HCN channel gating models: a re-evaluation based on how the voltage-sensing and cAMP-sensing domains regulate kinetics

by
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Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

in the
Department of Molecular Biology and Biochemistry
Faculty of Science

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SIMON FRASER UNIVERSITY
Spring 2017

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Abstract

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels contribute to rhythmic oscillations in the heart and brain. Upon membrane hyperpolarization, HCN channel pore opening is coupled to inward movement of the S4 helix within the transmembrane voltage sensing domain (VSD, helices S1-S4). The gating pathway is proposed to include an initial voltage-dependent VSD movement step followed by a voltage-independent pore movement step (a cyclic allosteric mechanism). Various other mechanisms influence open state stability: A cytosolic cyclic nucleotide-binding (CNB) fold destabilizes the open state when unliganded (an autoinhibition mechanism), whereas binding of the phospholipid PIP$_2$ to the transmembrane domain stabilizes the open state. After pore opening, the channel undergoes a mode-shift, presumed to include lateral movement of S4 towards S2, forming a more stable open state. Despite the knowledge of open state stabilization mechanisms, it remains unclear how these mechanisms affect the kinetics of the gating pathway. Do these mechanisms apply equally strongly to channel thermodynamics and kinetics? Do they apply under a variety of cellular conditions? And do they regulate the VSD movement step, the pore movement step, or both? In this work I examined both the thermodynamics and kinetics of the activation and deactivation pathways in a variety of HCN channel derivatives. I used two-electrode voltage clamp to determine that while channel thermodynamics follow the predictions of the autoinhibition model, a channel with an unliganded CNB fold has faster activation than a channel with autoinhibition relieved by CNB fold deletion. I propose this fast activation is promoted by a “quickening conformation” of the intact CNB fold. The quickening conformation is independent of PIP$_2$ in both autoinhibited and autoinhibition-free channels. I used voltage clamp fluorometry to determine the speed of a VSD movement during channel deactivation in relation to pore closure. The speed of this VSD movement did not limit the rate of the deactivation pathway at strong depolarizations and showed stronger voltage dependence than pore closure. The speed of this VSD movement was independent of both cAMP binding and mode shift. Together my results clarify the HCN gating mechanisms of cyclic allostery, autoinhibition, PIP$_2$ potentiation and mode shift, and produce novel models of both HCN channel activation and deactivation.
Keywords: HCN channels; cyclic nucleotides; voltage sensor; voltage clamp; S4 helix; kinetics
Dedication

For Cathy, who never stopped trying to infect me with her relentless optimism.

For Danny, whom I could always, without a doubt, turn to.

For Adam, who never ran out of patience.
Acknowledgements

I must first thank my senior supervisor Dr. Edgar Young, without whom I’d probably still be wandering aimlessly around the Faculty of Arts and Social Sciences. Applying for a summer job in your lab quite literally changed the direction of my life. I will always be grateful for the effort you put into my education, and that I had a supervisor that so purposefully encouraged my independence and growth as a scientist.

Thank you to my committee members, Dr. Nancy Forde and Dr. Lynne Quarmby, for their valuable input on the direction and scope of my projects from day one. I also thank Dr. Peter Ruben and Dr. Peter Larsson for taking time out of their busy schedules to be my examiners.

I am grateful for the financial support that I received from SFU and the Natural Sciences and Engineering Research Council of Canada (NSERC). I thank NSERC for their Canadian Graduate Scholarship (Master’s) and Postgraduate Scholarship (Doctoral). I thank SFU for their Graduate Fellowship, Weyerhaeuser Molecular Biology Graduate Scholarship, David L. Baillie Graduate Fellowship in Molecular and Cellular Biology, Bruce Brandhorst Award, Frank A. Linville Graduate Scholarship, and the Provost Prize of Distinction.

My time in graduate school was made much more enjoyable by the company of other members of the Young laboratory. Thank you for your friendship and enjoyable discussions – both those focused on science and those that were anything but. A special thank you to Dana Page and Matthew Brolich, fellow graduate students that were both classmates and confidants on this journey.

Outside of the lab, I interacted with wonderful staff. Thank you to the MBB office staff for always being willing to answer my countless questions. Thank you to all the Student Learning Commons staff; working with you as an SLC graduate facilitator was one of my most enriching experiences at SFU.
For the work in Chapter 2, I thank Dr. Tom Claydon and Dana Page for commenting on the manuscript. For the work in Chapter 3, I am indebted to Claydon lab members May Cheng, Christina Hull, Samrat Thouta, and Patrick Shi for sharing their oocytes, training me on VCF, and assisting with VCF-related troubleshooting. This project also would never have started were it not for Dr. Tom Claydon giving his advice and equipment for my foray into fluorescence.

Finally, to my family, I would not have reached this point without your encouragement. Thank you from the bottom of my heart.
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List of Acronyms

cAMP        cyclic adenosine 3’,5’-monophosphate
CNB         Cyclic nucleotide binding
CNG         Cyclic nucleotide gated
Cryo-EM     Cryo-electron microscopy
CSD         Cyclic AMP-sensing domain
DD          Diastolic depolarization
DMSO        Dimethyl sulfoxide
EAG         Ether à go-go
EC50        Half maximal effective concentration
ECG         Electrocardiogram
EPSP        Excitatory post synaptic potential
HCN         Hyperpolarization-activated cation
hERG        Human ether-à-go-go-related gene
hHCN        Human HCN
LTP         Long term potentiation
LVNC        Left ventricular noncompaction cardiomyopathy
mHCN        Mouse HCN
MTS         Methanethiosulfonate
MTSET       Methanethiosulfonate-ethyltrimethylammonium
NMR         Nuclear magnetic resonance
OST         Open state trapping
PCR         Polymerase chain reaction
PD          Pore domain
PI          Phosphatidylinositol
PIP₂        Phosphatidylinositol 4,5-bisphosphate
QA          Quick-activating
SA          Sinoatrial
SD          Standard deviation
SIDS        Sudden infant death syndrome
spHCN1      Sea urchin HCN
SUDEP       Sudden unexpected death in epilepsy
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TEVC</td>
<td>Two electrode voltage clamp</td>
</tr>
<tr>
<td>VCF</td>
<td>Voltage clamp fluorometry</td>
</tr>
<tr>
<td>VSD</td>
<td>Voltage-sensing domain</td>
</tr>
<tr>
<td>XC</td>
<td>Extreme-C region</td>
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Chapter 1.

Introduction

1.1. HCN channel structure and function

Transport of substances across the plasma membrane is essential for cells to receive materials from, and return materials to, their outside environment. These material species include water, ions, amino acids and metabolites. Channels are transmembrane proteins that form a hydrophilic pore to allow passage of selected species. Some channels are constitutively open, while others can be closed by a channel gate and allow permeation only upon specific cellular signals. The hyperpolarization-activated cation (HCN) channel is expressed in humans and many other organisms, is permeable to $K^+$ and $Na^+$ ions upon membrane hyperpolarization, and is regulated by intracellular ligands. This makes them an excellent model to study how channel structure and function can be modulated by cellular signals. My thesis will address five outstanding questions (see section 1.3) surrounding the regulation of HCN channel activation and deactivation pathways by cellular signals.

Section 1.1 introduces elements of HCN channel structure and function that will be relevant to my thesis. This section includes an introduction to basic HCN channel structure and function (1.1.1), the physiological roles of HCN channels (1.1.2), a discussion of HCN channel structures related to voltage gating (1.1.3), and a discussion of HCN channel structures related to cyclic adenosine 3′,5′-monophosphate (cAMP) modulation (1.1.4).
1.1.1. Overview of HCN channels

1.1.1.1. Overview of HCN channel structure

HCN channels are composed of four subunits and include four mammalian subtypes. A functional HCN channel consists of four subunits assembled with a four-fold symmetry around a cylindrical pore. The four mammalian subtypes are called HCN1-HCN4. The sequences of the HCN1-HCN4 subtypes were identified in the late 1990s in mice (mHCN1-mHCN4) and humans (hHCN1-hHCN4) (for review see (Clapham, 1998)). HCN channels were also found in sea urchins (spHCN1) at this time. See Table 1.1 for a summary of relevant HCN channel subtypes for this thesis. Based on sequence homology, HCN channels are most closely related to cyclic nucleotide-gated (CNG) channels and ether-à-go-go (EAG) channels (Jackson et al., 2007; Craven and Zagotta, 2006). Although the four subtypes have a high degree of sequence similarity (80–90 % between all mammalian subtypes (Jackson et al., 2007)) they have separate expression patterns in the human body and contribute to different physiological roles. A functional tetrameric channel is typically composed of at least two different subtypes (heterotetrameric), further expanding functional and structural diversity (Tran et al., 2002; Chen et al., 2001; Ulens and Siegelbaum, 2003).

HCN channels include three key domains: the voltage-sensing, pore, and cAMP-sensing domains. Like CNG and EAG channels, each HCN channel subunit has a cytosolic N-terminal region, a transmembrane region containing six α-helices known as S1-S6, and a cytosolic C-terminal region. The transmembrane region of the channel includes the voltage-sensing domain (VSD, S1-S4) and pore domain (PD, S5-S6). The cytosolic C-terminal region contains a cyclic nucleotide binding (CNB) fold that forms a pocket for the binding of cyclic nucleotides, including cAMP (see section 1.1.1.3). The CNB fold is connected to the PD via a C-linker structure, and together the C-linker and the CNB fold form the cAMP-sensing domain (CSD). C-terminal to the CNB fold are several hundred amino acids that form a final extreme-C region. The transmembrane VSD and PD and the cytosolic CSD are structures that are investigated in this thesis.
Table 1.1. Summary of relevant HCN channel subtypes

<table>
<thead>
<tr>
<th>Species</th>
<th>Subtype</th>
<th>Discovery</th>
<th>Residues in a subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>HCN1</td>
<td>(Santoro et al., 1998)</td>
<td>890</td>
</tr>
<tr>
<td>Human</td>
<td>HCN2</td>
<td>(Santoro et al., 1998)</td>
<td>889</td>
</tr>
<tr>
<td>Human</td>
<td>HCN3</td>
<td>(Stieber et al., 2005)</td>
<td>774</td>
</tr>
<tr>
<td>Human</td>
<td>HCN4</td>
<td>(Ludwig et al., 1999)</td>
<td>1203</td>
</tr>
<tr>
<td>Mouse</td>
<td>HCN1</td>
<td>(Santoro et al., 1997)</td>
<td>910</td>
</tr>
<tr>
<td>Mouse</td>
<td>HCN2</td>
<td>(Santoro et al., 1998)</td>
<td>863</td>
</tr>
<tr>
<td>Mouse</td>
<td>HCN3</td>
<td>(Santoro et al., 1998)</td>
<td>779</td>
</tr>
<tr>
<td>Mouse</td>
<td>HCN4</td>
<td>(Santoro et al., 1998)</td>
<td>1186</td>
</tr>
<tr>
<td>Sea urchin</td>
<td>HCN1</td>
<td>(Gauss et al., 1998)</td>
<td>767</td>
</tr>
<tr>
<td>Sea urchin</td>
<td>HCN2</td>
<td>(Galindo et al., 2005)</td>
<td>638</td>
</tr>
</tbody>
</table>

Note: Subunit length retrieved from UniProt database (www.uniprot.org).

This thesis uses two types of schematics to represent the HCN channel molecule topology, as shown in Figure 1.1. Figure 1.1A shows a single HCN subunit, while Figure 1.1B shows two subunits out of the four that would form the full tetrameric HCN channel. In Figure 1.1A, each transmembrane helix is shown individually. In Figure 1.1B, the S5 and S6 helices are grouped together as a single unit that contributes to the PD. A key question related to basic HCN channel structure that is addressed through this thesis is how the different channel regions interact with one another to regulate channel gating.
1.1.1.2. Overview of HCN channel voltage-gating

A key model of HCN channel movement in my thesis is the cyclic allostery model, which proposes that HCN channel gating pathways include a VSD movement step and a PD movement step (Chen et al., 2007). Further features and evidence for this model are addressed in section 1.2.1. A VSD movement is the initial step in both the activation and deactivation pathways. The VSD movement step for activation is proposed to involve an inward movement of S4, and might be a composite of multiple substeps where S4 helices from individual subunits in the tetramer move one by one. Similarly, channel deactivation is a two-step process involving an initial movement of the VSD (outward movement of the S4 helix) followed by a movement of the PD to close the channel pore. The S4-S5 linker is involved in transducing the movements of S4 to the gate. Though the activation and deactivation pathways contain movements in the same channel regions, the channel does not undergo deactivation by simply progressing through the activation pathway in reverse. The cyclic allostery model thus uses four distinct steps to describe
HCN channel gating (Figure 1.2-1.3). A schematic of the cyclic allostery four-step model is shown with a comparison between the activation and deactivation pathways in Figure 1.2, and with a comparison of channel structures during these two pathways in Figure 1.3.

**Figure 1.2.** The cyclic allostery model for voltage-gating identifies two steps in the activation pathway and two steps in the deactivation pathway

Note: Four channel states are included in the cyclic allostery model. C = channel closed state; O = channel open state. S4\text{out} and S4\text{in} notations refer to the position of the S4 helix. During activation, the VSD changes conformation from a resting (S4\text{out}) to active (S4\text{in}) state, and then the pore opens. During deactivation, the VSD changes conformation from an active state (S4\text{in}) to the resting state (S4\text{out}), and then the pore closes. The deactivation pathway is distinct from the activation pathway, as a reversal of the activation pathway is not energetically favourable.
Figure 1.3. **The conformational changes in the HCN channel during the steps of the cyclic allostery model**

Note: Same state diagram as in Figure 1.2. Within the HCN channel, S4\textsubscript{out}-S4\textsubscript{in} transitions involve a voltage-dependent movement of S4, while closed-open transitions involve movement of the PD. The S4\textsubscript{out}-S4\textsubscript{in} and S4\textsubscript{in}-S4\textsubscript{out} transitions may include several substeps (shown by four arrows) to account for the movement of multiple S4s in the tetrameric channel. Only relevant regions of the HCN channel are shown; full channel schematic shown in Figure 1.1.

The cyclic allostery model forms the basis of my five main thesis questions (summarized in section 1.3). The cyclic allostery model predicts that accurately describing an HCN channel gating pathway will require describing multiple channel states and steps. Accurate descriptions will also require an understanding of the relationship between steps in a pathway: knowing which step occurs fastest is essential for identifying the rate-limiting step of the pathway. To what extent does the cyclic allostery model accurately describe HCN channel kinetics? Is the present understanding of the two steps accurate? Are the two steps always sufficient to describe the activation and deactivation pathways? Each of my five main questions tests the accuracy of the cyclic allostery model in a specific set of conditions.
1.1.1.3. Overview of HCN channel potentiation by ligands

The ability of channels to pass ions is described in terms of conductance (G): a channel population with many open pores would have a high conductance. The relationship between voltage and conductance for voltage-gated channels is described by a sigmoidal G-V curve with a \( V_{1/2} \) value. The G-V curve describes the relative conductance of a population of channels for a given voltage. The \( V_{1/2} \) value represents the voltage at which 50% of the maximum achievable open probability has been reached; \( V_{1/2} \) is also often referred to as the midpoint voltage of activation. In HCN channels, the \( V_{1/2} \) ranges from approximately -60 mV to -100 mV, and this range is tightly linked to the physiological importance of HCN channels (see section 1.1.2).

Factors that alter \( V_{1/2} \) are known as modulators of channel thermodynamics, as the \( V_{1/2} \) value indicates the relative stability of open and closed channels. For HCN channels, two important examples of modulators are cytosolic cAMP and the minority lipid phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)). Both these molecules depolarize the HCN channel \( V_{1/2} \) (termed potentiation), and allow the channel to open with weaker hyperpolarizations (DiFrancesco et al., 1986; DiFrancesco, 1986; DiFrancesco and Tortora, 1991; Pian et al., 2006). Models of cAMP regulation of HCN channels are described in section 1.2.2, while a model of PIP\(_2\) regulation of HCN channels is described in section 1.2.3. Broadly, the potentiation of HCN channels by cAMP and PIP\(_2\) raises many questions about the kinetic effects of each molecule on the steps of HCN channel gating pathways under various conditions. Specifically, for this thesis: are there conditions where the kinetics of the movement steps do not change in parallel with thermodynamics when the channel is potentiated by PIP\(_2\) or cAMP?

1.1.2. Physiological roles of HCN channels

The activation of HCN channels upon membrane hyperpolarization leads to a net influx of Na\(^+\) in physiological conditions. This Na\(^+\) influx leads to membrane depolarization. HCN channels have a \( V_{1/2} \) near the typical cell resting membrane potential, and thus their activation or deactivation can occur with only small deviations in voltage. The following section outlines several key physiological roles of HCN channels. These physiological roles underlie the importance of having detailed models of the HCN
channel activation and deactivation pathways. Channels open and close to regulate not only heart and brain function, but the resting properties of cells within many different organs. Channel opening and closing must be precisely timed and occur at precise voltages to allow for correct tissue function, particularly in the sinoatrial (SA) node of the heart and in neurons. An absence of HCN channels, or channels that do not open and close at the correct instances, can result in disease. My thesis addresses whether present models of HCN channel activation and deactivation accurately describe the kinetics of HCN channel conformational changes under a variety of physiologically relevant conditions.

1.1.2.1. HCN channels contribute to spontaneous cardiac pacemaker activity

In the mammalian heart, HCN channels carry the ionic current originally designated $I_f$ (funny) ([Brown et al., 1979]), or $I_q$ (queer) ([Halliwell and Adams, 1982]). All four HCN channel subtypes are present in the mammalian heart but the dominant subtype is HCN4, accounting for approximately 80% of total HCN channel subunits (Ishii et al., 1999; Moosmang et al., 2001; Fenske et al., 2011). Within the heart, HCN channels are primarily expressed in the nodes of the cardiac conduction system, which generate and sustain the consistent firing of individual cardiac action potentials to produce the rhythmic heartbeat.

The SA node sets the pace of the heartbeat and has the highest level throughout the heart of $I_f$ current density (Shi et al., 1999). The SA node resides above the right atrial chamber, and possesses a group of specialized “pacemaker” cells where the spontaneous action potentials originate (Mangoni and Nargeot, 2008). An action potential initiated by the SA node propagates through the heart and triggers a precisely-timed contraction of first the atria and then the ventricles. The SA node contains primarily HCN4 and HCN1 subtypes (Wahl-Schott et al., 2014; Herrmann et al., 2011). Due to the roles (described below) of HCN channels in sustaining electrical conduction in the SA node pacemaker cells, they are often called the “pacemaker channel” (DiFrancesco, 1986).
HCN channels contribute to the diastolic depolarization (DD) of the cardiac action potential, which regulates the amount of time between subsequent action potentials. The DD results from a net influx of positively charged ions into the pacemaker cell that depolarizes the membrane potential towards the threshold for action potential firing (reviewed in (Baruscotti et al., 2010)). A faster DD shortens the time between sequential action potentials, and thus results in a quicker heart rate. Several types of ion channels contribute to the DD, such as calcium channels I_{CaT} and I_{CaL} (Stieber et al., 2003), and sustained inward current I_{st} channels (Mitsuiye et al., 2000). However, HCN channels are often identified as the primary regulator of the DD, because compared to these other channels they open at more hyperpolarized voltages (Baruscotti et al., 2010). The DD is also significantly slowed – though not completely stopped - by the presence of Cs^+, which is a known HCN channel blocker (Denyer and Brown, 1990). HCN channels thus play a critical role in establishing the speed of the SA node DD.

Cyclic AMP potentiates I_f currents in SA node cells. Cyclic AMP binding results in a depolarization of the V_{1/2} and stabilization of the channel open state by speeding activation kinetics and slowing deactivation kinetics (DiFrancesco and Tortora, 1991; Bois et al., 1997; DiFrancesco et al., 1986). A less hyperpolarized HCN channel V_{1/2} results in a DD that begins at a weaker hyperpolarization. This type of DD would reach the threshold for the subsequent action potential quicker, resulting in a more rapid output of action potentials from the SA node. Thus β-adrenergic stimulation that increases cAMP concentrations stabilizes the HCN channel open state, and results in a faster heart rate (Bucchi et al., 2003). The importance of cAMP modulation of HCN channels in regulating heart rate can be shown through mouse models. HCN4-deficient mice have primitive hearts that beat slower than those of wildtype mice, and their the heart rates cannot be sped by cAMP (Stieber et al., 2003). They die during embryonic development (Stieber et al., 2003). Mice also have atypically slow heart rates that are not sped by β-adrenergic stimulation when HCN4 is present but unable to bind cAMP; these mice also die in utero (Harzheim et al., 2008). It is possible other subtypes cannot compensate for a lack of HCN4 due to the high prevalence of HCN4 in the heart. Thus HCN channels are essential for not only maintaining the speed of automaticity in pacemaker cells, but also the ability of the SA node to quicken the pace of spontaneous cardiac action potentials when directed by specific cellular signals.
1.1.2.2. HCN channels help establish key neuronal properties essential for sensory processing

HCN channels allow for proper integration of electrical inputs within neurons. As neuronal dendrites receive excitatory post synaptic potentials (EPSPs) from synapses all along their length, EPSPs need to be temporally and spatially summed to generate a single sensory output at the soma to travel down the axon. This process is called dendritic integration, and is reviewed in (Magee and Johnston, 2005). As EPSPs from a distal dendrite have further to travel to reach the soma, they have been exposed to greater membrane resistance (see review (Magee, 2000)). Thus once they reach the soma, EPSPs from a distal dendrite should generate smaller depolarizations relative to EPSPs from a proximal dendrite. However, as the dendritic membranes extend away from the soma, the concentration of HCN channels increases up to six-fold (Lőrincz et al., 2002; Williams and Stuart, 2000; Magee, 1999). As HCN channels can be open at the resting membrane potential, they can increase membrane conductance. HCN channels in distal dendrites thus help counteract the increased membrane resistance EPSPs will encounter as they travel to the soma. This means that all initial sensory inputs from a dendrite, regardless of their position along that dendrite, can equally effect the temporal summation of EPSPs at the soma and the resulting action potential.

