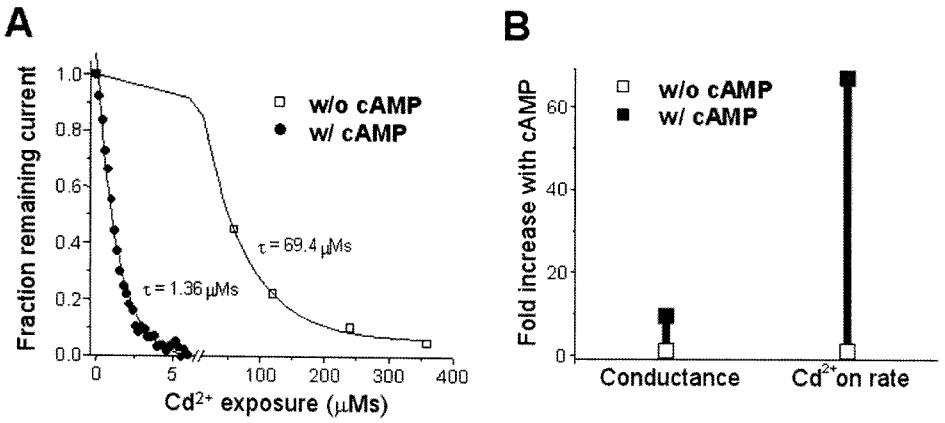


**Fig. 2.** cAMP-dependent $Cd^{2+}$ modification of 464C. (A) Time course of the normalized current decrease produced by repeated intracellular applications of $Cd^{2+}$ to 464C channels at −100 mV either in the presence or absence of cAMP. The lines are best fit to a mono-exponential function. (B) cAMP-dependence for the $Cd^{2+}$ modification rate of 464C channels. cAMP induced 9.35 ± 0.63-fold increase in steady state conductance of 464C channels. The rates were 1.11 ± 0.20 × $10^3$ $M^{-1}s^{-1}$ in the absence of cAMP and 7.45 ± 0.35 × $10^5$ $M^{-1}s^{-1}$ in the presence of cAMP.

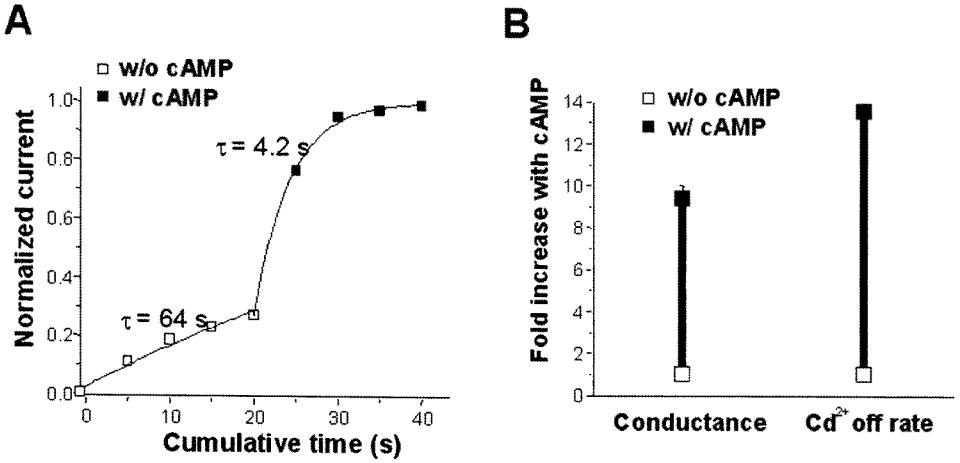


**Fig. 3.** cAMP dependence of recovery of $Cd^{2+}$-inhibited 464C channel with 1mM DMPS. (A) Time course of current recovery obtained by repeated applications of 1mM DMPS to $Cd^{2+}$-bound 464C channels from the intracellular side of the channel at −100 mV either in the presence or absence of cAMP. The lines are best fit to a mono-exponential function. (B) The recovery rates were calculated as shown in A. The $Cd^{2+}$ off rates with 1mM DMPS were 0.018 ± 0.003 $s^{-1}$ in the absence of cAMP and 0.25 ± 0.04 $s^{-1}$ in the presence of cAMP.

et al., 2002). Therefore, relative distance or position of four 464 cysteines as well as gated-access of $Cd^{2+}$ ion would determine the rate of irreversible $Cd^{2+}$ binding. The additional ~7 times difference in the rate of $Cd^{2+}$ block might reflect movement of lower S6 region upon cAMP binding. This movement might be able to locate the four 464 cysteines in right positions, thereby facilitating irreversible $Cd^{2+}$ binding.