HCN channels help limit neuronal long term potentiation (LTP) to avoid neuronal hyperexcitability. Like a muscle that strengthens with frequent exercise, a frequently used neuronal pathway will develop improved signal transmission. This synaptic plasticity-based process is called long term potentiation (LTP), and is an essential process in developing memories ((Bliss and Collingridge, 1993), reviewed in (Malenka and Bear, 2004; Fernandes and Carvalho, 2016)). Specifically, LTP results in strengthened synapses so that EPSPs can more effectively generate an action potential (Fan et al., 2005). LTP thus has the potential to render neurons overactive where they could generate unnecessary EPSPs, and thus this process must be carefully regulated. HCN1 channels inhibit LTP in the hippocampus (Nolan et al., 2004). Mice with a forebrain-specific HCN1 knockout had enhanced LTP, as shown by improved performance in spatial memory tasks (Tsay et al., 2007). Thus, when LTP increases neuronal excitation levels, HCN1 channels help limit this excitability, presumably to retain stability of the neuronal pathways (Fan et al., 2005).
HCN channels help neurons oscillate as a network, as reviewed in (Lewis and Chetkovich, 2011). Some distinct physiological states, such as sleep, require neurons to continuously undergo coordinated rhythmic oscillations. A particular property of non-rapid eye movement sleep is rhythmic “waves” of action potentials within the thalamus even without a sensory input (Lüthi and McCormick, 1998). Similar to its role in spontaneous action potentials in the heart, HCN channels in thalamocortical neurons open at hyperpolarized voltages and help depolarize the membrane potential toward the threshold for action potential firing (Kanyshkova et al., 2009). These pacemaker-like oscillations can become synchronized between different brain regions to create the electrical activity characteristic of different sleep stages.

1.1.2.3. HCN channels contribute to non-spontaneous electrical activity

Outside of the heart and the brain, HCN channels have roles within a variety of non-spontaneous cells. For example, they are expressed in the spinal cord (Milligan et al., 2006), the upper urinary tract (Hurtado et al., 2010; Klemm et al., 1999), photoreceptors (Demontis et al., 2002), and sour taste receptors (Stevens et al., 2001). HCN channels help stabilize the resting membrane potential of any cell in which they are expressed. Thus even in organs not typically associated with electrical oscillations, HCN channels contribute to proper cellular function.

1.1.2.4. HCN channels in disease

Mutant HCN channels have been found in human patients with several different types of cardiopathies and neuropathies, as shown in Table 1.2. All the mutations in Table 1.2 are in the HCN4 subtype, unless otherwise noted. HCN4 may be the subtype with the most known mutations as they often result in cardiac arrhythmias, and many arrhythmias can be found with a simple electrocardiogram. While all instances of HCN channel mutations in disease suggest the physiological importance of these channels, over half the mutations reside within the C-terminal region of the channel. This fact additionally suggests that proper structure of the C-terminal region, which contains the CSD, is essential for proper HCN channel gating.
### Table 1.2. Summary of select HCN channel mutations discovered in humans

<table>
<thead>
<tr>
<th>Patient conditions</th>
<th>Mutation</th>
<th>Functional Effect</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradycardia</td>
<td>G480R in PD</td>
<td>Negative shift in voltage dependence of activation</td>
<td>(Nof et al., 2007)</td>
</tr>
<tr>
<td>Bradycardia</td>
<td>A485V in PD</td>
<td>Negative shift in voltage dependence of activation</td>
<td>(Laish-Farkash et al., 2010)</td>
</tr>
<tr>
<td>Bradycardia</td>
<td>S672R in CNB fold</td>
<td>Negative shift in voltage dependence</td>
<td>(Milanesi et al., 2006)</td>
</tr>
<tr>
<td>Bradycardia</td>
<td>E695X in CNB fold</td>
<td>Insensitive to cAMP</td>
<td>(Schweizer et al., 2010)</td>
</tr>
<tr>
<td>Bradycardia; LVNC(^1)</td>
<td>A414G in S4-S5 linker</td>
<td>Negative shift in voltage dependence of activation</td>
<td>(Milano et al., 2014)</td>
</tr>
<tr>
<td>Bradycardia; LVNC</td>
<td>G482R in PD</td>
<td>Negative shift in voltage dependence of activation</td>
<td>(Milano et al., 2014)</td>
</tr>
<tr>
<td>Bradycardia; LVNC</td>
<td>T481H in PD</td>
<td>Negative shift in voltage dependence of activation</td>
<td>(Milano et al., 2014)</td>
</tr>
<tr>
<td>Bradycardia; Syncope</td>
<td>D553N in C-linker</td>
<td>Defective trafficking to membrane</td>
<td>(Ueda et al., 2004)</td>
</tr>
<tr>
<td>Brugada syndrome; atrial fibrillation; chronotropic incompetence</td>
<td>573X in CNB fold, mutant residues 544-572</td>
<td>Insensitive to cAMP</td>
<td>(Schulze-Bahr et al., 2003)</td>
</tr>
<tr>
<td>Brugada syndrome</td>
<td>447X in PD, mutant residues 404-446</td>
<td>Unknown</td>
<td>(Ueda et al., 2009)</td>
</tr>
<tr>
<td>Brugada syndrome</td>
<td>V492F in PD</td>
<td>Negative shift in voltage dependence of activation; slower activation</td>
<td>(Biel et al., 2016)</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>P257S in N-terminal region</td>
<td>Defective trafficking to membrane</td>
<td>(Macri et al., 2014)</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>K530N in C-linker</td>
<td>Negative shift in voltage dependence of activation; reduced C-linker oligomerization</td>
<td>(Duhme et al., 2013)</td>
</tr>
<tr>
<td>Atrioventricular block</td>
<td>G1097W in extreme-C region</td>
<td>Negative shift in voltage dependence of activation</td>
<td>(Zhou et al., 2014)</td>
</tr>
<tr>
<td>Idiopathic generalized epilepsy</td>
<td>R527Q in C-linker</td>
<td>Decreased slope of the conductance-voltage relationship</td>
<td>(Tang et al., 2008)</td>
</tr>
<tr>
<td>Idiopathic generalized epilepsy</td>
<td>A881T in extreme-C region</td>
<td>Unknown</td>
<td>(Tang et al., 2008)</td>
</tr>
<tr>
<td>SIDS(^2) at 4 months</td>
<td>A195V in N-terminal region</td>
<td>Unknown</td>
<td>(Evans et al., 2013)</td>
</tr>
<tr>
<td>SIDS at 5 weeks</td>
<td>V759I in extreme-C region</td>
<td>Unknown</td>
<td>Evans et al., 2013</td>
</tr>
<tr>
<td>Patient conditions</td>
<td>Mutation</td>
<td>Functional Effect</td>
<td>Author</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>---------------------------</td>
<td>----------------------------------------------------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>SUDEP3</td>
<td>F738C in extreme-C region</td>
<td>Unknown</td>
<td>(Tu et al., 2011)</td>
</tr>
<tr>
<td>SUDEP</td>
<td>P802S in extreme-C region</td>
<td>Unknown</td>
<td>(Tu et al., 2011)</td>
</tr>
<tr>
<td>SUDEP</td>
<td>G973R in extreme-C region</td>
<td>Unknown</td>
<td>(Tu et al., 2011)</td>
</tr>
<tr>
<td>Early infantile epileptic encephalopathy</td>
<td>S100F in N-terminal region</td>
<td>$V_{1/2}$ depolarized $\sim$27 mV; faster activation; slower deactivation</td>
<td>(Nava et al., 2014)</td>
</tr>
<tr>
<td>Early infantile epileptic encephalopathy</td>
<td>H279Y in S4-S5 linker</td>
<td>$V_{1/2}$ depolarized by $\sim$17 mV; slower deactivation</td>
<td>(Nava et al., 2014)</td>
</tr>
<tr>
<td>Early infantile epileptic encephalopathy</td>
<td>D401H in C-linker</td>
<td>$V_{1/2}$ depolarized $\sim$46 mV; faster activation; slower deactivation</td>
<td>(Nava et al., 2014)</td>
</tr>
</tbody>
</table>

1 LVNC = Left Ventricular Noncompaction Cardiomyopathy  
2 SIDS = Sudden Infant Death Syndrome  
3 SUDEP = Sudden Unexpected Death in Epilepsy  

Note: Table is sorted into cardiopathies (top) and neuropathies (bottom). Genetic variations within HCN channels with less severe physiological symptoms are further discussed in (Evans et al., 2013; Tu et al., 2011; Nava et al., 2014). All mutations are in HCN4 except HCN2 R527Q and HCN1 A881T (Tang et al., 2008); HCN2 F738C and HCN2 P802S (Tu et al., 2011); HCN1 S100F, HCN1 D401H, HCN1 H279Y (Nava et al., 2014). D553N mutation further investigated in (Netter et al., 2012); S672R mutation further investigated in (Xu et al., 2012); G482R further investigated in (Millat et al., 2015).

Knockout and mutation of HCN channels in adult mice have helped elucidate the physiological roles of individual HCN channel subtypes. With the HCN1 subtype, null mice were used in multiple studies to examine the roles of these subtypes in different mouse tissues. See Table 1.3 for a summary of key studies that found physical abnormalities or diseased states in HCN channel mouse models. Although it is perhaps unsurprising that a global knockout of a protein would have physiological consequences, even a single mutation within the HCN4 CNB fold can result in death of the mouse in utero, again highlighting the importance of proper CSD structure for proper HCN channel function (Harzheim et al., 2008).
Table 1.3. Summary of select HCN channel mouse models that produce disease phenotypes

<table>
<thead>
<tr>
<th>Type of mouse model</th>
<th>Description</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global HCN1 knockout</td>
<td>Mice had congenital SA node dysfunction with bradycardia, sinus dysrhythmia, and sinus pauses</td>
<td>(Fenske et al., 2013)</td>
</tr>
<tr>
<td>Global HCN1 knockout</td>
<td>Mice had lower seizure thresholds and increased dendritic excitability</td>
<td>(Huang et al., 2009)</td>
</tr>
<tr>
<td>Global HCN1 knockout; forebrain-specific HCN1 knockout</td>
<td>Both types of mice had higher seizure severity and increased seizure-induced mortality</td>
<td>(Santoro et al., 2010)</td>
</tr>
<tr>
<td>Global HCN2 knockout</td>
<td>Mice has absence seizures and cardiac sinus dysrhythmia</td>
<td>(Ludwig et al., 2003)</td>
</tr>
<tr>
<td>Spontaneous four base pair insertion in HCN2 CNB fold, resulting in inability of HCN channels to bind cAMP in the brain</td>
<td>Mice had ataxia, tremors, and absence seizures</td>
<td>(Chung et al., 2009)</td>
</tr>
<tr>
<td>Global HCN3 knockout</td>
<td>Mice had abnormally long T-wave amplitudes on ECGs</td>
<td>(Fenske et al., 2011)</td>
</tr>
<tr>
<td>Global HCN4 knockout</td>
<td>Mice died between embryonic days 9.5 and 11.5. Heart rate could not be accelerated by cAMP</td>
<td>(Stieber et al., 2003)</td>
</tr>
<tr>
<td>Global mHCN4 R669Q mutation which prevents cAMP binding</td>
<td>Homozygous mice die in utero. Heterozygous mice develop sinus pauses upon β-adrenergic stimulation</td>
<td>(Harzheim et al., 2008)</td>
</tr>
<tr>
<td>Tamoxifen-inducible HCN4 knockout</td>
<td>Mice had cardiac arrhythmias with recurrent sinus pauses</td>
<td>(Herrmann et al., 2007)</td>
</tr>
<tr>
<td>Tamoxifen-inducible and cardiac specific HCN4 knockout</td>
<td>Mice has severe bradycardia and atrioventricular block and died within 5 days</td>
<td>(Baruscotti et al., 2011)</td>
</tr>
</tbody>
</table>

Note: See (Herrmann et al., 2012) for a review of many of these studies.

1.1.3. Structures related to the voltage-gating of HCN channels

Homology to other voltage-gated channels suggests a model of the HCN channel transmembrane domain. Although there is currently no crystal structure of an HCN channel transmembrane region, crystal structures exist of the transmembrane region of the homologous voltage-gated K⁺ channel Kv1.2 ((Long et al., 2005), PDB 2A79), a Kv1.2/2.1 chimera channel ((Long et al., 2007), PDB 2R9R), and the bacterial voltage-gated K⁺ channel KvAP ((Jiang et al., 2003), PDB 1ORQ). These crystal structures,
together with HCN channel functional studies, give us a model of the secondary to quaternary structures of the HCN channel transmembrane region. The first four α-helices following the N-terminal region (S1-S4) form the VSD, while the last two (S5-S6) form the PD. These six α-helices are joined together by linkers, which are mostly loops. A notable exception is the S4-S5 linker which is presumed to be not a loop but an α-helix. There is also a pore helix between the S5-S6 linker and the S6 helix. A selectivity filter adjacent to the pore helix allows HCN channels to be selective for only $K^+$ and $Na^+$ ions.

The following section is divided into three parts: a discussion of the VSD S4 helix (1.1.3.1), a discussion of the VSD peripheral helices S1-S3 (1.1.3.2) and a discussion of the PD (1.1.3.3). Both the VSD and PD are discussed in terms of the conformational changes they undergo during HCN channel gating.

1.1.3.1. S4 is the HCN channel voltage sensor

Among vertebrate HCN channels, there is a high sequence similarity in the VSD region (Jackson et al., 2007), and all mammalian HCN channel subtypes have the same S4 primary structure. The S4 helix has nine or ten positively charged amino acids (depending on the presumed length of the S4 helix: this thesis assumes all ten). A basic residue occurs every third residue in S4, with the exception of a central serine that disrupts this pattern (Figure 1.4).

Figure 1.4 also describes the notation of the S4 residue positions that are used throughout this thesis. I refer to S4 residues using a negX/posX notation for easy comparison across subtypes. I selected a conserved lysine near the centre of the helix to be residue “zero” (spHCN1 K335; mHCN1 K250; mHCN2 K303). Any residue N-terminal to Kzero will have a “neg” notation, while any residue C-terminal to Kzero will have a “pos” notation. The positively charged residues on S4 thus span from residues numbers neg12 to pos18. Kzero is suggested to be near the interface of the extracellular leaflet and aqueous environment (see Figure 1.7); Kzero thus may also represent the transition from residues in the N-terminal region of S4 to S4 residues buried in the membrane (Bell et al., 2004).
Charged residues within the transmembrane domain play a critical role in causing voltage-based conformational changes in voltage-gated channels (see paragraph below). S4 is widely accepted and referred to as the primary HCN channel voltage sensor in relation to other parts of the VSD. The following paragraphs summarize the experiments and results that suggest S4 is the primary voltage-sensing structure in HCN channels.

Figure 1.4. Alignment of S4 and relative position of S4 positive charges across subtypes

Note: Alignment of mouse and sea urchin HCN channel S4 regions. Relevant positions are bolded and coloured by residue. The neg/pos identification below the alignment will be used throughout this thesis to make discussing S4 simpler when comparing different subtypes. The pos18 histidine residue is not always included in the S4 (it may be within the S4-S4 linker) depending on the study, but I have included it in S4 in this thesis. There are 3.4 residues per helical turn, suggesting all the basic residues do not align on one side of the helix. The residues shown are completely conserved between mHCN1-mHCN4 and also hHCN1-hHCN4. XC = extreme-C region.

Proteins sense voltage due to the presence of charged residues crossing the membrane. VSDs in voltage-gated channels allow the protein to act as a switch that can turn on or off due to the difference of a few millivolts. VSDs exist in traditional voltage-gated ion channels, channels without pore domains (Ramsey et al., 2006; Sasaki et al., 2006), and a voltage-sensing intracellular enzyme (Murata et al., 2005; Liu et al., 2012). VSDs register changes in membrane potential through at least one charged amino acid within the plasma membrane. Upon membrane depolarization, a positively charged residue will move outward while a negatively charged residue will move inward. A charged VSD residue whose movement leads to opening of the channel gate is known as a gating charge. Movements of gating charges are associated with an increase or
decrease in protein activity, rendering the protein’s function voltage-dependent. The strength, or steepness, of this voltage dependence is correlated to the number of gating charges that move across the membrane: a greater number of charges produces stronger voltage dependence. A strong voltage dependence of channel activation results in a steep G-V relationship. Together $V_{1/2}$ and G-V steepness represent parameters that will describe the dependence of HCN channel activation on voltage throughout this thesis.

Mutating basic S4 residues of HCN channels disrupts channel gating. The Sanguinetti group performed a glutamine scan of the basic residues of S4 and Spos3 in mHCN2 (Chen et al., 2000). Mutation of the four N-terminal basic residues (Kneg12, Rneg9, Rneg6, Rneg3) hyperpolarized channel $V_{1/2}$. Mutation of Kzero substantially hyperpolarized the $V_{1/2}$ to the extent that they could not study the channel, and mutations in Spos3, Rpos6, Rpos12 and Rpos15 caused loss of channel function. Mutation of Rpos9 substantially reduced HCN channel surface expression. These results led the authors to suggest that the four most N-terminal basic residues are outside the membrane, while residues Kzero to Rpos15 are embedded in the membrane. These results were similar to those of Vaca (Vaca et al., 2000), who found that neutralizing Kneg12, Rneg9, Rneg6, and Rneg3 led to large shifts in channel voltage dependence but neutralization of charged residues Kzero to Hpos18 caused loss of channel function. Overall this data suggests that the HCN channel S4 has an extended N-terminal region and the positively charged residues within the central and C-terminal regions of the helix are the gating charges and are required for proper channel function. The N-terminal region of S4 still contributes to the voltage dependence of the channel, and thus understanding the structure and conformational changes within this region would advance the understanding of HCN channel gating.

Although most voltage-gated potassium channels such as Kv channels are depolarization-activated rather than hyperpolarization-activated, the S4 helix of both types of channels moves inward upon hyperpolarization and outward upon depolarization. The Larsson research group measured accessibility of introduced cysteines to internally and externally applied methanethiosulfonate- (MTS) ethyltrimethylammonium at various spHCN1 S4 positions (Männikkö et al., 2002). They
found that spHCN1 Spos3C was accessible to internal reagent at a hyperpolarized voltage but accessible to external reagent at a depolarized voltage. This suggests that this residue crosses the length of the membrane during gating, specifically moving inward upon hyperpolarization and outward upon depolarization (Figure 1.5). The authors thus predict that it is the electromechanical coupling between the voltage sensor and the gate that is reversed between the two homologous types of channels. This study also found that after replacing the two most N-terminal arginines (Rneg9 and Rneg6) with cysteines, these residues were modified by external reagent at both hyperpolarized and depolarized voltages. This confirms that the N-terminal region of S4 protrudes into the extracellular space outside the membrane at all voltages.
Figure 1.5. S4 movements are conserved between spHCN1 channels and Shaker channels

Note: A: Schematic showing implied transmembrane movement of S4 in spHCN1 channels. Coloured circles represent individual residues as indicated on right side. Charged S4 residues pos12 and pos15 and pos18 are not shown. B: Schematic showing that inward S4 movement opens HCN channels, but closes Shaker channels. This implies there is a different coupling mechanism between S4 and the activation gate in HCN channels and Kv channels. Adapted from (Männikkö et al., 2002) with permission.

The Larsson and Siegelbaum groups followed this report with further cysteine accessibility studies in mHCN1 channels. Interestingly, the studies produced different
models of mHCN1 S4 movements. The Larsson group found that residue Spos3 crossed the membrane completely, as it was accessible to an internal MTS reagent upon hyperpolarization and an external methanethiosulfonate (MTS) reagent upon depolarization (Vemana et al., 2004) (Figure 1.6). The Siegelbaum group found that S4 N-terminal residues (up to and including Rneg3) were accessible to an external MTS reagent regardless of the voltage (Bell et al., 2004) (Figure 1.7). The C-terminal residues of mHCN1 S4 (Spos3 to Rneg15) were inaccessible at a depolarized voltage but accessible to an internal MTS reagent at a hyperpolarized voltage. They did not find any residues that crossed the membrane completely. The Siegelbaum group results suggested that the N-terminal region of S4 does not move much during voltage-gating, while the C-terminal region of S4 undergoes substantial changes in environment during voltage-gating. These two groups used different background channels, which might explain their different results. The Larsson group mHCN1 channel retained a C-linker, while the Siegelbaum group mHCN1 channel lacked the C-linker. They also tested accessibility of intracellular residues using slightly different MTS reagents (methanethiosulfonate-ethyltrimethylammonium (MTSET) for the Siegelbaum group and methanethiosulfonate-ethylammonium for the Larsson group). One model that the Siegelbaum group put forth to explain these results was a movement of the C-terminal region of S4 into and out of the membrane during voltage-gating (Bell et al., 2004) (Figure 1.8). This model is termed a "swinging tail" model. The swinging of the C-terminal region of S4 into an accessible region is coupled to pore opening, while the swinging of S4 into an inaccessible region is coupled to pore closing. The extent of HCN channel S4 movements during gating are thus still disputed.
Figure 1.6.   The Larsson group found Spos3 crosses the entire membrane during gating

Note:   Schematic showing implied transmembrane movement of S4 in mHCN1. Coloured circles represent individual residues as indicated on right side. Adapted from (Vemana et al., 2004) with permission.
Figure 1.7. The Siegelbaum group found the N-terminal region of S4 does not move relative to the membrane during gating

Note: Schematic summarizing the reactivity of S4 residues when substituted for cysteines and modified by internal or external MTSET. Alpha carbons of the cysteine substitutions are blue when modified by external MTSET and red when modified by internal MTSET. The gray areas delineate the region of the S4 helix that is protein and/or lipid buried. Positively charged residues are highlighted by plus symbols: black and white plus symbols indicate buried or accessible residues, respectively. The image on the right includes the neg/pos notation used throughout this thesis. Black underlined residues in this image were mutated to cysteines and tested for accessibility to MTSET. Grey residues in this image were not tested but are relevant residues in this thesis. Adapted from (Bell et al., 2004) with permission.
The Siegelbaum group proposes a swinging-tail model to account for the stationary N-terminal region of S4 during gating movements. Note: Schematic of the swinging-tail model. The C-terminal tail of S4 is buried within the lipid or protein interface (gray area) in the closed state and swings into an aqueous environment in the open state. Listed residues are from mHCN1. Coloured circles represent individual residues as indicated on right side. Adapted from (Bell et al., 2004) with permission.

1.1.3.2. S1-S3 helices contribute to HCN channel voltage-gating

The S1-S3 helices of HCN channels also have a role in voltage-sensing and voltage-gating. HCN channels have two D residues in S2 (mHCN2 D225 and D231) and two D residues in S3 (mHCN2 D267 and D275) that are conserved with four D residues in hERG and Shaker channels (Chen et al., 2000). The results of the original glutamine scan of S4 suggested that Rpos6, Rpos12, and Rpos15 may electrostatically pair with
acidic residues on S2 and S3 as mutating the acidic S2 and S3 residues resulted in loss of channel function (Chen et al., 2000). S1 helps determine HCN channel activation speed, as introducing an HCN4 S1 into an otherwise HCN1 channel slowed activation (Ishii et al., 2001). A tryptophan scan of the S1 helix found four S1 residues where the W mutation caused loss of channel function (Ishii et al., 2007). Two residues in S1 have also been suggested to face S4 and interact with it (Ishii et al., 2007). These results suggest that interactions between the primary voltage-sensing helix and the remainder of the VSD are essential for proper HCN channel gating. These results highlight the importance of understanding the movements of not only S4 but also the peripheral VSD helices during HCN channel activation and deactivation.

The paddle model put forth by the MacKinnon research group places an additional importance on the S3 helix (Jiang et al., 2003). A crystal structure of the bacterial voltage-gated K⁺ channel KvAP showed that the S3 helix is divided in the middle by a small loop into S3a (the intracellular portion) and S3b (the extracellular portion). S3b and the N-terminal region of S4 are packed tightly together and are proposed to move as a single unit called a “paddle”. Though no other crystal structures have shown such a distinctive division of S3, this model broadly suggests that the N-terminal region of S4 may be of particular importance: the environment of S3b, the S3-S4 linker, and the extracellular portion of S4 (charged residues Kneg12 to Kzero) would all be highly relevant in voltage-gating.

An occluded binding site formed by S2 and S3 is proposed to shield S4 charges in the membrane (Tao et al., 2010). This addresses the problem of how the positive charges can be stable while moving through the hydrophobic membrane. The occluded binding site was identified in the Kv2.1/1.2 chimera channel but is suggested to be conserved within potassium channel VSDs (Tao et al., 2010). The binding site involves an E and an F residue in S2 and a D residue in S3. At any given time, an S4 gating charge is positioned within this occluded binding site. When S4 moves, the gating charges each pass successively through this binding site. The occluded binding site thus may serve as a catalytic centre to lower the energy associated with transferring each HCN S4 charge through the membrane. The possibility of an HCN channel occluded binding site raises many questions about S4 gating movements. Do all the gating charge
movements through the binding site have similar speeds? Does the movement of each gating charge represent its own pathway step? The presence of the occluded binding site suggests more than two steps may be needed to fully describe a gating pathway.

1.1.3.3. The C-terminal portion of S6 forms a HCN channel gate

The C-terminal ends of the S6 helices in the HCN channel tetramer form a bundle which acts as intracellular gate (Rothberg et al., 2002; Flynn and Zagotta, 2003; Shin et al., 2001)(Figure 1.9). This contrasts with the narrow selectivity filter which is at the extracellular end of the pore. The HCN channel selectivity filter sequence of C-I-GYG is similar to the sequence of T/S-V/I/L/-GYG found in Kv channels (Macri et al., 2012). However, homology modeling with KcsA channels (Zhou et al., 2001), KirBac1.1 channels (Kuo et al., 2003) and MthK channels (Jiang et al., 2002) suggest relatively greater flexibility of the HCN selectivity filter because of a reduced number of hydrogen bonds may be the reason that HCN channels are able to pass both Na⁺ and K⁺ (Giorgetti et al., 2005). The following paragraph will summarize the evidence for an intracellular gate. The intracellular positioning of the gate suggests the conformations of the adjacent C-linker and CNB fold could easily modulate the opening and closing of the HCN channel pore.
By using specific HCN blocker compound ZD7288 (Shin et al., 2001) and general blocker Cd$^{2+}$ (Rothberg et al., 2002), the Yellen group showed that the HCN channel gate resides on the intracellular and not extracellular side of the pore. When a blocker was applied to the intracellular side of the channel it entered the pore only after a hyperpolarizing voltage opened the gate. Were the gate on the extracellular side of the pore, the blocker should have been able to enter the intracellular end of the pore regardless of the voltage. Upon subsequent depolarization, the blocker became trapped in the channel pore. The blocker was presumably prevented from leaving the pore on the extracellular side by the narrow selectivity filter, and prevented from leaving the pore on the intracellular side by the closed gate. By introducing cysteine mutations in the C-terminal region of S6 that presumably lined the central pore, the authors also identified residues predicted to be within the gate (Shin et al., 2001). The intracellular portion of S6 is thus a region of focus for modulation of HCN channel gating.
1.1.4. Structures related to the cAMP-modulation of HCN channels

The C-terminal region of HCN channels includes sequentially a C-linker, a cyclic nucleotide binding (CNB) fold, and an extreme-C region. The C-linker is a unique structure to HCN channels and their close homologs, the CNG and KCNH channel families (Kv10.1, Kv10.2 and Kv11.1) (Puljung et al., 2014). The CNB fold is an ancient and highly conserved structure common in many types of proteins (for review see (Berman et al., 2005)). Interestingly, the sequences amongst different CNB folds are quite varied, but the tertiary structures are strongly conserved (Jackson et al., 2007; Berman et al., 2005). Cyclic AMP and cGMP (Zagotta et al., 2003), and cCMP (Zong et al., 2012) have all been shown to bind to HCN channels, but this thesis will focus on cAMP as HCN channels are selective for cAMP over cGMP, and cCMP is only a partial HCN channel agonist (Zhou and Siegelbaum, 2007; Zong et al., 2012).

The first crystal structure of an HCN channel CSD (C-linker and CNB fold) was solved in 2003 by the Zagotta group (Zagotta et al., 2003) (Figure 1.10). Today a variety of CSD structures have been solved for several HCN channel subtypes (Table 1.4). The C-linker is composed of six α-helices (αA’ to αF’). Of note, the αA’ helix is presumed to be nearly parallel to the intracellular leaflet of the membrane and the S4-S5 linker, allowing for the possibility of protein-protein and protein-lipid interaction sites. The CNB fold has four α-helices (αA, αP, αB, αC) and a β-roll. The β-roll forms a jelly-roll motif, where eight antiparallel β-strands form a barrel-like shape. Cyclic AMP was inside the binding pocket formed by the β-roll and the distal αC helix in the crystal structure, and interacted with residues within the β-roll, within the αC helix, and also in the loop between them. A CSD with no cAMP bound to the CNB fold is in the apo (unliganded) state, while a CSD with cAMP bound to the CNB fold is in the holo (liganded) state. This information forms the basis of my thesis questions that concern the conformational changes that occur in the HCN channel upon cAMP binding.
Figure 1.10. Crystal structure of the mouse HCN2 CSD in the holo state

Note: A: Ribbon representation of the crystal structure of the mHCN2 CSD (residues 443-645) with cAMP. Cyclic AMP is shown bound inside the β-roll. Adapted from (Zagotta et al., 2003) with permission. B: The S4–S6 region of one subunit of Kv1.2 ((Long et al., 2005), PDB 2A79) is shown (residues 288–416), with the S4-S5 linker coloured pink and the S5 and pore helix coloured blue. Three subunits of the HCN2 C-linker and CNB fold as in A are shown, with one subunit coloured green. The relative position of the PD and CNB fold is not known. Gold star represents the approximate position of residue R591, which is mutated in this thesis. Adapted from (Prole and Yellen, 2006) with permission.
Table 1.4. Crystal structures of the HCN channel CSD

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Mutation</th>
<th>Ligand</th>
<th>Author, PDB ID</th>
<th>Type of Structure</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>mHCN1</td>
<td>None</td>
<td>cAMP</td>
<td>(1), 3U0Z</td>
<td>X-ray Diffraction, 2.9 Å</td>
<td></td>
</tr>
<tr>
<td>mHCN2</td>
<td>None</td>
<td>None</td>
<td>(2), 5JON</td>
<td>X-ray Diffraction, 2.04 Å</td>
<td>Only includes C-linker helices αD’, αE’, and αF’. Part of a fusion protein with the maltose binding protein.</td>
</tr>
<tr>
<td>mHCN2</td>
<td>C508N, C584S, C601S</td>
<td>None</td>
<td>(3), 1Q43</td>
<td>X-ray Diffraction, 2.0 Å</td>
<td></td>
</tr>
<tr>
<td>mHCN2</td>
<td>E502K</td>
<td>cAMP</td>
<td>(3), 1Q50</td>
<td>X-ray Diffraction, 2.3 Å</td>
<td></td>
</tr>
<tr>
<td>mHCN2</td>
<td>I636D</td>
<td>cGMP</td>
<td>(3), 1Q3E</td>
<td>X-ray Diffraction, 1.9 Å</td>
<td></td>
</tr>
<tr>
<td>hHCN2</td>
<td>None</td>
<td>cAMP</td>
<td>(4), 3ETQ</td>
<td>X-ray Diffraction, 1.9 Å</td>
<td></td>
</tr>
<tr>
<td>hHCN2</td>
<td>None</td>
<td>None</td>
<td>(7), 2MPF</td>
<td>Solution NMR</td>
<td>Only includes C-linker helices αD’, αE’, αF’</td>
</tr>
<tr>
<td>hHCN2</td>
<td>None</td>
<td>cAMP</td>
<td>(1), 3U10</td>
<td>X-ray Diffraction, 2.3 Å</td>
<td></td>
</tr>
<tr>
<td>hHCN4</td>
<td>None</td>
<td>None</td>
<td>(8), 2MNG</td>
<td>Solution NMR</td>
<td>Only includes C-linker helices αC’, αD’, αE’, αF’</td>
</tr>
<tr>
<td>hHCN4</td>
<td>None</td>
<td>cAMP</td>
<td>(9), 3OTF</td>
<td>X-ray Diffraction, 2.4 Å</td>
<td></td>
</tr>
<tr>
<td>hHCN4</td>
<td>None</td>
<td>cAMP</td>
<td>(6), 3U11</td>
<td>X-ray Diffraction, 2.5 Å</td>
<td></td>
</tr>
<tr>
<td>hHCN4</td>
<td>None</td>
<td>7-CH-cAMP</td>
<td>(10), 4NVP</td>
<td>X-ray Diffraction, 2.5 Å</td>
<td>7-CH-cAMP has a replacement of the heterocyclic nitrogen at position 7 to a CH-moiety</td>
</tr>
<tr>
<td>hHCN4</td>
<td>None</td>
<td>cGMP</td>
<td>(11), 4KL1</td>
<td>X-ray Diffraction, 2.7 Å</td>
<td></td>
</tr>
<tr>
<td>spHCN1</td>
<td>None</td>
<td>cAMP</td>
<td>(6), 2PTM</td>
<td>X-ray Diffraction, 1.93 Å</td>
<td></td>
</tr>
</tbody>
</table>

(1) (Lolicato et al., 2011); (2) (Goldschen-Ohm et al., 2016); (3) (Zagotta et al., 2003); (4) (Taraska et al., 2009); (5) (Craven et al., 2008); (6) (Flynn et al., 2007); (7) (Saponaro et al., 2014); (8) (Akimoto et al., 2014); (9) (Xu et al., 2010); (10) (Möller et al., 2014); (11) (Lolicato et al., 2014).

Note: Accession numbers and crystal structure type were taken from the Protein Data Bank (www.rcsb.org). NMR = nuclear magnetic resonance.

Mutation studies identified which of the seven mHCN2 residues that interact with cAMP impart ligand selectivity, efficacy, or affinity (Zhou 2007). These seven residues are listed below in Table 1.5. Of particular note is the residue HCN2 R591, as the mutation HCN2 R591E is used throughout this thesis to prevent cAMP binding and study HCN channel structure in the apo state.
Table 1.5. Summary of residues relevant for cAMP binding in the mHCN2 CNB fold

<table>
<thead>
<tr>
<th>Residue</th>
<th>Location</th>
<th>Description</th>
<th>Mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>E582</td>
<td>β-roll</td>
<td>Forms a salt bridge with αC helix residue R632, which helps stabilize the ligand within the binding pocket</td>
<td>E582A: lower affinity for both cAMP and cGMP.</td>
</tr>
<tr>
<td>R591</td>
<td>Loop between β-roll and αC helix</td>
<td>Forms an electrostatic interaction with cAMP, potentially through a ionic bond formed with the cyclic phosphate, which contributes to high affinity binding</td>
<td>R591E: elimination of all ligand based effects R591A: mutation reduces the affinity of the HCN channel to cAMP</td>
</tr>
<tr>
<td>T592A</td>
<td>Loop between β-roll and αC helix</td>
<td>Forms three hydrogen bonds with cGMP and two with cAMP</td>
<td>T592A: loses selectivity, more so for cGMP than cAMP</td>
</tr>
<tr>
<td>R632</td>
<td>αC helix</td>
<td>Forms ionic interactions with the ligand to help stabilize the position of the helix αC as the innermost cap of the binding pocket. Essential for high efficacy</td>
<td>R632A: still binds cAMP, but cAMP can no longer potentiate channel activation</td>
</tr>
<tr>
<td>R635/ I636/ K638</td>
<td>αC-helix</td>
<td>Contributes to cAMP selectivity</td>
<td>I636A: 15-fold loss in cAMP selectivity I636D: prefers cGMP over cAMP 635A or 638A: 6-8 fold loss in cAMP selectivity</td>
</tr>
</tbody>
</table>

Note: Table formed from results of (Zhou and Siegelbaum, 2007). Residue numbers are from mHCN2.

The first HCN channel crystal structure showed that C-linker regions in adjacent subunits interact with one another to form a tetrameric gating ring (Zagotta et al., 2003). In fact, all the intersubunit interactions arose in the C-linker; the CNB folds of each subunit were surprisingly independent. Specifically, C-linkers form “elbow-shoulder” interactions, where the αA’ and αB’ helices of one subunit generate an antiparallel helix–turn–helix motif which interacts with the αC’ and αD’ helices of the adjacent subunit (Zagotta et al., 2003) (Figure 1.11). Both intrasubunit and intersubunit salt bridges between the αB’ and αD’ helices stabilize the closed state of the channel, but are proposed to be broken upon the rearrangement of the quarternary structure as these helices move farther apart upon ligand binding (Craven and Zagotta, 2004; Ulens and Siegelbaum, 2003; Zhou et al., 2004). Thus it is not just the CNB fold within the CSD that can regulate HCN channel gating, but the C-linker as well.
Figure 1.11. Cyclic AMP binding forms a tetrameric gating ring in the C-linker that shifts the equilibrium towards the open state

Note: Schematic of an unliganded closed channel (left) and a liganded open channel (right). Lower cubes represent the C-linker and CNB fold. Cyclic AMP (green circles) binding to the CNB fold induces a significant rearrangement of the tertiary structure of the elbow–shoulder interactions and hence the quaternary structure of the whole C-terminal region. This conformational change is coupled to pore opening. The elbow-shoulder term arises as the C-linker looks to have an elbow (αC’ and αD’) of one subunit resting on the shoulder (αA’ and αB’) of its neighbour. Adapted from (Zagotta et al., 2003) with permission.

A key conformational change in the CNB fold upon cAMP binding is the outward movement of the helix αC. The apo channel CSD was presumed to be substantially different from that of the holo channel. Surprisingly, the first apo CSD crystal structure (Taraska et al., 2009) showed few differences between apo and holo channels. However, this crystal structure also contained two bromide ions within the CNB fold, and thus may not represent entirely the physiological structures of the apo channel. Further studies using transitional metal ion fluorescence resonance energy transfer (Taraska et al., 2009) and double electron electron resonance methods (Puljung et al., 2014) suggest several ångströms of movement in the proximal end of the αC helix upon ligand binding. The mHCN2 CSD showed up to 10 Å of movements of the αC helix towards the β-roll when cAMP binds, and a rotation of the αC helix which would bring multiple residues into contact with the cAMP purine ring (Puljung et al., 2014). These αC helix
movements are presumed to transduce through the CNB fold and αF’-αC’ helices to result in movements of the αA’ and αB’ helices in the adjacent subunit C-linker (Puljung et al., 2014). The outward movements and tilting movements of the αC helix upon cAMP binding thus may act as a trigger that both further stabilizes cAMP in the binding pocket, and initiates the conformational changes that form the tetrameric gating ring. If the conformational changes induced by cAMP binding begin in the αC helix and transduce all the way to the αA’ and αB’ helices of the C-linker, it is plausible they transduce into further regions, such as the VSD.

1.2. Models of HCN channel gating

This section presents four models that describe different aspects of HCN channel gating. These models are independent of one another, and may all simultaneously contribute to regulation of HCN channel gating. The applicability of these models under different conditions will be tested throughout my thesis. This section also introduces the five key questions of my thesis, as these questions relate to unclear elements of the models discussed below. This section has one part for each model: the cyclic allostery model (1.2.1; Questions #3 to #5), the autoinhibition model (1.2.2.; Questions #1 and #5), the PIP$_2$ potentiation model (1.2.3; Question #2), and the mode shift model (1.2.4; Question #5).

1.2.1. The cyclic allostery model

The cyclic allostery model proposes an inherently voltage-independent closed-open step that is regulated by both ligand binding and VSD movement. Physiologically, the presence of a voltage-independent HCN channel gate-opening step may help limit the negative effects of extreme hyperpolarizations (Chen et al., 2007). For example, an extreme hyperpolarization in the SA node might encourage such rapid HCN channel activation that an arrhythmia results, but a voltage-independent step would impose a limit on HCN channel opening speed.
The Yellen group showed that movement of the VSD is still possible with a immobile PD, which supports the proposal of weak coupling between the VSD and PD (Ryu and Yellen, 2012). This group introduced cysteine mutations to spHCN1 channels that caused the channel to be locked in either the open or closed state upon intracellular Cd\(^{2+}\) application. They then measured the gating currents (the rate of movement of the gating charge) of these channels to determine how S4 moves in locked-open or locked-closed channels. Locking the spHCN1 channels open depolarized the gating current \(V_{1/2}\), while locking the spHCN1 channels closed hyperpolarized the gating current \(V_{1/2}\). In neither case were gating currents blocked, indicating S4 was still able to move. An S4 that can move independently of a fixed pore supports an allosteric coupling model where the inward movement of each S4 increases the likelihood of pore opening (Ryu and Yellen, 2012). Allosteric coupling between the VSD and PD of HCN channels thus may allow cAMP to regulate the conformational changes of the VSD and the PD independently.