Because $Cd^{2+}$ binding to 464C is too tight, $Cd^{2+}$ cannot be released by itself. Instead, its release could be achieved by applying the dithiol reagent DMPS to the hyperpolarized channel from the intracellular side (Liu et al., 1997 and Rothberg et al., 2002). As shown in Fig. 3, the current recovery of $Cd^{2+}$-blocked channels with 1mM DMPS occurred more slowly in the absence of cAMP than in the presence of cAMP. The cAMP-induced difference in the recovery rates appeared to be comparable to the cAMP-induced difference in conductance (Fig. 3B), which suggested that access of DMPS to the $Cd^{2+}$ binding site (464C) was also impeded by closure of an intracellular inactivation gate in the absence of cAMP.

The crystal structure of bacterial KcsA channel pore is thought to be similar to that of other P region-containing ion channels. KcsA structure reveals two plausible regions that act as a gate for permeant ions; glycine-tyrosine-glycine (GYG) containing selectivity filter, located extracellular to the central pore cavity, and a crossing bundle of pore-lining helices, forming the intracellular entrance to the cavity (Doyle et al., 1998). For example, the Shaker voltage-gated potassium channel appears to gate at the intracellular entrance of the pore. Metal ions such as $Cd^{2+}$ and $Ag^{+}$ as well as small MTS reagents applied from the intracellular

side can enter the pore only when the intracellular gate is open upon membrane depolarization (Liu et al., 1997; del Camino and Yellen, 2001).

Conformational changes in the outer mouth of the pore, including the selectivity filter, contribute to C-type inactivation of voltage-gated potassium channel (Liu et al., 1996). The selectivity filter has also been considered a gate in inward rectifier potassium channels and CNG channels (Lu et al., 2001; Becchetti and Roncaglia, 2000; Liu and Siegelbaum, 2000). In CNG channels, conformational changes of CNBD induced by cyclic nucleotide binding are allosterically coupled to opening conformational changes in the channel pore (Tibbs et al., 1997; Varnum and Zagotta, 1996). It has been proposed that movement of S6 helices is coupled to conformational changes in the selectivity filter (Flynn and Zagotta, 2001).

Although HCN channels are activated by membrane hyperpolarization and not by depolarization, they appear to have an intracellular voltage-dependent gate like Shaker voltage-gated potassium channel. We found that the blocker ZD7288 applied from intracellular side can enter and leave the pore only in open state at a hyperpolarized voltage (Shin et al., 2001). Voltage-dependent $Cd^{2+}$ access to one of the substituted cysteines in S6, T464C, further supports the existence of the voltage-dependent activation gate intracellular to this residue (Rothberg et al., 2002). Because the voltage sensor movements and the conformational changes induced by cyclic nucleotide binding may be quite different, it is not surprising that the different activation modalities between voltage-gated channel and CNG channel exert their effects on different parts of the pore.

HCN channels have similar structural elements to both Shaker voltage-gated potassium channel (six transmembane regions, strongly charged S4, GYG-containing selectivity filter) and CNG channel (six transmembane regions, CNBD). Thus, besides the voltage dependent gating, spHCN channels are also strongly gated by direct binding of cAMP to CNBD in C-terminal region of spHCN channel.

In mammalian HCN channel, it has been demonstrated that the C-terminal CNBD inhibits activation of the core transmembrane region and that cAMP binding relieves this inhibition (Wainger et al., 2001). We also found that the CNBD deletion removed the inactivation in spHCN channels (our unpublished observation). Therefore, spHCN channel might be an exaggerated version of mammalian HCN channels; in the absence of cAMP, CNBD strongly inhibits spHCN gating, which might produce observable inactivation. Binding of cAMP to CNBD might also relieve this strong inhibition, resulting in an ~8-fold increase of current. As a coupling mechanism between voltage gating and CNBD inhibition, Waigner et al. (2001) proposed that C linker between S6 region and CNBD may constrain movement of S6 helices, which is considered to form the voltage gate. In CNG channel, upon cyclic nucleotide binding to CNBD, S6 helices undergo a conformational change to enlarge the helix bundle, although it is not the activation gate for the permeant ions in CNG channel (Flynn and Zagotta, 2001).

Our present study demonstrates that both voltage and cAMP that control the gating process of spHCN channel appear to exert their effects on the intracellular entrance to the pore. So far, it is not clear whether voltage and cAMP use the same structure as a gate. Based on the observations in both HCN and CNG channels, however, we suggest that the binding of cAMP may stabilize the open conformation of the intracellular voltage-dependent gate in spHCN channels.

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