The closed-open step is proposed to include movement of the pore and follow the VSD movement step. As with any step, there could theoretically be conditions where the voltage-independent pore movement step is the rate-limiting step of an HCN channel gating pathway. The Siegelbaum research group found that mHCN2 channels had a maximum opening speed at strong hyperpolarizations (Chen et al., 2007). This suggested that a voltage-dependent step in the pathway was rate-limiting at weak hyperpolarizations (less negative than \(-150\) mV), but at strong hyperpolarizations becomes so fast as to not significantly contribute to the speed of activation. The voltage-independent step of pore movement is thus rate-limiting at strong hyperpolarizations. They also completed HCN1/HCN2 chimera studies, which suggested that the kinetics of the voltage-independent rate-limiting step are determined in the pore region, further supporting the notion that the voltage-independent step arises from movements of the channel gate (Chen et al., 2007). Thus while the activation gate movements are triggered by the voltage-dependent S4, they are themselves presumed to represent a voltage-independent step in the HCN channel activation pathway.

Tracking S4 movement with voltage clamp fluorometry (VCF) suggests that S4 movement was the rate-limiting step at a \(-120\) mV hyperpolarization (Bruening-Wright et
VCF involves covalently attaching a fluorophore to a channel, and then monitoring the changes in fluorescence emission as the channel changes conformation with standard voltage-clamp experiments ((Mannuzzu et al., 1996), see review (Zhu et al., 2016)). Changes in fluorescence emission are presumed to reflect changes in the environment surrounding the fluorophore. The Larsson group covalently attached a fluorophore to various residue positions within the N-terminal region of the spHCN1 S4 (Bruening-Wright and Larsson, 2007; Bruening-Wright et al., 2007). They verified that their fluorescence deflections superimposed with charge movement traces derived from gating currents, suggesting their fluorophore was tracking the inward movement of S4 upon hyperpolarization (Bruening-Wright et al., 2007). The authors proposed that the S4 inward movement step is the rate-limiting step in activation for hyperpolarizations between -120 mV and -160 mV, as opposed to the pore opening step (Bruening-Wright et al., 2007). VCF also allowed study of the relative kinetics between the VSD and the pore; pore closure might occur immediately after a tracked VSD movement, or there might be a substantial lag time between a tracked VSD movement and pore movement. The authors specifically proposed that two S4s move inward within a tetramer before the pore opens (Bruening-Wright et al., 2007). They also suggested cooperativity of the different S4s across subunits – where the activation of one S4 facilitates the activation of the second – is not required for HCN channel gating. These two joint studies were the first to track VSD movements in HCN channels using VCF. They also highlighted the fact that S4 inward movements can be rate limiting, despite a previous model that predicted the pore opening step was rate-limiting (Altomare et al., 2001). The pore opening step has previously been predicted to be rate limiting as this might account for the lower sigmoidicity of HCN traces relative to channels which follows the Hodgkin-Huxley model of activation (Altomare et al., 2001). These VCF studies were able to show the pore was not the rate limiting step because VCF allows simultaneous tracking of both VSD movement and pore opening, unlike patch clamp used for previous studies (Altomare et al., 2001). The speed of VSD movements under all conditions and in all subtypes, however, remains unclear.

Since cAMP binding is presumed to cause conformational changes in the CSD, the adjacent S6 gate is a logical structure to be regulated by these voltage-independent effects of cAMP on gating. Cyclic AMP binding continues to increase the open probability
of HCN channels even at strongly hyperpolarized voltages, when voltage-dependent steps should be already completed (Chen et al., 2007). Additionally the presence of cAMP sped the maximum speed of the HCN2 channels during activation and slowed the maximum speed upon deactivation (Chen et al., 2007). These results suggest cAMP binding can regulate the voltage-independent pore movement step. It is still possible, however, that cAMP additionally regulates a voltage-dependent step. The pore movement step is thus voltage-independent, but cAMP-dependent and the effect of cAMP on the voltage-independent step can be further investigated.

The Siegelbaum group put forth an eight-state cube model to fully represent cyclic allostery with cAMP potentiation (Chen et al., 2007) (Figure 1.12). The front face of the cube represents the voltage-dependent and voltage-independent steps of apo channels for both activation and deactivation (equivalent to the steps shown in Figure 1.2). The back face of the cube represents the same four steps, but with the additional regulation by cAMP. This model represents the present understanding of the dual regulation of HCN channels by voltage and cAMP through two-step activation and deactivation pathways.
Figure 1.12. The cyclic allostery model combines the transitions of resting and active VSDs with apo and holo CNB folds to regulate gating

Note: An eight-state cubic schematic for HCN channel gating. R = resting (outwards) position of S4; A = activated (inwards) position of S4. The four unliganded states form the vertices on the front face of the cube (in black). The four cAMP-bound states form the four vertices on the back face of the cube (in blue). cAMP binding and unbinding steps are shown in red. Transitions and states lying on hidden edges and corners of the cube are drawn with dashed lines and lighter shades. Adapted from (Chen et al., 2007) with permission.

Although the maximum speed of HCN2 channels for both activation and deactivation is well defined, the speed of the VSD movement step is unknown. This gap in the understanding of the cyclic allostery model limits the extent to which it can describe the allosteric VSD to PD coupling. Identifying the absolute speed of the VSD movement step in relation to the speed of the PD movement step will clarify how tight the coupling is, which is essential for an accurate gating model. Question #3 of my thesis thus asks: Is there a voltage where speed of VSD movement during deactivation is no longer rate-limiting for the deactivation pathway, as predicted by the cyclic allostery mechanism? Identifying the absolute speed of the VSD movement step across a range of voltages would characterize the voltage-dependence of this step. Question #4 of my thesis thus asks: Does the speed of VSD movement during deactivation have stronger
voltage-dependence than the speed of the pore closure step, as assumed by the cyclic allosteric mechanism? Knowing the conditions where a voltage-dependent step has slow rate-limiting kinetics would suggest when electrical oscillations in the human body would be most vulnerable to arrhythmias from sudden voltage changes.

Much like how it limits the understanding of VSD and PD coupling, not knowing the speed of the VSD movement step also limits the understanding of cAMP modulation of HCN channels. Without quantifying the rates of VSD movements, it is impossible to measure whether those rates are cAMP-dependent. There may be conditions where the VSD movement step and pore movement step are affected differently by cAMP potentiation (Ryu and Yellen, 2012). Question #5 of my thesis thus asks: as cAMP binding can slow deactivation, does the speed of VSD movement during deactivation decrease when the channel binds cAMP?

1.2.2. The autoinhibition model

The autoinhibition model proposes that the presence of the apo CNB fold stabilizes the HCN channel closed state. The apo channel is thus considered intrinsically inhibited. Autoinhibition results in a relatively hyperpolarized $V_{1/2}$, slow activation kinetics, and fast deactivation kinetics. Relief of autoinhibition can be achieved two ways: cAMP can bind to the CNB fold to induce conformational changes that alleviate this autoinhibition, or the entire CNB fold can be truncated to remove the autoinhibitory structure. Either of these actions would depolarize the $V_{1/2}$, speed activation kinetics, and slow deactivation kinetics. Question #1 and Question #5 of my thesis are questions regarding the kinetics of the conformational changes in autoinhibited and autoinhibition-free channels.

The Tibbs research group (Wainger et al., 2001) first elucidated the molecular mechanism of cAMP potentiation of $I_f$ currents. Using excised membranes allowed the authors of the study to measure mHCN2 channel $V_{1/2}$ and activation kinetics when cAMP was absent, or present in high concentrations in the bath. They found that application of cAMP to the cytosolic side of the excised membrane depolarized the HCN2 $V_{1/2}$ by over 17 mV, and sped activation 3.5-fold. This $V_{1/2}$ shift upon cAMP binding remained
unchanged when testing an HCN2 channel lacking the extreme-C region. However, a channel with both the CNB fold and extreme-C region removed was no longer potentiated in response to application of cAMP. This HCN2 ΔCNB channel had a relatively depolarized $V_{1/2}$ value both when cAMP was present and when cAMP was absent. This suggests that a channel with the CNB fold removed has similar open-state stability to a wildtype channel with cAMP bound. Digestion of the CNB fold by application of internal pronase had led to similar shifts in $V_{1/2}$ for $I_\text{f}$ currents in SA node myocytes (Barbuti et al., 1999). Wainger (Wainger et al., 2001) also examined a channel with the extreme-C region and the final $\alpha C$ helix of the CNB fold truncated. This channel had a relatively hyperpolarized $V_{1/2}$ value in the presence or the absence of cAMP. This distal $\alpha C$ helix is required for proper interaction with cAMP, but truncation of this helix alone does not relieve autoinhibition; the presence of the proximal CNB fold up until the end of the $\beta$-roll is sufficient to impose autoinhibition. The proposal of a truncation of the extreme-C region and the $\alpha C$ helix not relieving autoinhibition is supported by later crystal structures that show the $\alpha C$ helix is not very structured in the apo state (Saponaro et al., 2014; Akimoto et al., 2014). Without a rigid structure, the $\alpha C$ helix cannot contribute strongly to the autoinhibition effect and thus produces little relief of autoinhibition when removed. Thus a key concept of the autoinhibition model is that in order to relieve autoinhibition, the entire CNB fold must be removed.

Truncation of the full C-terminal region generates autoinhibition relief similar to truncation of the full CNB fold. An HCN2 channel with the entire C-terminal region truncated (C-linker, CNB fold, and extreme-C region) attempted in the initial autoinhibition study did not express, but a corresponding version in HCN1 (HCN1 ΔC) did express (Wainger et al., 2001). HCN1 ΔC showed nearly identical characteristics to HCN1ΔCNB: they both had similar $V_{1/2}$ values without cAMP present, and no shift of $V_{1/2}$ upon cAMP binding. This suggests the C-linker structure does not relate to the relief or presence of autoinhibition. An HCN channel truncated to remove the entire CNB fold is thus viewed as a minimal unit supporting fully uninhibited voltage-gating function (Wainger et al., 2001). Later studies suggest that in the apo state, the CNB fold generates steric clashes with the tetrameric C-linker. Thus C-linker tetramerization, which facilitates channel activation (see section 1.1.4), is disfavoured in the apo state (Akimoto et al., 2014). By truncation of the entire CNB fold, the steric clashes that
normally prevent tetramerization have also been removed and tetramerization is allowed even in the apo state (Akimoto et al., 2014). Though not the region of ligand binding, the C-linker thus must be in the tetrameric conformation to allow autoinhibition relief to properly transduce to the pore.

The mechanisms of the autoinhibition model are summarized in Figure 1.13. In this figure three channel constructs included in the original autoinhibition study are shown (HCN2 WT, HCN2 ΔαC, and HCN2 ΔCNB) (Wainger et al., 2001). The figure shows that HCN2 WT channels are able to transition between autoinhibited (left) and autoinhibition-free states (right) depending on the concentration of cAMP that is present. Independent of cAMP concentration, the channel still can exist in a closed (top) or open (bottom) state. When cAMP is present and channels are in the holo form, the channel open state is favoured. When cAMP is absent and channels are in the apo form, the channel closed state is favoured. As the removal of the distal αC helix prevents the channel from binding cAMP, the HCN2 ΔαC channel is unable to reach the autoinhibition-free channel states. The HCN2 ΔCNB channel must be autoinhibition-free as the inhibiting CNB fold structure is absent, and thus is unable to reach the autoinhibited channel states.
Figure 1.13. Intrinsic channel autoinhibition can be relieved by binding of cAMP to the CNB fold or removal of the compete CNB fold

Note: A: The closed and open channel states in the presence or absence of cAMP in HCN2 WT channels. These four states represent the either side the cube shown in Figure 1.12. The VSD is represented as a white circle with a plus sign, and VSD movements are not shown in this schematic. Auto = autoinhibition is present; auto free = autoinhibition is not present. Thickness and length of transition lines reflect the favoured direction of each equilibrium. Autoinhibition represented by interactions in the C-linker (green rectangle) and distal αC helix (blue zigzag). B: The closed and open channel states in the absence of cAMP in HCN2 ΔαC channels. C: The closed and open channel states in the absence of cAMP in HCN2 ΔCNB channels.
Although autoinhibition has been well characterized over many years, the majority of data has been focused on $V_{1/2}$ values; the kinetic regulation by autoinhibition is less clear. An example of how the autoinhibition model previously failed to predict kinetics was in the deactivation pathway. An open state trapping (OST) model for deactivation proposes that holo channels have slow deactivation relative to a channel with a truncated CNB fold (Wicks et al., 2009, 2011). Both these channels are thermodynamically autoinhibition-free, and thus would be predicted to have similar kinetics by the autoinhibition model. However the OST mechanism additionally stabilizes the open state of holo channels kinetically so they have ultra-sustained activation (Wicks et al., 2009, 2011) (Figure 1.14). A charge reversal mutation in the HCN4 channel S4 (K381E) dramatically enhances the OST mechanism, but OST is presumed to affect wildtype channels as well (Wicks et al., 2009, 2011). Thus this cAMP-dependent and K381E-enhanced OST mechanism shows that the CSD and the VSD are proposed to cooperate to generate an ultra-sustained activation phenotype distinct from autoinhibition.

**Figure 1.14.** Open state trapping prolongs deactivation of HCN4 channels in the holo state

Note: Schematic of the deactivation pathway for a channel with OST. Orange bars represent a possible interaction between the C-linker and S4-S5 linker that stabilizes holo channels. This channel also contains a K381E mutation in S4 that enhances OST. OST slows the $O_{S4\text{in}}$-$O_{S4\text{out}}$ step resulting in ultra-sustained activation of the channel. Enhanced OST is not present in apo or truncated channels. An additional step in the deactivation pathway where the closed channel dissociates from cAMP is not shown.

Advancing the understanding of the speeds of autoinhibited and autoinhibition-free channels will help characterize HCN channel gating kinetics to the same extent as
HCN channel gating thermodynamics. Further characterizing the kinetics of channels with a truncated CNB fold may be particularly useful, as this may clarify the roles of the C-linker. The forming of the tetrameric gating ring suggests the C-linker plays an important role in opening the channel pore, yet it is unable to independently impart autoinhibition on the channel. Channel regulation by the C-linker is thus unclear, particularly in apo channels when the channel is autoinhibited and the gating ring is not formed. To characterize the kinetics of truncated channels in more detail, kinetic parameters can be compared to thermodynamic parameters. Question #1 of my thesis thus asks: does autoinhibition relief by truncation speed opening kinetics to the same degree that it depolarizes the $V_{1/2}$?

1.2.3. The PIP$_2$ potentiation model

In mammalian HCN channels, application of exogenous PIP$_2$ to excised membrane patches depolarizes the $V_{1/2}$ of (or potentiates) HCN1, 2 and 4 channels (Pian et al., 2006; Zolles et al., 2006). Question #2 of my thesis addresses an outstanding question regarding the activation kinetics of the PIP$_2$ potentiation model.

PIP$_2$ potentiation can occur with an apo state CNB fold, indicating that it occurs through a mechanism separate from cAMP regulation (Pian et al., 2006). The Siegelbaum group showed that in HCN channels, PIP$_2$ depletion is a main factor for the hyperpolarization of the channel $V_{1/2}$ seen in excised patches that occurs as the patch loses cellular components, termed rundown (DiFrancesco et al., 1986)(Pian 2006). Though PIP$_2$ itself is embedded in the membrane and thus is not a typical cytosolic component, the kinases that generates PIP$_2$ from phosphatidylinositol are cytosolic enzymes; depletion of PIP$_2$ within the membrane thus occurs when cytosolic enzymes are not present to replenish it (Ford et al., 2003).

In spHCN1 channels, PIP$_2$ has two effects: it potentiates the voltage dependence of activation (as in mammalian channels), and it inhibits cGMP-activated currents at strongly hyperpolarized voltages (Flynn and Zagotta, 2011). These two separate phenomenon have been associated with two separate PIP$_2$ binding sites in spHCN1: one in the transmembrane domain which produces the potentiation, and one in the C-
linker that produces the current inhibition (Flynn and Zagotta, 2011). Although the transmembrane domain binding site has yet to be isolated, the potentiation is due in part to stabilization of the inward state of S4 (Flynn and Zagotta, 2011). Two positively charged residues in the spHCN1 C-linker A’ helix (R478 and K482) serve as the PIP₂ binding site (Flynn and Zagotta, 2011). The inhibitory effect of PIP₂ in the C-terminal region thus may be due to the stabilization of the autoinhibited state (Flynn and Zagotta, 2011). This inhibitory effect of PIP₂ is not seen in all mammalian channels, perhaps because R478 is not conserved (Flynn and Zagotta, 2011). Electrostatic interaction between R478 and K482 and the negatively charged PIP₂ on the inner leaflet thus may destabilize the C-linker gating ring, supporting autoinhibition.

As with autoinhibition, the kinetics of the effect of PIP₂ on HCN channels are less well described than the effect of PIP₂ on thermodynamics. The potentiation of V₁/₂ by PIP₂ in HCN2 channels could reasonably be accompanied by the speeding of activation kinetics or slowing of deactivation kinetics, as both of these would stabilize the channel open state. Yet the binding site for PIP₂ within the spHCN1 C-linker suggests that PIP₂ may stabilize the closed state, which could reasonably be accompanied by the slowing of activation kinetics and speeding of deactivation kinetics. Question #2 of my thesis thus asks: as PIP₂ can potentiate channel V₁/₂ values, does PIP₂ also speed channel activation?

1.2.4. The mode shift model

The mode shift model proposes that prolonged activation of HCN channels can promote the formation of a secondary open state. The two HCN channel open states (Oᵢ and Oᵢᵢ) each have their own distinct voltage dependence. Channels that reach the Oᵢᵢ state during activation will have slower deactivation, as the Oᵢ state is more stable than the Oᵢᵢ state. Question #5 of my thesis will address an outstanding question regarding the deactivation kinetics of HCN channels before and after mode shift.

The study of HCN channel gating currents initially suggested there are two HCN channel open states with different deactivation kinetics. Mutations within the pore region and HCN channel blocker compounds allow researchers to prevent the flow of ionic
currents and study gating currents in isolation. These types of experiments allow for direct study of the movements of the VSD. After introducing a pore mutation that blocked ionic currents, the Larsson group (Männikkö et al., 2005) found spHCN1 channels showed voltage-dependent gating currents. The gating currents associated with deactivation became less as longer activation pulses were used, indicating slower charge movement during deactivation (Männikkö et al., 2005). This phenomenon was seen in spHCN1 ionic current traces as well, with deactivation transients following a short activation pulse fitting to a single exponential equation, while transients following a long activation pulse were better fit to a double exponential equation after an initial delay (Männikkö et al., 2005). Channels previously exposed to a long activation pulse underwent a depolarizing shift in the $V_{1/2}$ of up to 50 mV (Männikkö et al., 2005). This $V_{1/2}$ shift was cAMP-independent, suggesting that it arises from a different mechanism than autoinhibition (Männikkö et al., 2005; Elinder et al., 2006). Mammalian HCN1, HCN2, and HCN4 channels had similar results, though the effects of longer activation pulses were less prominent in the slower HCN2 and HCN4 channels (Elinder et al., 2006). Broadly, this is all a form of hysteresis, where channel behaviour depends on the previous activity (in this case the length of activation) of the channel.

HCN channel hysteresis suggests that there may be more than one channel open state. The past channel activity may determine which open state is reached and thus the behaviour of the channel (Figure 1.15). Short activation pulses will promote the C-O$_i$ transition, and longer activation pulses promote the O$_i$-O$_{ii}$ transition. The transition from the O$_i$ to O$_{ii}$ states was termed mode shift.
HCN channels undergo a mode shift after initial activation to reach a more stable open state

Figure 1.15. HCN channels undergo a mode shift after initial activation to reach a more stable open state

Note: A four-state model with two different modes (i and ii). Each mode has a different voltage dependence and one open state (left) and one closed state (right). The gating charge movement and the channel opening occur at very negative potentials in mode i, but they are shifted to more depolarized potentials in mode ii. The mode i-mode ii transition is favoured in the open states, while the mode ii-mode i transition is favoured in the closed states. Adapted from (Männikkö et al., 2005) with permission.

Hysteresis is potentially caused by lateral S4 movement. In structural terms, the transition from the C to O_i state is proposed to permit additional conformational changes in the channel which can lead to the mode shift to the more stable O_ii state. Later VCF experiments isolated a VSD movement that occurs after pore opening (Bruening-Wright and Larsson, 2007). As the authors used a fluorescent reporter covalently linked to the extracellular portion of S4, this VSD movement presumably occurred on or around the N-terminal region of S4. The VCF experiments led to a model where the transition from the O_i to O_ii state involved a shift of S4. This conformational change would be independent from any gating movements undergone by S4 before pore opening. The authors propose this additional conformational change is a lateral transition of S4 towards the negative charges on S2, which would lead to increased electrostatic interactions between the two
helices and thus a more stable open state (Bruening-Wright and Larsson, 2007) (Figure 1.16).

**Figure 1.16.** Mode shift is presumed to involve a lateral shift of S4 towards S2

Note: Model for VSD movement during mode shift in HCN channels. The transmembrane domains of HCN were placed after homology modeling with the crystal structure of Kv1.2 channel (Long et al., 2005). Only two of the four PDs and VSDs are shown. Schematic shows that S4 undergoes a conformational change during mode shift (red arrows) that brings the S4 positive charges closer to one or both of the two S2 negative charges, thereby stabilizing the inward position of S4. Adapted from (Bruening-Wright and Larsson, 2007) with permission.

Mode shift is physiologically relevant, as computer simulations predict mode shift causes HCN channels to open at voltages that permit proper cardiac function. HCN channel mode shift has been modeled to prevent arrhythmic firing of action potentials in the SA node (Männikkö et al., 2005). Mode shift can occur within a few hundred milliseconds in HCN1 channels, indicating HCN channels involved in cardiac pacemaker electrical oscillations would be open long enough to reach the OII state (Männikkö et al., 2005). Computer simulations of SA node activity have shown that if HCN channels do not reach the OII state and undergo the accompanying shift in voltage-dependence, cardiac arrhythmias would occur. This may be because without mode shift, HCN channels would open too quickly and not allow the action potential to undergo sufficient hyperpolarization to close hERG channels (Männikkö et al., 2005). However, computer simulations that allow for hysteresis that depolarizes the HCN channel V1/2 by 40 mV eliminates all arrhythmic activity. HCN channel mode shift is thus predicted to stabilize
cardiac rhythmic firing events, and potentially could have a similar role in neuronal wave oscillations.

Many questions remain regarding the proposed movement of S4 towards S2 during mode shift. Difficulties in tracking VSD movements directly have limited the ability to study this shift from the O\textsubscript{i} to O\textsubscript{ii} state in detail. Does the entire helix move, or does part remain stationary? Do other helices move or is S4 the sole VSD helix moving during mode shift? The kinetics of VSD deactivation movements when the channel begins deactivation from the O\textsubscript{i} or O\textsubscript{ii} states is unknown in three out of four mammalian subtypes. Mode shift thus represents a physiologically relevant model with mechanism not yet fully defined that would benefit greatly from further study. Question #5 of my thesis thus asks: as mode shift can slow deactivation, does the speed of VSD movement during deactivation decrease when the channel undergoes mode shift?

1.3. Hypotheses and specific objectives

Before the research completed in this thesis, the understanding of the HCN channel activation and deactivation pathways can be summarized by Figure 1.17 and Figure 1.18.

Figure 1.17 shows the three steps of the activation pathway for HCN2 WT channels in the apo and holo form, along with channels with a partial or full CNB truncation. The presence of a full (apo HCN2 WT) or partial (HCN2 ΔαC) CNB fold results in a channel with an autoinhibited open state. The touching C-linkers in the closed state represent the stabilization of the closed state by the autoinhibition mechanism. The binding of cAMP (holo HCN2 WT) to the CNB fold or removal of the full CNB fold (HCN2 ΔCNB) results in a channel with an autoinhibition-free open state. In these two channels, the conformational changes in the CNB fold have prevented the C-linkers from touching, and thus prevented autoinhibition from stabilizing the closed state.

Figure 1.18 shows the deactivation pathway for apo mode i channels, apo mode ii channels, and holo mode ii channels. Isolating holo mode i channels in intact cells is challenging, and was not attempted in this thesis (see section 3.4.2.4). The VSD
movement step involves both a C-terminal swing of S4 into the membrane (proposed in (Bell et al., 2004)) and an outwards movement of S4 (proposed in (Vemana et al., 2004)). Channels initiating deactivation from the mode ii state have an S4 that is positioned closer to S2, but the same swinging-tail and outward movement in the VSD movement step. Channels initiating deactivation from the holo mode ii state additionally have autoinhibition-relief and OST, resulting in slower deactivation.
Figure 1.17. The HCN channel activation pathway before the research in this thesis.

Note: Schematic showing the activation pathways for HCN2 WT, HCN2 ΔαC and HCN2 ΔCNB channels at the beginning of my thesis work. VSD represented by white circle with a plus sign, and S4 inward movement occurs in the first step. Both cAMP binding to HCN2 channels or removal of the full CNB can relieve autoinhibition to result in a more stable state. Autoinhibition is represented by the touching C-linkers in the first and third rows. In this pathway all channels reached the mode ii state during activation, and PIP₂ is present equally in all membranes. Only relevant channel region shown, full channel schematics are in Figure 1.1.
Figure 1.18. The HCN channel deactivation pathway before the research in this thesis

Note: Schematic showing the deactivation pathways for apo O\textsubscript{i}, apo O\textsubscript{ii}, and holo O\textsubscript{ii} channels at the beginning of my thesis work. Only relevant channel parts shown. Black wavy line eliminates C-terminal region for clarity. See Figure 1.17 for what the C-terminal region would look like in the liganded (holo) and unliganded (apo) states. Orange lines represent the presence of the OST mechanism (see Figure 1.14). The S4 of channels in the O\textsubscript{ii} state moved laterally towards S2 upon mode shift (pink arrows, see Figure 1.16). A final step in the deactivation pathway for holo channels is not shown where the channels in the C\textsubscript{S4out} state dissociate from cAMP.
Despite the numerous advancements in the understanding of HCN channel structure and function since their discovery in the 1990s, questions still remain regarding their gating behaviours. In particular, kinetics of HCN channel gating pathways require further study, for both activation and deactivation. My thesis selects some key questions to target, described in section 1.2 and summarized here:

**Question #1:** Does autoinhibition relief by truncation speed opening kinetics to the same degree that it depolarizes the $V_{1/2}$?

**Question #2:** As PIP$_2$ can potentiate channel $V_{1/2}$ values, does PIP$_2$ also speed channel activation?

Question #1 and #2 Rationale: Modulators such as cAMP and PIP$_2$ are often characterized by their effects on channel $V_{1/2}$ values. Yet models such as the autoinhibition model have been shown to be a poor predictor of channel kinetics, even when their effects on thermodynamics are well understood. Thus I cannot assume that modulators known to depolarize $V_{1/2}$ values would necessarily speed channel activation.

**Question #3:** Is there a voltage where speed of VSD movement during deactivation is no longer rate-limiting for the deactivation pathway, as predicted by the cyclic allosteric mechanism?

**Question #4:** Does the speed of VSD movement during deactivation have stronger voltage-dependence than the speed of the pore closure step, as assumed by the cyclic allosteric mechanism?

**Question #5:** As the mode shift and cAMP binding can slow deactivation, does the speed of VSD movement during deactivation decrease when the channel undergoes mode shift or binds cAMP?

Questions #3 - #5 Rationale: The cyclic allosteric model is prevalent in the HCN channel field. Yet basic elements of this model remain untested, due to difficulties in measuring the kinetics of voltage-dependent steps. Thus not only are the absolute
speeds of voltage-dependent steps unknown, but it is also unknown if and how these steps are regulated by channel modulators.

These various questions can be summarized as one general overarching question: to what extent does my present working model, a cyclic allostery model including autoinhibition, mode shift, cAMP and PIP₂ potentiation, accurately predict HCN channel activation and deactivation kinetics?

My overarching objective is to clarify to what extent each of the mechanisms correctly explains HCN channel behaviours for both activation and deactivation. Answering this question would advance the understanding of the regulation of HCN channel gating by the VSD and CSD, and the conformations of the HCN channel in different states of the activation and deactivation pathway. The mechanisms described above thus need to be more thoroughly investigated to ensure they correctly predict all behaviours of the HCN channel VSD and CSD. For example, the mechanisms may be investigated under novel conditions (such as in cells with PIP₂ depletion); or investigated using different HCN channel derivatives (such as channels with different degrees of CSD truncation).

My overall approach is to examine the kinetics of movements of the VSD and the PD in HCN2-based derivatives with a variety of VSD and CSD conformations.

Chapter 2 investigates the applicability of the autoinhibition and PIP₂ potentiation mechanisms to gating kinetics in order to answer Questions #1 and #2. Wainger (Wainger et al., 2001) showed that truncation of the C-terminal domain can both depolarize HCN2 channel \( V_{1/2} \) values and speed HCN2 channel activation kinetics in excised membranes. Pian (Pian et al., 2006) showed endogenous PIP₂ can potentiate HCN2 channel \( V_{1/2} \). But do these mechanisms speed channel activation in intact cells? In Chapter 2, I determine, through two electrode voltage clamp (TEVC) of *Xenopus* oocytes expressing HCN2 channels with varied C-terminal regions, that autoinhibition correctly predicts the \( V_{1/2} \) thermodynamics of all channels studied but does not correctly predict activation kinetics. My key conclusions are: (#1) there is a conformation of the apo CNB fold that promotes faster channel activation than a truncated autoinhibition-free channel, which I coin the “quickening conformation”; (#2) endogenous PIP₂ in the plasma
membrane does not significantly speed activation kinetics in autoinhibited or autoinhibition-free channels. Collectively, these results suggest a new mechanism regulating HCN2 activation kinetics independent of autoinhibition and PIP$_2$ that still requires an intact CNB fold.

Chapter 3 will determine the speed of a VSD movement in order to answer Questions #3, #4, and #5. All present models of HCN channel deactivation possess at least a VSD movement step and a pore closure step. However, due to difficulties in obtaining HCN channel gating currents, there have been few experiments monitoring movements of the VSD directly (Männikkö et al., 2002, 2005; Bruening-Wright and Larsson, 2007). Present models are thus limited in their ability to predict whether factors influencing the speed of the pore movement step also influence the speed of the VSD movement step. In Chapter 3, I measure the speed of a VSD movement step during deactivation using voltage clamp fluorometry and a fluorophore covalently linked to the N-terminal region of the HCN2 derivative S4 in both apo and holo channels. The fluorophore tracked a voltage-dependent VSD movement during deactivation that preceded pore closure. My key conclusions are: (#3) the speed of the tracked VSD movement does not substantially limit the rate of the overall deactivation pathway at strong depolarizations; (#4) the speed of the tracked VSD movement step during deactivation has stronger voltage dependence than speed of the pore closure step; (#5) neither cAMP binding nor mode shift slow the tracked VSD movement during deactivation. Together these results suggest a new model of the HCN deactivation pathway. This new model introduces absolute speeds of VSD movement, supports the existence of two potential rate-limiting steps that was proposed in the cyclic allostery model, and clarifies that there is a mode shift-dependent and a mode shift-independent VSD step in the HCN channel deactivation pathway.
Chapter 2.

HCN channel C-terminal region speeds activation rates independently of autoinhibition

Parts of this chapter have been published as (and are reprinted from):

Contributions to this chapter:

I designed and performed all work and all analysis, with the exception of the following:
Zarina Madden contributed to experimental set-up of TEVC equipment.
Edgar C. Young constructed the channel HCN2 ΔXC.
Stephen Saad assisted with sequencing HCN2 R591E and HCN2 ΔCNB.
The abstract of the published work is reprinted as follows:

Hyperpolarization- and cyclic nucleotide-activated (HCN) channels contribute to rhythmic oscillations in excitable cells. They possess an intrinsic autoinhibition with a hyperpolarized $V_{1/2}$, which can be relieved by cAMP binding to the cyclic nucleotide binding (CNB) fold in the C-terminal region or by deletion of the CNB fold. We questioned whether $V_{1/2}$ shifts caused by altering the autoinhibitory CNB fold would be accompanied by parallel changes in activation rates. We used two electrode voltage clamp on *Xenopus* oocytes to compare wildtype (WT) HCN2, a constitutively autoinhibited point mutant incapable of cAMP binding (HCN2 R591E), and derivatives with various C-terminal truncations. Activation $V_{1/2}$ and deactivation $t_{1/2}$ measurements confirmed that a truncated channel lacking the helix $\alpha C$ of the CNB fold ($\Delta \alpha C$) had autoinhibition comparable to HCN2 R591E; however, $\Delta \alpha C$ activated approximately two-fold slower than HCN2 R591E over a 60-mV range of hyperpolarizations. A channel with a more drastic truncation deleting the entire CNB fold ($\Delta \text{CNB}$) had similar $V_{1/2}$ values to HCN2 WT with endogenous cAMP bound, confirming autoinhibition relief, yet it surprisingly activated slower than the autoinhibited HCN2 R591E. Whereas CNB fold truncation slowed down voltage-dependent reaction steps, the voltage-independent closed-open equilibrium subject to autoinhibition in HCN2 was not rate-limiting. Chemically inhibiting formation of the endogenous lipid PIP$_2$ hyperpolarized the $V_{1/2}$ of HCN2 WT but did not slow down activation to match $\Delta \text{CNB}$ rates. The findings suggest a “quickening conformation” mechanism, requiring a full length CNB, that ensures fast rates for voltage-dependent steps during activation regardless of potentiation by cAMP or PIP$_2$. 
2.1. Introduction

In the absence of cAMP, the HCN channel is believed to be in an "autoinhibited" state, since removing the CNB fold by proteolysis or gene truncation (Barbuti et al., 1999; Wainger et al., 2001) has a potentiating effect similar to that of cAMP binding; an HCN channel truncated to remove the CNB fold is thus viewed as a minimal unit supporting fully uninhibited voltage-gating function (Vemana et al., 2004; Wainger et al., 2001). The presence of the N-terminal subregion of the CNB fold up to helix αB is sufficient to impose autoinhibition, whereas the final helix αC is necessary for cAMP-mediated relief of the autoinhibition (Wainger et al., 2001). Presumably rearrangement of the C-linker due to loss of cAMP from the CNB fold causes autoinhibition by inducing a strain on the S6 pore gate, hence destabilizing the open state relative to the closed state. This structural model focused on S6 and the C-linker provides a straightforward prediction of how autoinhibition regulates the thermodynamics of the gate-opening closed-to-open step, but it is less straightforward to predict how autoinhibition regulates the kinetics of voltage-dependent activation governed by the transmembrane S4 segment.

One possibility is that the autoinhibition and relief mechanisms would simply modify kinetics parallel to the modifications in $V_{1/2}$ values: that is, relief conditions that produce depolarized $V_{1/2}$ values would produce faster activation, while autoinhibition conditions that produce hyperpolarized $V_{1/2}$ values would produce slower activation. But the thermodynamic and kinetic effects of autoinhibition in principle are not constrained to be parallel, depending on the particular nature of the transition state in question. And if a different gating step is rate limiting (not the steps regulated by autoinhibition), then autoinhibition may have no effects on the activation kinetics at all.

In this study, I set out to clarify whether the prevailing model of CNB fold-mediated autoinhibition would suitably describe gating kinetics of HCN2 channels. To ensure I knew the exact composition of the tetramers I heterologously expressed HCN channel derivatives as homomers in intact Xenopus oocytes (Santoro et al., 2000; Chen et al., 2000, 2001; Wang et al., 2002). Specifically, I sought to test the notion that the thermodynamics and kinetics should be regulated in a parallel fashion: Does the
introduction of an intact unliganded CNB fold slow activation kinetics in parallel to its known effect of hyperpolarizing $V_{1/2}$ values? In other words, does autoinhibition relief by truncation speed opening kinetics to the same degree that it depolarizes the $V_{1/2}$? This is Question 1 of the five main questions addressed in my thesis. As autoinhibition can also be imposed by introducing only the N-terminal subregion of the CNB fold, would this introduction equally induce slow activation kinetics? Does speeding of deactivation accompany the slowing of activation due to autoinhibition? Does the voltage-independent gate-opening step of HCN2 restrict the activation rate in autoinhibited channels, and are these kinetic restrictions removed when autoinhibition is relieved by CNB fold deletion? My findings suggest that any slowing effect of autoinhibition on activation kinetics must be restricted to steps that are not rate-limiting, and suggest there is an additional mechanism which can preserve a high rate of activation only when the full CNB fold sequence is present. This mechanism results in a “quickening conformation” that accounts for a kinetic difference between channels whose autoinhibition is relieved in different ways: by CNB fold deletion or by addition of cAMP. This new mechanism does not contradict autoinhibition, but introduces an additional element to consider when examining channel activation speeds to further characterize the gating of the HCN channel family.

I further investigated whether another form of HCN channel potentiation, PIP$_2$ binding, might regulate thermodynamics and kinetics in a parallel fashion. Specifically, as PIP$_2$ can potentiate channel $V_{1/2}$ values, does PIP$_2$ also speed channel activation? This is Question 2 of the five main questions addressed in my thesis. I found the quickening conformation mechanism operates equally with and without PIP$_2$ potentiation of HCN channel $V_{1/2}$ values, which provides a further example where thermodynamics and kinetics are differently regulated.

### 2.2. Materials and Methods

#### 2.2.1. Composition and subcloning of DNA constructs

All channels in this investigation were subcloned into the high-expression vector pGEM-HE (Liman et al., 1992) to allow in vitro transcription of RNA from linearized DNA
with the T7 promoter (Ambion mMessage Machine Kit). All channels were expressed in *Xenopus* oocytes (see below) as homomers. "HCN2" or "HCN2 WT" in this report denotes full-length wild-type mouse HCN2 (863 amino acids) (Ludwig et al., 1998). The HCN2 R591E derivative (Chen et al., 2001) is identical to HCN2 with the exception of the Arg to Glu mutation at residue 591. Composition of deletion derivatives were as follows: \(\Delta XC\) consists of HCN2 amino acids 1-645, previously reported as "HCN2 \(\Delta 645\)"(Zagotta et al., 2003); \(\Delta \alpha C\) consists of HCN2 amino acids 1-617, previously reported as HCN2\(\Delta \alpha C\) (Wainger et al., 2001); \(\Delta CNB\) consists of HCN2 amino acids 1-525, previously reported as HCN2\(\Delta CNBD\) (Wainger et al., 2001). HCN2, HCN2 R591E and HCN2 \(\Delta CNB\) were generous gifts from S. A. Siegelbaum, HCN2 \(\Delta XC\) had been previously constructed by Edgar Young, and I reconstructed HCN2 \(\Delta \alpha C\) using polymerase chain reaction (PCR) mutagenesis. Regions of constructs which were involved in PCR mutagenesis during construction of these clones were verified by us using dideoxy sequencing (Eurofins Operon).

### 2.2.2. TEVC recordings

Oocytes were obtained through ovariectomies of *Xenopus laevis* frogs following guidelines from the Canadian Council on Animal Care. Oocytes were then defolliculated by collagenase (Sigma Aldrich) as previously reported (Wicks et al., 2009) and washes after collagenase treatment used either ND96 (Cedar Lane Laboratories) or “Magnesium Oocyte Ringer 2” (in mM: 96 NaCl, 2 KCl, 20 MgCl\(_2\), 5 HEPES, pH 7.4), either of which was then followed by “Oocyte Ringer” (in mM: 1.8 CaCl\(_2\), 2 KCl, 115 NaCl, 10 HEPES, pH 7.3). Both before and after RNA injection, oocytes were stored at 16°C in Barth’s Saline solution (Modified without Ficoll 400, Cedar Lane) with 100 ng/µL of the antibiotic gentamicin (Sigma Aldrich) with at least one daily change to fresh solution after injection. Oocytes were injected with 15 - 40 ng channel RNA within 24 hours of collagenase treatment, and recordings were obtained 3-4 days after injection.

TEVC recordings reported in this work were generated from the oocytes of at least two frogs for each channel type or condition, and each batch of oocytes extracted for this study was used for testing at least two channel types or conditions. Thin-walled glass capillaries with filaments (World Precision Instruments Category # TW150F-3)
were pulled to form TEVC electrodes with a resistance between 0.2 MΩ and 2.2 MΩ when filled with 3M KCl. TEVC experiments were completed at room temperature (19 - 23°C) using a GeneClamp 500B amplifier and Digidata 1440A (both Axon Instruments) and the computer program Clampex 10.2. Current was sampled at 10 kHz and filtered at 1 kHz to improve signal to noise ratio; datapoints have been undersampled in Figures for clarity of printing. The TEVC Bath solution consisted of (in mM): 96 NaCl, 3 KCl, 5 HEPES (pH 7.4), 1 MgCl₂, 0.75 CaCl₂. For wortmannin treatments, a stock solution of 30 mM wortmannin (Sigma) was prepared in dimethyl sulfoxide (DMSO) and stored at -20°C; this stock solution (or an equal volume of DMSO alone) was then diluted into TEVC Bath to produce a 45 μM wortmannin test solution, with maximum 6 hours wait before application. Wortmannin effects were measured by first recording from the oocyte in TEVC Bath without wortmannin, then removing the electrodes and incubating the oocyte in the 45 μM wortmannin test solution for 35 min, then returning to TEVC Bath without wortmannin for immediate re-impalement and recording. The 45 μM wortmannin concentration was deemed saturating since trials with 120 μM did not produce appreciably greater hyperpolarizations of $V_{1/2}$.

Voltage-clamp holding potential was -40 mV and oocytes were excluded from analysis if they exhibited inward leak current greater than 200 nA at this voltage. The activation protocol (Figure 2.1) included a hyperpolarizing activation epoch (-40 mV to -170 mV, every 10 mV) followed by a tail epoch (-120 mV) and a deactivation epoch (+20 mV). A “staircase” of steps to +40 mV, 0 mV, and -40 mV was applied at the end of every sweep, to verify stability of time-independent currents. Channel behaviour was stable for tens of minutes, judged by reproducibility of repeated recordings.
At the beginning of the activation epoch, a small lag segment with concave-negative shape consistently occurred in the trace, with an inflection point leading to the concave-positive activation transient as reported previously for HCN currents (DiFrancesco, 1999; Wainger et al., 2001). In some recordings, time-dependent currents not typical of HCN channel behaviour were observed in the later portion of the activation epoch, with either a decrease in inward current ("sag") or a second inflection point leading to concave negative curvature ("post-plateau instability"). These behaviours were not systematically observed but occurred more often at -150 mV or more negative (see Figure 2.1 for examples at -170 mV). Oocytes were excluded from analysis altogether if either of these behaviours occurred at -130 mV or less negative; additionally, individual sweeps at -150 mV or beyond were excluded if either behaviour appeared within the first 800 ms of activation.

2.2.3. Analysis

For determining $V_{1/2}$, tail current values at -120 mV were measured using Clampfit 10.2 at the local peak following the capacitance clearance (typically < 25 ms). Using Sigmaplot 10.0, activation voltages ($V$) and tail current values ($I$) were fit to the four parameter sigmoid Boltzmann equation $I = y0 + a / (1 + exp[-(V - V_{1/2}) / s])$. The floating parameters were $a$ (positive, maximum time-dependent HCN current amplitude), $s$ (positive, reciprocal slope), $V_{1/2}$ (negative, midpoint activation voltage), and $y0$ (negative, total maximum current), from which the $I_{\text{leak}}$ parameter (negative, "leak" current) was calculated as $I_{\text{leak}} = y0 + a$. $I_{\text{leak}}$ includes both the instantaneous component of HCN conductance (Proenza et al., 2002) and non-HCN sources of leak; the average value of $|I_{\text{leak}}| / a$ for each channel type was in the range 0.02 to 0.08, so I can conclude that a majority of HCN current observed from the channels was time-dependent HCN current. As described below, in many cases and especially for HCN2 and $\Delta XC$ which can
bind endogenous cAMP, the absolute inward current did not reach a steady state during the activation epoch (see Figure 2.1 for typical recordings). Thus the isochronal tail currents generally underestimate the steady-state conductance levels, which limits the precision of the estimates of $V_{1/2}$ and $s$.

For kinetics of the activation epoch, the concave-negative lag segments, as well as portions of traces at -150 mV showing sag or post-plateau instability, were truncated to isolate concave positive datasets for exponential fitting. Activation tau ($\tau$) values were determined using Clampfit 10.2 by Levenberg-Marquardt fitting of each activation trace to the double exponential equation $I(t) = A_{\text{early}} \exp[-t / \tau_{\text{early}}] + A_{\text{late}} \exp[-t / \tau_{\text{late}}] + C$, where $\tau_{\text{early}} < \tau_{\text{late}}$. Amplitudes ($A$) and time constants ($\tau$) were forced positive on all fits. For quantitation of the lag duration, $d$, an iterative fitting procedure based on ref. (Wicks et al., 2009) was used. The lag component presumably represents the time required for initial voltage dependent steps before the pore movement step. An initial overestimate of $d$ was made that encompassed some of the early concave positive region, and fitting was done using only time points after the overestimated $d$. Then the earliest time point before $d$ exhibiting good match to the fit curve was found by visual inspection, and this became the new smaller estimate of $d$. Fitting and reduction of $d$ was then repeated until $d$ could no longer be reduced without compromising fit quality.

I judged that two exponential terms were generally sufficient because the fit residual $R = (\text{Sum of Squared Errors}) / (\text{Sum of Data Squared})$ for -130 mV activation traces improved significantly when the number of terms was increased from one to two, but improved markedly less upon addition of a third term, with no difference in fit quality on visual inspection (see Figure 2.2 and Figure 2.3). Although double-exponential fitting was applied in all cases, some traces (particularly at voltages -90 mV to -110 mV) have only $\tau_{\text{early}}$ reported, for the following reasons: In some cases, one component was insignificant with $A / (A_{\text{early}} + A_{\text{late}}) < 0.03$, indicating a single exponential was sufficient. In other traces, the $\tau_{\text{late}}$ was >10 s, indicating a quasi-linear component. Because traces did not all reach endpoint, the fractional contribution of the early component to total activation trace amplitude ($f_{\text{early}}$) was calculated over the fitted time interval ($\Delta t$) as $f_{\text{early}} = A_{\text{early}} (1 - \exp[-\Delta t / \tau_{\text{early}}]) / \{ A_{\text{early}} (1 - \exp[-\Delta t / \tau_{\text{early}}]) + A_{\text{late}} (1 - \exp[-\Delta t / \tau_{\text{late}}]) \}$. 
Figure 2.2. Autoinhibited HCN2 R591E and ΔαC both exhibit biphasic activation transients at strong hyperpolarizations

Note: Example transients at -130 mV from HCN2 R591E and ΔαC are shown with fits formatted as in Figure 2.7 using one (left) or two (right) components, with residuals plotted directly below. For residual plots, dotted line indicates zero, and data points in the excluded lag interval are shown in gray. Note, fits in right panels are the same as Figure 2.7.
HCN2 WT in intact oocytes exhibits a late phase of activation that persists at both weak and strong hyperpolarizations

Note: Example transients from HCN2 WT (same trace as in Figure 2.5) at voltages indicated, fit with one, two, or three components and formatted as in Figure 2.7. For -130 mV transients, residuals are plotted below corresponding fits, formatted as in Figure 2.2.

The deactivation protocol (Figure 2.4) contained an activation epoch (-140 mV) followed by a deactivation epoch of either +40 mV, +20 mV, or 0 mV. Because deactivation currents always reached a steady endpoint, kinetics were quantified by a t1/2 value, determined as the time required to reach the midpoint of deactivation current (starting from the beginning of the deactivation epoch). Starting (lag plateau) values of current were visually identified following capacitance clearance (<25 ms). Oocytes for
which the $V_{1/2}$ value and activation $\tau$ calculations were both excluded were also removed from the deactivation $t_{1/2}$ data set.
2.3. Results

2.3.1. Two constitutively inhibited HCN2 derivatives exhibit different activation with different kinetics

I used the Xenopus oocyte expression system with two-electrode voltage clamp (TEVC) to record hyperpolarization-activated currents from homomeric mouse HCN2 channels and HCN2 derivatives (Figure 2.5 upper panels, Figure 2.1). Autoinhibition relief in full-length HCN channels requires not only cAMP binding but also conformational changes, so there should be a variety of ways to disrupt the relief mechanism. I hypothesized that, based on the autoinhibition model, changes to the C-terminal region should influence thermodynamics and kinetics in a parallel manner. This hypothesis predicts that regardless of how autoinhibition relief is disrupted, fully autoinhibited channels should show identical kinetic behaviours. I sought to test this hypothesis in intact oocytes by comparing two examples of HCN2 derivatives with constitutive autoinhibition imposed in two different ways. Previous TEVC studies have examined HCN2 R591E, which has its autoinhibitory CNB fold mutated to remove the positively charged arginine residue that contacts cAMP (Chen et al., 2001; Wang et al., 2002). I compared HCN2 R591E to the derivative ΔαC which has its CNB fold truncated (Figure 2.5 upper right): the N-terminal "β-roll" moiety region of the CNB is still present (preserving the cAMP-contact arginine 591 (Zagotta et al., 2003)) and is sufficient to impose autoinhibition, but the truncation removes the helix αC which is critical for
contacting cAMP during autoinhibition relief (Wainger et al., 2001; Zhou and Siegelbaum, 2007).

Figure 2.5. Two mutant HCN2 derivatives exhibit constitutive autoinhibition in intact oocytes

Note: Upper panels show representative examples of hyperpolarization-activated inward currents of mouse HCN2 WT and mutant derivatives. Subunit structures are depicted in schematic icons including subregions of C-terminal region: C-linker (dashed line), CNB fold (bold line) including helix \( \alpha_C \) (zigzag), and extreme-C region (oval); R591E point mutation is shown with a cross. Channels were expressed in Xenopus oocytes and studied using two-electrode voltage-clamp; only activation and tail epochs are shown, and the \(-160 \text{ mV} \) and \(-170 \text{ mV} \) traces of \( \Delta \alpha_C \) were excluded due to post-plateau instability (see Methods and Figure 2.1 for protocol and example traces in full). Traces for \(-70 \text{ mV} \) and \(-150 \text{ mV} \) activation are highlighted for clarity. Lower panels show conductance-voltage relations from tail currents (points, data from corresponding upper panels) after correction by leak-subtraction and normalization to maximal amplitude determined from a Boltzmann equation fit (solid curves, see Methods). For comparison, the example HCN2 WT curve is repeated as a black dashed line in the panels of mutant derivatives. Boltzmann equation fit parameters were as follows: HCN2 WT, \( V_{1/2} = -84.9 \text{ mV} \), \( s = 16.9 \text{ mV} \); HCN2 R591E, \( V_{1/2} = -98.7 \text{ mV} \), \( s = 11.9 \text{ mV} \); \( \Delta \alpha_C \), \( V_{1/2} = -96.2 \text{ mV} \), \( s = 6.48 \text{ mV} \). See Figure 2.6 for mean values.

Activation traces at weakly hyperpolarized voltages show evidence that activation is indeed inhibited in both HCN2 R591E and \( \Delta \alpha_C \) compared to HCN2 WT in the
presence of endogenous cAMP. For instance, for HCN2 WT the trace at -70 mV is the first to indicate an observable increase of inward current across the 3-s epoch, while this "threshold" activation does not appear in HCN2 R591E or ΔαC until -90 mV (Figure 2.5, highlighted -70mV curves). This suggests that HCN2 WT requires approximately 20 mV less hyperpolarization than HCN2 R591E or ΔαC to achieve comparable channel activation. This suggestion was quantitatively confirmed by the mean \( V_{1/2} \) values recorded from the tail epoch in the activation protocol. The mean \( V_{1/2} \) values of HCN2 R591E (-99.4 ± 3.1 mV, \( n = 10 \)) and ΔαC (-101.1 ± 5.5 mV, \( n = 10 \)) were both significantly more hyperpolarized (one-sided \( p < 10^{-5} \)) compared to that of HCN2 WT (-83.4 ± 5.0 mV, \( n = 49 \); see Figure 2.5 lower panels, and Figure 2.6 for \( V_{1/2} \) means). The \( V_{1/2} \) values of the two autoinhibited channels were not significantly different from each other (two-sided \( p > 0.3 \)). The \( V_{1/2} \) values matched previous reports (-80 mV for HCN2 WT (Zolles et al., 2006); -97 mV to -102 mV for HCN2 R591E (Chen et al., 2001; Wang et al., 2002)) and are consistent with HCN2 R591E and ΔαC possessing similarly strong autoinhibition which is constitutive, i.e., it is not relieved by endogenous cAMP.

![Figure 2.6. Voltage-dependence of the activation properties of truncated HCN2 derivatives](image)

**Figure 2.6. Voltage-dependence of the activation properties of truncated HCN2 derivatives**

*Note:* Mean values of \( V_{1/2} \) and reciprocal slope \( s \) for derivatives in this study, showing autoinhibition of HCN2 R591E and ΔαC but not ΔCNB. Error bars show SD and number of recordings is shown as (n).

The hypothesis of parallel thermodynamic and kinetic effects implies that the autoinhibited HCN2 R591E and ΔαC with similar \( V_{1/2} \) values should have similar slow activation kinetics. Surprisingly contrary to this prediction, these two channels did not exhibit identical kinetic behaviour: rather, superimposition of traces at the same voltage
suggested that activation was perceptibly slower for \( \Delta \alpha C \) than HCN2 R591E (Figure 2.7 left panels). After exclusion of an initial concave-negative lag period \((d)\), HCN2 R591E and \( \Delta \alpha C \) transients at -90 mV were fitted well to a single exponential curve as previously noted for HCN2 R591E (Wang et al., 2002). Although a double exponential fit was more suitable at stronger hyperpolarizations (see next paragraph), I confirmed that the quantitation outcome for -90 mV transients did not substantially change upon introducing a second exponential component for fitting, because the faster "early" component represented almost the entire fit - its relative contribution to decay amplitude \((f_{\text{early}})\) was almost 1 (see Figure 2.7, fits at -90 mV). Moreover, the time constant for the slower "late" component \((\tau_{\text{late}})\) grew markedly longer as hyperpolarization grew weaker, so that even if this late component had detectable amplitude, its time course was so slow as to be nearly linear (see Figure 2.8 for \( f_{\text{early}}, \tau_{\text{late}} \) data). At -90 mV, the time constant for the dominant early component \((\tau_{\text{early}})\) was \( 1710 \pm 380 \text{ ms} \) \((n = 9)\) for \( \Delta \alpha C \), which was over 2.2-fold slower than \( \tau_{\text{early}} \) for the comparably autoinhibited HCN2 R591E \( (760 \pm 240 \text{ ms}, n = 10) \).
Figure 2.7. Two constitutively autoinhibited HCN2 derivatives exhibit different activation kinetics

Note: Left panels show superimpositions of representative activation transients of each derivative at indicated voltages. Transients have been normalized to the total decay amplitude observed over the 3-s activation epoch. Middle and right panels show double exponential fits for activation transients from corresponding left panels. Current datapoints are shown as points; open circles show currents calculated at selected times for the fitted decay equation. Extension of fitted equation to times before the fit boundary is shown by dashed curves. For -130 mV traces, dotted grey curves show individual components of the double exponential fit, with an arbitrary vertical offset for clarity. Lag duration ($d$), time constants ($\tau_{early}$ and $\tau_{late}$), and fractional contribution of the earlier component ($f_{early}$) are indicated; note late components at -90 mV are negligible.
Figure 2.8. Mean values of all activation kinetics parameters for HCN2 WT and derivatives

Note: Points plot means and error bars show ranges of values (for plots of time constants and $d$) or SD (for $f_{early}$). Dataset for HCN2 R591E is shown in gray in all panels for comparison. In the plots of time constants (upper panels), open points show $\tau_{early}$ and solid points show $\tau_{late}$; for transients with a quasi-linear component, the $\tau_{late}$ was $>10$ s and was excluded from calculation of mean and range. Numerals in parentheses at the top of each upper panel indicate, for each voltage, the number of transients fitting a single exponential ($m_1$), the number of transients with quasi-linear components ($m_2$), and the total number of transients analysed ($n$), formatted as ($m_1, m_2 / n$).

I extended the comparison of autoinhibited channels to stronger hyperpolarizations, as different mechanistic steps may be rate-limiting at different voltages. Again, $\Delta\alpha C$ opened significantly slower than HCN2 R591E. For both derivatives, a double exponential fit was superior to a single exponential fit at stronger...
hyperpolarizations (Figure 2.7, fits at -130 mV; Figure 2.2) which might reflect slow formation of secondary open states after the initial fast closed-open transition (Altomare et al., 2001). However, the early component accounted for the majority of activation transient amplitudes (mean \( f_{\text{early}} > 0.5 \) for all derivatives in this study at all voltages), so I focused on this parameter for all future kinetic comparisons. The mean \( \tau_{\text{early}} \) for \( \Delta \alpha \) was slower than that of HCN2 R591E by a factor of >1.5-fold at all voltages studied (one-sided \( p < 0.001 \), for -90 mV through -150 mV; Figure 2.9). There was also a noticeable lengthening of the lag duration \( d \) in \( \Delta \alpha \) by a factor of >1.3-fold compared to HCN2 R591E at hyperpolarizations -110 mV and more negative (one-sided \( p < 0.002 \) for all voltages except -90 mV).

Thus while the different modifications of the C-terminal region produce autoinhibition to a similar degree as indicated by \( V_{1/2} \) in \( \Delta \alpha \) and HCN2 R591E, the two inhibition mechanisms are not exactly coincident, because only HCN2 R591E is capable of a relatively fast response to hyperpolarization (e.g. \( \tau_{\text{early}} < 100 \) ms at -150 mV and < 1 s at -90 mV; Figure 2.9).
Figure 2.9. Difference in early phase of activation between constitutively autoinhibited channels persists over a range of voltages

Note: Points plot mean values of $\tau_{\text{early}}$ and $d$ for HCN2 R591E (blue squares) and $\Delta\alpha C$ (green circles). Error bars on log-axis plots show ranges of values. See Figure 2.8 for $f_{\text{early}}$ and $\tau_{\text{late}}$ values characterizing the minority late phase. For each derivative a total of 10 recordings was analysed, except that one transient of $\Delta\alpha C$ at -90 mV was excluded because its total amplitude was < 60 nA. The HCN2 R591E $\tau_{\text{early}}$ is significantly smaller than HCN2 $\Delta\alpha C$ at all tested voltages (one-sided $p < 0.001$ for all voltages). The HCN2 R591E $d$ value is significantly smaller than HCN2 $\Delta\alpha C$ at all tested voltages except -90 mV (one-sided $p < 0.002$ for -110 mV, -130 mV, and -150 mV).

A previous study (Wang et al., 2002) showed time constants generally slower than this study for full length channels at comparable voltages, which suggests differences in bath solutions or endogenous levels of modulating factors in oocytes could lead to different rates for the same mechanism. Thus I should exercise caution in comparing absolute numerical values of kinetic constants between studies. Rather, the hypothesis testing is based on comparisons between channels in one set of oocytes.
2.3.2. The helix αC but not the extreme-C region is necessary to preserve a fast early phase of activation similar to unliganded full-length HCN2 derivatives

The difference in kinetics of HCN2 R591E and ΔαC raises the issue of which one of these derivatives more accurately mimics the behaviour of an unliganded HCN2 WT channel. In one scenario, ΔαC could reflect the mechanism and kinetics of a full length unliganded HCN2 channel, and HCN2 R591E could possess a non-canonical structure and mechanism due to its charge-reversing mutation, that made its activation faster than unliganded HCN2 WT. In an opposing scenario, HCN2 R591E could reflect the mechanism and kinetics of unliganded HCN2, and the presence of the helix αC or extreme-C region or both could be important for the channel to maintain a specific conformation enabling fast activation. This "quickening" conformation would be preserved in HCN2 R591E and unliganded HCN2 WT but would be compromised by truncation of the CNB fold in ΔαC. I favour this latter scenario based on comparison of the TEVC recordings of HCN2 WT and HCN2 R591E, as explained below.

HCN2 WT channels exist mostly in unliganded form during the early activation period in TEVC experiments, because the concentration of cAMP is low in the *Xenopus* oocyte as for other typical cells under basal conditions (DiFrancesco and Tromba, 1988; Wang et al., 2002). Thus when a strong hyperpolarization is applied to HCN2 WT in a TEVC recording, the early phase of the activation transient predominantly reflects the opening of unliganded channels, and substantial cAMP binding to accumulate liganded channels will occur only during a later phase (Figure 2.3). In contrast, when a weak hyperpolarization is applied to HCN2 WT, the activation rate of unliganded channels is slow enough that the observable activation transient is dominated by the small number of cAMP-liganded channels with faster activation. With a longer activation, such as 3 s, cAMP will have bound to HCN2 WT regardless of the activation voltage; this is why the $V_{1/2}$ value still reflects HCN2 WT in the liganded form.

This contrast allows us to look for evidence of anomalously rapid activation of HCN2 R591E by comparing its early phase of activation to that of HCN2 WT (Figure 2.3) specifically at strongly hyperpolarized voltages where both time constants reflect opening of unliganded channels. If the charge-reversing mutation in HCN2 R591E
confers anomalously rapid activation, I would expect $\tau_{\text{early}}$ for HCN2 R591E to be faster than $\tau_{\text{early}}$ for HCN2 WT at the same strong hyperpolarization. However, I saw no evidence for this: the $\tau_{\text{early}}$ of HCN2 R591E was not significantly faster than that of HCN2 WT at any voltages (one-sided $p > 0.08$, Figure 2.8). At -110 mV or weaker the $\tau_{\text{early}}$ of HCN2 R591E was significantly slower than that of HCN2 WT (one-sided $p < 0.009$), as expected since the HCN2 WT recordings at weak hyperpolarizations reflect cAMP-ligated channels. Overall, the comparison of early phases in activation of HCN2 WT and HCN2 R591E show no evidence for HCN2 R591E activating anomalously fast compared to unliganded HCN2 WT.

I further observed comparable $\tau_{\text{early}}$ values in another derivative, $\Delta XC$, which lacks the extreme-C region but has an intact CNB fold along with arginine at position 591. The half maximal effective concentration (EC50) for cAMP binding to $\Delta XC$ is ~10-fold higher than for HCN2 WT (Chen et al., 2001; Zagotta et al., 2003). This strongly suggests a weaker overall cAMP affinity in $\Delta XC$ compared to HCN2 WT, so that $\Delta XC$ is even more likely than HCN2 WT to be unliganded in the early phase of activation because of the low levels of endogenous cAMP in the oocyte. I found that $\Delta XC$ exhibited gating properties that were not noticeably inhibited relative to full length HCN2 WT (Figure 2.10). This included a similar $V_{1/2}$ (for $\Delta XC$, -86.2 ± 9.2 mV, $n = 9$; not significantly more negative than for HCN2 WT, one-sided $p > 0.1$), and qualitatively similar biphasic activation kinetics. There was moreover no significant slowing found for any of the fitted gating parameters of $\Delta XC$ relative to HCN2 WT, one-sided $p > 0.2$ for all comparisons) including time constants of either the early or late components ($\tau_{\text{early}}, \tau_{\text{late}}$), and duration of the lag phase ($d$) (Figure 2.8). Even the fractional contribution of the early component ($f_{\text{early}}$) was not significantly different (two-sided $p > 0.07$, all voltages). This is consistent with both HCN2 and $\Delta XC$ being primarily in the unliganded state at the holding voltage preceding the activation epoch in the protocol.
Figure 2.10. Activation properties of ΔXC in intact oocytes closely resemble those of HCN2 WT

Note: Top panel shows example conductance-voltage relation of ΔXC formatted as in Figure 2.5 (data points and fitted solid curve), including HCN2 WT relation for comparison (dashed line, from Figure 2.5). Boltzmann equation fit parameters for ΔXC were $V_{1/2} = -85.1 \text{ mV}, s = 13.2 \text{ mV}$. Lower panels show activation transients of ΔXC at indicated voltages, with superimposed HCN2 WT transients for comparison. Superimpositions and double exponential fits are formatted as in Figure 2.7. Note that the late phase kinetics are presumably dominated by the second-order off-rate of cAMP; the similarity in its rate and relative amplitude in ΔXC and HCN2 WT suggests that the higher EC50 for cAMP in ΔXC derives from a faster off-rate with no change in on-rate.
Thus ΔXC has an intact CNB fold and activates with a fast early phase (e.g. $\tau_{\text{early}} \sim 500$ ms at -90 mV) whose dominant contribution is likely to be from unliganded channels resembling HCN2 R591E or unliganded HCN2 WT. But the fast response is significantly slowed on deletion of the helix $\alpha_C$ ($\tau_{\text{early}} \sim 1700$ ms at -90 mV). The helix $\alpha_C$ can therefore be viewed as a critical element not only for relief of autoinhibition upon cAMP binding, but also for maintaining a "quickening" conformation that supports efficient activation kinetics when the channels are still unliganded.

2.3.3. **Relief of autoinhibition by deletion of the CNB fold fails to preserve the fast activation kinetics of full length HCN2**

Deleting the inhibitory CNB fold altogether should relieve autoinhibition and, if thermodynamic and kinetic effects are parallel, might be expected to speed up activation compared to that of intact autoinhibited channels; on the other hand, the loss of helix $\alpha_C$ in such a deletion might be expected to prevent the quickening conformation and thus slow down activation. To investigate the relative importance of autoinhibition and the quickening conformation for determining activation kinetics, I examined ΔCNB, the same derivative which was originally used to establish the autoinhibition model (Wainger et al., 2001); this derivative contains the C-linker but lacks the entire CNB fold and the extreme-C region.

ΔCNB had a mean $V_{1/2}$ value of -82.9 ± 7.4 mV ($n = 6$; Figure 2.11A-B) which was significantly more positive than that of autoinhibited HCN2 R591E (one-sided $p < 0.002$) but not that of HCN2 WT (one-sided $p > 0.4$; see Figure 2.6 for summary of $V_{1/2}$ means). As with HCN2 R591E, activation transients of ΔCNB showed a late component (Figure 2.11C) whose contribution decreased with progressively weaker hyperpolarizations, as distinct from the slow cAMP-dependent phase persisting at all voltages typical of HCN2 WT (Figure 2.3, Figure 2.8). This confirms that as expected, ΔCNB had autoinhibition relieved in a manner different than HCN2 WT, in that it did not involve slow cAMP binding during the activation transient.
Figure 2.11. Channel lacking the CNB fold exhibits slower activation than intact channels with or without cAMP binding

Note: A: Representative example of activation currents for $\Delta$CNB with traces at -70 mV and -150 mV highlighted. Schematic icon at top shows truncated structure retaining only C-linker in C-terminal region. B: Conductance-voltage relation from panel a formatted as in Figure 2.5 (data points and fitted solid curve), including HCN2 WT relation for comparison (dashed line, from Figure 2.5). Boltzmann equation fit parameters for $\Delta$CNB were $V_{1/2}$ = -79.4 mV, $s$ = 19.2 mV. C,D: $\Delta$CNB activation transient at -130 mV with double exponential fit (c, formatted as in Figure 2.7 right) and with superimposed HCN2 R591E transient (d, normalized as in Figure 2.7 left). See Figure 2.12 for further example at -90 mV and comparison with HCN2 WT. E,F: Mean parameters for early phase of $\Delta$CNB activation (points, formatted as in Figure 2.9; see Figure 2.8 for $\tau_{\text{late}}$ and $f_{\text{early}}$); a total of 6 recordings were analysed. For comparison, mean values without error bars are shown for HCN2 WT (black dashed lines) and HCN2 R591E (blue dashed lines) are shown (see Figure 2.8 for error bars).

Despite the relief of autoinhibition, $\Delta$CNB failed to show markedly faster kinetics compared to unliganded full-length channels HCN2 R591E and HCN2 WT (Figure 2.11D-F, Figure 2.12). The mean $\tau_{\text{early}}$ of $\Delta$CNB was significantly slower than that of the two full-length channels at all voltages (Figure 2.11E, one-sided $p < 0.05$). The slowness
of ΔCNB activation is especially remarkable considering that $V_{1/2}$ of ΔCNB is at least 15 mV more depolarized than that of the autoinhibited derivatives, indicating a stronger driving force for inward voltage-sensor movement in ΔCNB at a fixed voltage. This suggests that the removal of autoinhibition by deleting the CNB fold was insufficient to ensure fast activation kinetics. I propose that just as the loss of the helix αC in ΔαC compromised the "quickening conformation" of the intact CNB fold found in ΔXC, a similar distortion of conformation occurs upon deletion of the entire CNB fold to produce ΔCNB.
Figure 2.12. Comparison of ΔCNB activation to that of full-length derivatives

Note: Activation transients of ΔCNB at voltages as indicated, with superimposed transients of full-length derivatives as indicated for comparison. Double exponential fit of -90 mV trace is formatted as in Figure 2.7. Note the dominant exponential $\tau_{\text{early}}$ for ΔCNB is intermediate between the $\tau_{\text{early}}$ and $\tau_{\text{late}}$ from the double exponential fit for HCN2 WT (Figure 2.3 top); to illustrate this, the two components of the HCN2 WT fit are shown with their starting point placed on the fit boundary datapoint (black circle). The fit boundary datapoint for the ΔCNB transient is marked with a red circle.

The effect on activation kinetics due to losing the quickening conformation appears similar in severity across a range of activation voltages. Comparing HCN2 WT and ΔCNB, which show similar $V_{1/2}$ values characteristic of relieved autoinhibition, the
HCN2 WT channels with the quickening conformation had $\tau_{\text{early}}$ values at least 2 fold faster across all voltages studied from -90 mV to -150 mV (Figure 2.11E). While I typically made comparisons between channel types at a fixed test hyperpolarization, the kinetic difference between channel types due to the quickening conformation is even more dramatic if I take the alternative approach of selecting test voltages a fixed difference from each channel's $V_{1/2}$ value. Thus, choosing a test voltage approximately 30 mV negative of $V_{1/2}$, the $\tau_{\text{early}}$ of HCN2 R591E at -130 mV (140 ± 30 ms, $n = 10$) is >3.8 times faster than the $\tau_{\text{early}}$ of $\Delta$CNB at -110 mV (530 ± 100 ms, $n = 6$). The difference in activation speeds between $\Delta$CNB and full-length derivatives is also sizable compared to the relatively shallow dependence of kinetics on activation voltage. The mean $\tau_{\text{early}}$ at -150 mV for $\Delta$CNB is similar to $\tau_{\text{early}}$ at -110 mV for HCN2 WT (Figure 2.11E): thus truncating the CNB fold from an unliganded full-length HCN2 channel has roughly the same effect on kinetics as weakening the applied hyperpolarization by 40 mV. For comparison, I note that 40 mV is much larger than the approximately 20 mV positive shift in the $V_{1/2}$ that results from binding of cAMP to a full-length HCN2 channel (Wainger et al., 2001).

### 2.3.4. The voltage-independent gate-opening step is not rate-limiting, whereas the quickening conformation but not autoinhibition controls a voltage-dependent rate-limiting step

The gate-opening step in unliganded HCN2 has been shown to be a voltage-independent equilibrium step: notably, the open probability reaches a limiting maximum value less than 100% even at strong hyperpolarizations that should drive voltage-dependent equilibria maximally forward (Craven and Zagotta, 2004). This limited open probability was apparent in the early phase of activation of HCN2 WT in my TEVC recordings (Figure 2.3): unliganded channels opened during the early phase to a maximum open probability less than one, before cAMP binding in the late phase led to further increases in open probability (Wang et al., 2002). However, while this demonstrated the thermodynamic influence of the voltage-independent gate-opening step, my recordings found little evidence of a voltage-independent limit to activation kinetics at strong hyperpolarization. Instead, the value of $\tau_{\text{early}}$ continued to decrease with
stronger hyperpolarizations for all the channels studied (Figure 2.9 for HCN2 R591E and ΔαC, Figure 2.11E for ΔCNB). This means that over a large voltage range, the voltage-independent gate-opening step of HCN2 channels must be rapidly equilibrating compared to the rate-limiting voltage-dependent steps that are presumed to involve inward S4 charge movement in all HCN channels (Männikkö et al., 2002; Bruening-Wright et al., 2007).

Since HCN2 R591E and ΔαC exhibited voltage-dependent \( \tau_{\text{early}} \) values of 80 - 120 ms at -150 mV, I can infer the voltage-independent gate-opening step has a time constant not more than ~100 ms in these channels. A previous study (Wang et al., 2002) also failed to detect a plateau in the \( \tau \) vs. \( V \) relationship, but that study did not test voltages more than 25 mV negative of the \( V_{1/2} \). My observations extend to -150 mV, which in the case of ΔCNB is a full 70 mV more negative than \( V_{1/2} \). While there are possibly multiple voltage-dependent steps in the activation pathway that are rate-limiting, my results show that a subset of such steps (i.e., at least one step) must be sensitive to the presence or absence of the quickening conformation: for concision in further discussion, I will refer to this key subset of voltage-dependent steps as the Quick-Activating (QA) steps. A quantitative indicator of voltage dependence in each derivative was calculated as the ratio of \( \tau_{\text{early}} \) values for pairs of activation voltages spaced 20 mV apart, e.g. \( \tau_{\text{early}(-130 \text{ mV})} / \tau_{\text{early}(-150 \text{ mV})} \) (Figure 2.13). As hyperpolarization grew stronger, the voltage-dependence did grow somewhat weaker (ratio values closer to unity), but even at the strongest hyperpolarization the ratio \( \tau_{\text{early}(-130 \text{ mV})} / \tau_{\text{early}(-150 \text{ mV})} \) was > 1.7 for both HCN2 R591E and ΔαC. The \( \tau_{\text{early}} \) voltage-dependence ratios for ΔCNB were slightly lower than corresponding ratios for HCN2 R591E and ΔαC (significant, one-sided \( p < 0.04 \) for all comparisons) but were still well above unity.
Thus my discovery of the quickening conformation provides an answer to Q1 of my thesis. Deletion of the CNB fold from an unliganded HCN channel can be understood to have different effects on different steps of the activation pathway. The voltage-independent gate-opening step likely has its equilibrium shifted towards the open state due to removal of autoinhibition, producing the positively shifted $V_{1/2}$. Quite possibly, the gate-opening step also has its rate increased in parallel with the thermodynamic effect, but this step is too fast to be rate-limiting for the overall activation rate. On the contrary, disruption of the quickening conformation in ΔCNB decreases the rate of one or more QA steps, producing slower voltage-dependent activation overall. This scenario can be contrasted with the "relief" of autoinhibition by a different means, that is, addition of cAMP to a channel with a complete CNB fold. The voltage-independent gate-opening step would be similarly shifted in thermodynamic terms, enabling higher maximal open probability and positively shifted $V_{1/2}$. But additionally, cAMP binding accelerates the QA steps with the quickening conformation always present; this effect might more properly be viewed as "enhancement" rather than "relieving autoinhibition", since the effect does not mimic the consequences of altogether removing the autoinhibitory CNB fold.
2.3.5. Deactivation is slowed down by cAMP binding to an intact CNB fold more than by autoinhibition relief through CNB fold deletion

Another feature of the autoinhibition mechanism I wished to test was the speeding of deactivation, which has been reported to be more pronounced than effects on activation kinetics (DiFrancesco, 1999). I tested deactivation using a protocol with a 3-s activation epoch at a strong hyperpolarization which should enable HCN2 WT channels to accumulate in the cAMP-liganded state before deactivation is tracked. I would thus expect that among the various derivatives, relief of autoinhibition as indicated by $V_{1/2}$ ($\Delta CNB$ or cAMP-liganded HCN2 WT) should be reliably associated with slower deactivation. Because the endpoint of deactivation transients was reliably achieved for all experiments, I measured deactivation using the parameter $t_{1/2}$ (time to reach midpoint of total decay amplitude); $t_{1/2}$ should take into account both the initial lag phase and the time for current decay.

Both HCN2 R591E and $\Delta \alpha C$ both deactivated faster than HCN2 WT (Figure 2.14A): at a fixed voltage of 0 mV, the mean $t_{1/2}$ values were $140 \pm 22$ ms ($n = 10$) and $170 \pm 14$ ms ($n = 10$) for HCN2 R591E and $\Delta \alpha C$ respectively, significantly faster than that of HCN2 WT (290 $\pm 58$ ms, $n = 39$; one-sided $p < 10^{-6}$ for both comparisons with HCN2 WT). These findings confirm the well-known effect of cAMP to slow down deactivation. I considered that the $V_{1/2}$ of HCN2 WT was 15 to 17 mV more depolarized than that of other derivatives, indicating a weaker driving force for outward voltage-sensor movement in HCN2 WT at a fixed voltage. However, the weaker driving force cannot be the sole explanation for the slower deactivation of HCN2 WT: testing deactivation with greater driving force at +40 mV did accelerate HCN2 WT deactivation, but the effect was $< 1.4$-fold and the kinetics still did not match the fast deactivation of the autoinhibited channels at 0 mV (Figure 2.14B). Therefore, autoinhibition relief by cAMP binding has an effect on deactivation that is disproportionately large compared to its effect on $V_{1/2}$. 

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Figure 2.14. cAMP-ligated HCN2 channels exhibit open state trapping with slower deactivation compared to autoinhibited HCN2 derivatives and truncated ΔCNB

Note: A: Representative examples of deactivation currents at 0 mV, each normalized to total decay amplitude. Traces are from HCN2 WT (black) and HCN2 R591E (blue) in each panel, and ΔαC (green, left panel) and ΔCNB (red, right panel). Deactivation $t_{1/2}$ values for each example trace are indicated, with HCN2 R591E showing the smallest $t_{1/2}$ value. Axis break indicates change in plot timescale for clarity; no data was omitted. See Figure 2.4 for full deactivation protocol and traces at different voltages. B: Mean $t_{1/2}$ values for all channels with error bars showing SD; for some channels the bars are drawn wider for clarity. Symbols are: HCN2 WT, black up-triangles with wider error bars (number of recordings $n = 39$); ΔXC, purple down-triangles ($n = 9$); HCN2 R591E, blue squares ($n = 10$); ΔαC, green circles ($n = 10$); ΔCNB, red diamonds with wider error bars ($n = 6$).

For the truncated ΔXC, which retains a CNB fold capable of mediating cAMP potentiation, slow deactivation was observed matching HCN2 WT (Figure 2.14B). In contrast ΔCNB, with autoinhibition constitutively relieved, did not deactivate slowly like cAMP-ligated HCN2 WT: ΔCNB deactivation kinetics were >1.7 fold faster than HCN2 WT at all voltages studied (significant, one-sided $p < 10^{-4}$; Fig. 2.14A right, and Figure
Thus, the slow deactivation characteristic of cAMP-liganded HCN2 WT requires not only relief of autoinhibition (successful in ΔCNB) but also requires an intact and cAMP-liganded CNB fold (unavailable to ΔCNB). This provides an example of "open state trapping" which has been observed in HCN4 derivatives (Wicks et al., 2009, 2011), similarly reliant on the presence of an intact CNB fold with bound cAMP. Open state trapping illustrates well that the extent of autoinhibition as measured by $V_{1/2}$ alone is not a reliable predictor of deactivation kinetics. As with activation kinetics, it provides another case where "relief of autoinhibition" is not an apt description of the effect of cAMP binding.

Conversely, while the two autoinhibited derivatives HCN2 R591E and ΔαC had very similar $V_{1/2}$, they showed differences in deactivation kinetics. The deactivation $t_{1/2}$ values for ΔαC were slower than those of HCN2 R591E with a factor difference between 1.2 and 1.4 for voltages from 0 to +40 mV (Figure 2.14B). This difference was less dramatic than was found for $\tau_{\text{early}}$ of activation (>1.5 fold for voltages from -90 to -150 mV) but it was statistically significant (one-sided $p < 0.002$) for all three voltages studied. Thus of these two constitutively autoinhibited derivatives, the full length HCN2 R591E shows faster kinetics for both activation and deactivation.

### 2.3.6. Inhibition of HCN2 by PIP$_2$ depletion fails to slow down activation kinetics

The above work addressing Question 1 of my thesis showed that the extent of autoinhibition as indicated by $V_{1/2}$ was a poor predictor of both activation and deactivation kinetics. To test an example of HCN channel modulation that does not involve the CNB fold directly, I examined the effect of the lipid messenger PIP$_2$. The binding sites for PIP$_2$ in the transmembrane and C-linker regions of HCN channels (Flynn and Zagotta, 2011) are present in all the derivatives studied in this work. Hence I investigated Q2 of my thesis: I tested whether PIP$_2$ depletion would slow activation kinetics of HCN2 WT in a fashion parallel to the hyperpolarization of $V_{1/2}$. I used wortmannin, an inhibitor of phosphatidylinositol (PI) 4-kinase (Vanhaesebroeck et al., 2001), to decrease the available levels in the oocyte of phospho-PI derivatives including PIP$_2$ (Pian et al., 2006; Zolles et al., 2006).
HCN2 WT activation was tested in paired measurements made in the same oocyte before and after treatment with 45 μM wortmannin (Figure 2.15). A negative shift in $V_{1/2}$ was observed with a mean $\Delta V_{1/2}$ of $-16.6 \pm 7.9$ mV ($n = 11$) which was significantly different from zero (one sided $p < 10^{-4}$), indicating a degree of gating inhibition similar to that observed in previous reports (Pian et al., 2006; Zolles et al., 2006). Notably the $\Delta V_{1/2}$ from depletion of phospho-PI derivatives was similar in magnitude to the hyperpolarizing shift in $V_{1/2}$ observed from eliminating cAMP binding (HCN2 R591E or $\Delta\alpha_C$ vs. HCN2, difference of 16 - 18 mV in mean $V_{1/2}$). In analogous treatment with bath solutions containing the vehicle solvent DMSO without wortmannin, the mean $V_{1/2}$ shift was not significantly different from zero ($\Delta V_{1/2} = -3.0 \pm 7.9$ mV, $n = 11$, two-sided $p > 0.2$).
Figure 2.15. Treatment with wortmannin to deplete PIP$_2$ results in hyperpolarization of $V_{1/2}$ without slowing of activation kinetics

Note:  
A: Conductance-voltage relation for an example oocyte expressing HCN2 WT before and after treatment with PIP$_2$. See Figure 2.16 for full traces. Boltzmann equation fit parameters were $V_{1/2} = -83.0$ mV, $s = 16.2$ mV before wortmannin, and $V_{1/2} = -96.8$ mV, $s = 17.2$ mV after wortmannin ($\Delta V_{1/2} = -13.8$ mV). B: Points plot mean wortmannin effects for $\tau_{\text{early}}$ and $d$ for HCN2 WT and $\Delta \alpha C$ at different voltages. Effect on a parameter is calculated as the ratio of the value after wortmannin treatment to the value before treatment. Error bars show SD, with bars for $d$ drawn wider for clarity; number of paired recordings $n = 11$ for HCN2 WT and $n = 4$ for $\Delta \alpha C$, except one recording $\Delta \alpha C$ at -90 mV was excluded because its amplitude was < 60 nA. C-F: Superimpositions of normalized example transients before and after wortmannin treatment, for channels and voltages as marked, with wortmannin effects for $\tau_{\text{early}}$ and $d$ indicated. HCN2 WT examples are from the oocyte in panel a; $\Delta \alpha C$ examples are from an oocyte where the shift in $V_{1/2}$ due to wortmannin treatment was $\Delta V_{1/2} = -6.7$ mV.
Although wortmannin treatment clearly inhibited gating, there was no statistically significant increase in $\tau_{\text{early}}$ or lag duration $d$ at all voltages studied (one-sided $p > 0.06$; Figure 2.15B left panel) with the exception of a 10% increase in $d$ at -150 mV (significant, one-sided $p < 0.03$); in fact, for -90 mV there was a slight reduction in mean $\tau_{\text{early}}$. I confirmed the same lack of effect on activation kinetics in $\Delta\alpha C$ where cAMP binding would not be a factor. Wortmannin treatment hyperpolarized the $V_{1/2}$ of $\Delta\alpha C$, albeit to a lesser extent than seen in HCN2 WT (mean $\Delta V_{1/2}$ for $\Delta\alpha C$, -4.8 ± 2.5 mV, $n = 4$; significantly different from zero, one-sided $p < 0.02$). Just as for HCN2 WT, there was no associated significant increase in $\tau_{\text{early}}$ or $d$ over all voltages studied (one-sided $p > 0.1$; Figure 2.15B right panel) with the exception of a 9% increase in $\tau_{\text{early}}$ at -130 mV (significant, one-sided $p < 0.02$). This provides as answer to Q2 of my thesis: potentiation of $V_{1/2}$ by PIP$_2$ is not associated with a parallel increase in activation speed.
Notably, transients at each test voltage had a similar shape before and after wortmannin treatment (Figure 2.15C-F), and in fact were visually indistinguishable (superimposable) after being scaled to the absolute transient amplitude. Thus was true even at weak hyperpolarizations near the $V_{1/2}$ value, where the total amplitude of the 3-s activation transient was reduced substantially upon wortmannin treatment, leading to hyperpolarized $V_{1/2}$. This is consistent with a scenario where any steps influenced by PIP$_2$ are rapidly equilibrating (not rate-limiting); those steps would have equilibrium shifted towards the reverse direction, resulting in a hyperpolarizing shift of $V_{1/2}$ without altering the activation kinetics. Moreover, quite obviously the negligible effect of PIP$_2$ depletion on activation kinetics of full length HCN2 is distinct from the slowing of activation kinetics resulting from removal of the quickening conformation to produce $\Delta$CNB.

### 2.4. Discussion

#### 2.4.1. Insufficiency of simple autoinhibition model based on $V_{1/2}$ assessment

Consequences for HCN channel modulation from truncations of the C-terminal region are likely to have physiological relevance, since C-terminal truncations have been identified in human patients with cardiac sinus node defects (Schulze-Bahr et al., 2003; Schweizer et al., 2010) and even in myocardial tissues of healthy mice (Ye and Nerbonne, 2009). Thus it is essential to generate a cohesive biophysical theory of how the molecular structure can determine kinetic properties. Although the autoinhibition model has provided a valuable organizing framework for understanding the mechanistic consequences of C-terminal truncation, my results addressing Question #1 of my thesis show the limits of the simplest form of this model in regards to predicting activation speeds.

Beyond just answering Question #1, my work also unexpectedly uncovered a new mechanism distinct from autoinhibition. The most important mechanistic insights from my findings are that (a) for activation kinetics in particular, there is a quickening effect derived from having a CNB fold which works in opposition to the hyperpolarization...
of $V_{1/2}$ mediated by the autoinhibition mechanism, and (b) overall the presence or absence of a full-length CNB fold including helix $\alpha$C is a more important determinant of kinetics than the presence or absence of autoinhibition. Unexpectedly I found that an autoinhibited derivative activates faster than a derivative with autoinhibition fully abolished by CNB fold deletion (HCN2 R591E activates faster than $\Delta$CNB). Among the five channels studied, the presence of a full-length CNB fold was associated with a faster early phase of activation compared to channels whose CNB fold was partial or missing; this held whether or not the full-length CNB fold was capable of binding endogenous cAMP to relieve autoinhibition during a later phase of activation. I propose that the presence of a full-length CNB fold ensures the preservation of a “quickening conformation” required for fast activation kinetics. Deletion of the extreme-C region is tolerated, but deletion of the helix $\alpha$C or the entire CNB fold compromises the quickening conformation resulting in slower activation. For deactivation kinetics, I found HCN2 WT with cAMP bound exhibits slower deactivation than the autoinhibition-free $\Delta$CNB, providing another example of the "open-state trapping" effect (Wicks et al., 2009, 2011) first seen in HCN4 channels containing an S4 mutation. Thus although $V_{1/2}$ is typically taken as the primary indicator of the inhibition status, it fails to correlate in a general fashion with either activation kinetics or deactivation kinetics, so that even derivatives with similar $V_{1/2}$ due to equivalent strength of constitutive autoinhibition can exhibit different kinetics ($\Delta\alpha$C kinetics are slower than HCN2 R591E for both activation and deactivation).

Imagining the minimal voltage-gating unit to consist of an HCN channel truncated to remove the CNB fold, the autoinhibition theory envisions addition of the C-terminal region as introduction of a repressive straining element disfavouring the activated open state. However, this work shows this view holds reliably only in thermodynamic terms for the voltage-independent gate-opening step: for activation kinetics, addition of an unliganded C-terminal region actually speeds up the voltage-dependent "Quick-Activating" (QA) step that is rate-limiting. Consequently, for voltage-dependent activation and deactivation kinetics, cAMP binding to the C-terminal region enables the formation of new channel structures that kinetically favour the open state (faster activation and slower deactivation) beyond what could be achieved by the minimal unit.
2.4.2. Importance of a complete CNB fold

The CNB fold is not believed to be proximal to the transmembrane region which presumably controls voltage-dependent channel gating (Zagotta et al., 2003), so how might the CNB fold form a structural requirement for the "quickening conformation" enabling fast kinetics? It is probable that at least one of the elements involved in the quickening conformation is the C-linker immediately N-terminal to the CNB fold. The C-linker has been proposed to influence gating in two ways. First, the C-linker is directly connected to the S6 gate that regulates pore opening, and changes in intersubunit interactions governing C-linker self-association have been proposed to alter S6 orientation (Wu et al., 2012; Craven and Zagotta, 2004; Zhou et al., 2004; Lolicato et al., 2011). Second, the C-linker has been proposed to interact directly with the S4-S5 linker that transduces S4 movement to pore gate opening (Decher et al., 2004; Prole and Yellen, 2006; Kwan et al., 2012). Thus, the conformation and orientation of the four C-linkers within the tetramer can potentially influence both the voltage-independent gate-opening step and voltage-dependent steps such as the QA step.

Although many mechanisms are possible, I can propose a simple model for the structural effects of CNB fold truncation, where the rate of S4 movement in the QA step is sensitive to the relative orientation of the C-linker between neighbouring subunits. See Figure 2.17 for a schematic showing a hindering C-linker conformation (shown as wavy block) that slows down activation; this conformation can be avoided given the correct "quickening" conformation of the CNB fold (shown as horseshoe, Figure 2.17 first row). Even though the helix \( \alpha C \) is not in direct contact with the C-linker, it is quite plausibly important for maintaining C-linker orientation, because intersubunit self-association interactions have been demonstrated (Matulef and Zagotta, 2002) for the helix \( \alpha C \) in cyclic nucleotide-gated channels (homologues of HCN channels). Loss of the helix \( \alpha C \) to produce \( \Delta \alpha C \) (Figure 2.17 second row) preserves the straining structures critical for autoinhibition within the remaining portion of the C-terminal region, but might also re-orient that region; this re-orientation mildly disrupts the quickening conformation which slows down the rate-limiting QA step. A more dramatic loss of the entire CNB fold to produce \( \Delta \text{CNB} \) (Figure 2.17 third row) eliminates critical C-linker-CNB fold contacts; this could create a more dramatic disruption of the quickening conformation so that activation
becomes slower than in HCN2 R591E. At the same time, removal of the straining structure responsible for autoinhibition would thermodynamically enhance the voltage-independent gate-opening step (giving depolarized $V_{1/2}$ values) and might even speed up this step, but this step would not be rate-limiting in ΔCNB.
Figure 2.17. *Schematic summarizing how a deleterious conformation hindering activation could be avoided through the presence of the helix αC in the CNB fold, independent of the autoinhibition mechanism*

Note: Schematics show participating elements conceptually and are not intended to depict detailed structures. Two of four subunits are depicted with transmembrane domains shown as tilted rectangles. The QA step that is rate-limiting for activation involves hyperpolarization-dependent inward movement of S4 voltage-sensor (circle with plus sign), whereas the step that opens the S6 gate (reorientation of rectangles) is voltage-independent and rapidly equilibrating relative to the QA step; the pathway contains multiple other steps which are omitted. The C-terminal region is shown with C-linker (wavy block), cAMP binding site (horseshoe) and helix αC (zigzag); extreme-C region and tetramerization interactions of the transmembrane and C-linker regions are omitted. Differently truncated HCN channels have an intact CNB fold (first row) which is unliganded in intact oocytes before hyperpolarization, a CNB fold truncated before the helix αC (second row), or no CNB fold (third row). The gate-opening step is thermodynamically disfavoured by CNB-dependent autoinhibition (first and second rows). However, the presence of the helix αC (only first row) maintains the quickening conformation required for the channel to quickly proceed through the rate-limiting QA step. In channels missing helix αC (second and third rows) the C-linker enters an alternative “hindering” arrangement producing slow activation.
2.4.3. Comparison of TEVC results with previous excised patch studies

This study focuses on how the cytoplasmic CNB fold influences activation when it is unliganded, in contrast with numerous previous studies focused on understanding the cAMP regulation mechanism. Typical studies of cAMP regulation in HCN channels control the cAMP concentration by using either the inside-out excised patch configuration (Santoro et al., 1998; Gauss et al., 1998; Ludwig et al., 1998; DiFrancesco and Tortora, 1991; DiFrancesco, 1999; Wainger et al., 2001) or "whole-cell" patch-clamp of small mammalian cells (Qu et al., 2001), where cellular components are exchanged with the contents of the bath (excised patch) or pipette lumen (whole cell). One consequence of losing cellular components is a "rundown" suppression of HCN channel activity (DiFrancesco et al., 1986), most prominently including a hyperpolarization of the $V_{1/2}$ that has been shown to be caused by PIP$_2$ depletion (Zolles et al., 2006; Pian et al., 2006). The truncated HCN2 derivatives ΔCNB and ΔαC were first characterized in excised patch studies which were essential for establishing the autoinhibition model of cAMP regulation (Wainger et al., 2001); this study is the first to describe the kinetics of these truncation derivatives in intact oocytes before rundown has occurred.

A dramatic slowing of activation kinetics is another feature of rundown (DiFrancesco et al., 1986) that at first glance seems to parallel the hyperpolarization of the $V_{1/2}$. However, my findings addressing Question #2 of my thesis show the slowing effect is in fact unlikely to be due to PIP$_2$ depletion like the $V_{1/2}$ shift, since even a 20 mV hyperpolarizing shift in $V_{1/2}$ induced by wortmannin did not significantly slow down the early phase of activation of HCN2 WT. My findings complement those of a previous study where PIP$_2$ derivatives were added back to unliganded HCN2 in excised patches after rundown had occurred: a positive $V_{1/2}$ shift was obtained without accelerating activation kinetics (Pian et al., 2006). Thus the slowing of activation during rundown is most likely due to a modulatory factor distinct from PI derivatives like PIP$_2$.

There are two aspects of gating behaviour observed in the TEVC findings which differ from behaviours in previous excised patch experiments on the same channels; these differences suggest additional influences of cellular factors lost during rundown in the excised patch experiments. One difference concerns the detection of the voltage-
independent gate-opening step; the other difference concerns the relative activation rates of uninhibited ΔCNB vs. autoinhibited channels like HCN2 R591E and ΔαC. Closer examination shows that my TEVC experiments do not contradict the previous findings derived from excised patches, but rather provide more detail on relevant mechanistic steps, specifically regarding their operating rates in an intact cell and their susceptibility to rundown.

The voltage-independent gate-opening step is one example of a mechanistic step slowed by rundown: in intact oocytes, I found its time constant was shorter than ~100 ms in unliganded full-length channels (HCN2 R591E or HCN2 WT \( \tau_{\text{early}} \)), whereas it was ~500 ms in unliganded HCN2 WT in excised patches after rundown (Chen et al., 2007). The voltage-dependent QA step also appears to be slowed by rundown, such that in excised patches, time constants were in excess of 1500 ms for hyperpolarizations weaker than -130 mV (Chen et al., 2007). I note that rundown appears to slow down the gate-opening step preferentially over the QA step: in intact oocytes, the voltage-independent gate-opening step remains rapidly equilibrating compared to voltage-dependent steps even at 50 mV to 70 mV negative of \( V_{1/2} \), whereas in excised patches after rundown, the voltage-independent gate-opening step became rate-limiting at only 30 mV negative of \( V_{1/2} \) (Chen et al., 2007). This illustrates how in principle, different mechanistic steps can show different sensitivities to different cellular factors.

In intact oocytes, HCN2 R591E (autoinhibition imposed) showed faster activation than ΔCNB (autoinhibition removed); this is a reversed relationship to that found in excised patches (Wainger et al., 2001). This suggests that rundown affects the two derivatives differently. To be specific, I propose that the loss of cellular factors during excised patch rundown could cause a disproportionately large slowing effect on activation of HCN2 R591E and unliganded HCN2 WT, in comparison with ΔCNB where the slowing effect would be slight or none. Then excised patch measurements could show ΔCNB had faster kinetics than the full length constructs. Therefore taking my new findings with the previous excised patch results together, I propose that the activation pathways of HCN2 R591E and ΔCNB have different rate-limiting steps. The voltage-dependent QA step that is rate-limiting for HCN2 R591E in intact oocytes is strongly sensitive to rundown in excised patches, whereas a distinct step whose forward rate is
most severely compromised by CNB deletion is rundown-insensitive. The requirement of the QA step for a cellular factor (distinct from PIP$_2$) would be in addition to the requirement for the quickening conformation. Thus the cellular factor modulating the QA step might in fact still bind properly to ΔCNB or other truncated channels, but its presence would no longer be sufficient to preserve fast kinetics in those mutant channels due to lack of the quickening conformation.

I do not exclude the possibility that the observed TEVC vs. excised patch differences arise from other factors like bath solutions or even protein biogenesis. However, any such responsible factor apparently acts differently on ΔCNB than it acts on longer channels like HCN2 R591E, ΔXC, or HCN2 WT, and moreover acts differently on the voltage-dependent QA step than on the voltage-independent gate-opening step.
Chapter 3.

HCN channel deactivation includes two distinct VSD movement steps

An abstract based on the work in this chapter has been accepted for poster presentation at the Annual Meeting of the American Society for Biochemistry and Molecular Biology in Chicago, Illinois in April 2017. The abstract authors and title are as follows:

Magee, K.E.A., Claydon, T.W., and Young, E.C. “Fluorescence investigations of the rate-limiting step in the HCN ion channel deactivation pathway”.

The work in this chapter is also being prepared as a manuscript with intent to publish.

Contributions to this chapter:

Max Kobelev subcloned HCN2F ΔCNB. For HCN2F-based channels Max Kobelev completed ~5% of TEVC recordings and ~40% of TEVC analysis under my direction.

Dr. Tom Claydon provided the VCF equipment for the work in this chapter, assisted with my VCF training, and provided comments on the abstract.

For this chapter I completed all the work except the tasks listed above. This includes subcloning the channels HCN2F and HCN2F R591E, completing all VCF recordings, completing all analysis of VCF recordings, and generating all figures. I completed ~95% of TEVC recordings of all HCN2F-based channels and ~60% of the analysis of TEVC recordings of HCN2F-based channels.
3.1. Introduction

3.1.1. The kinetics of VSD movements in HCN2 derivatives are unknown

The speed of VSD movements is an outstanding question for HCN channels. There is evidence from accessibility studies that S4 moves inward upon hyperpolarization (Männikkö et al., 2002; Vemana et al., 2004), but several features of gating movements remain unclear. For example, how fast are VSD movements relative to other steps? How strongly voltage-dependent are the speeds of VSD movements? How tight is the coupling between the VSD and the PD? Answers to these questions would provide substantial advances to present models of HCN channel gating and thus their physiological roles.

I will address the speed of a VSD movement in HCN2 derivative channels in this chapter to answer Questions #3, #4, and #5 of my thesis (see summary in section 1.3). Question #3 asks if there is a deactivation voltage where VSD movement is not rate-limiting for the deactivation pathway, and Question #4 asks whether the VSD movement step has stronger voltage dependence than the pore movement step. The cyclic allostery model predicts that the answer to both these question is yes, as the pore movement step is voltage-independent (Chen et al., 2007). However the Larsson group found a weak voltage-dependence of the pore opening step, and proposed a slow VSD movement step before a faster pore opening step at from -120 mV to -160 mV for spHCN1 channels (Bruening-Wright et al., 2007). Question #5 asks whether cAMP binding results in a change in the speed of VSD movements. Previous structural studies have suggested the C-linker comes in close proximity to the S4-S5 linker (Decher et al., 2004; Prole and Yellen, 2006), which could allow conformational changes in the CSD upon cAMP binding to transduce through the S4-S5 linker to the VSD. Question #5 also asks whether the speed of the VSD movement step is dependent on mode shift. The VSD presumably has different starting conformations for the deactivation pathway depending on the open state reached during activation. Different starting conformations of the VSD in mode i and mode ii for the deactivation pathway could result in different speeds of VSD movements. Directly measuring the speed of the VSD movement step
would clarify discrepancies and uncertainties regarding the cyclic allostery model and provide answers to Questions #3, #4, and #5.

3.1.2. Voltage clamp fluorometry is well suited for tracking slow VSD movements in HCN2 channels

VSD movements in HCN channels have previously proven difficult to track. Measuring gating current generated as gating charges move across the membrane is often used to study VSDs, but HCN channels prove difficult subjects for these experiments. Accessibility studies have found only two uncharged residues (spHCN1 Spos3 (Männikkö et al., 2002) and mHCN1 Spos3 and Lpos4 (Vemana et al., 2004)) that cross fully from the intracellular to the extracellular side of the membrane during gating. Although it is not necessary for a gating charge to cross the entire membrane, this suggests there might be less movement of S4 in HCN channels that other Kv channels. HCN channels thus may have only have one gating charge per subunit for a total of four gating charges, as opposed to the predicted 12-14 total gating charges in Shaker channels (Aggarwal and MacKinnon, 1996; Seoh et al., 1996) or 10 total gating charges in Kv1.2 channels (Ishida et al., 2015). Additionally, HCN channel gating kinetics are much slower than typical voltage-gated potassium channels such as Shaker or Kv1.2 channels. Gating currents in HCN channels thus provide small amplitudes with slow kinetics, rendering these experiments challenging to perform and interpret.

Consequently, only the fast spHCN1 subtype of HCN channels have had their VSD movements successfully tracked by gating current measurements. The Larsson group measured spHCN1 channel gating currents by blocking ionic currents with an HCN channel-specific blocker (Männikkö et al., 2002), or a P435Y mutation that renders the pore non-conducting (Männikkö et al., 2005; Bruening-Wright et al., 2007). The gating charge movements of the spHCN1 channel had a time constant of approximately 25 ms at a -120 mV hyperpolarization. However, gating currents have not been detected in slower subtypes such as HCN2 and HCN4 (Männikkö et al., 2005). These subtypes have functions where the speed of the gating pathway is important for proper tissue function, such as the contribution of HCN4 to the DD in spontaneous cardiac action potentials in the SA node (see section 1.1.2.1). The speed of VSD movements in the
HCN2 and HCN4 subtypes is thus a physiologically relevant outstanding question for all HCN channel gating models.

Voltage clamp fluorometry (VCF) is a technique that could circumvent the difficulties of gating current studies (reviewed in (Zhu et al., 2016)). VCF adds a fluorescence component to standard electrophysiology experiments to track conformational changes that occur in voltage-gated channels. By mutating an extracellular residue to a cysteine and removing any other extracellular cysteines, researchers can tag a channel region of interest with a sulphydryl-reactive fluorophore. As the channel changes conformation during gating, the environment around the fluorophore reporter may change. For example, the reporter may move towards a residue that quenches fluorescence such as a tryptophan. The number of photons emitted from the reporter would thus change, in which case registering fluorescence emission during gating would suggest the speed of gating movements. VCF protocols generate voltage-dependent fluorescence and current transients simultaneously, thus additionally allowing for a comparison of the speeds of VSD and pore gate movements.

VCF was first established in the late 1990s to show that S4 is the voltage sensor in Shaker channels (Mannuzzu et al., 1996; Cha and Bezanilla, 1997). VCF also showed S4 residues buried in the resting state can become more extracellularly-exposed with membrane depolarization, helping establish the direction of S4 gating movements (Mannuzzu et al., 1996). VCF has been completed in two joint studies on spHCN1 channels (Bruening-Wright et al., 2007; Bruening-Wright and Larsson, 2007). VCF is thus a previously successful technique that should enable the study of VSD movements in the slower subtypes of HCN2 and HCN4.

3.1.3. Summary of work and conclusions in this chapter

In this study I aimed to track the speed of a VSD movement during deactivation in HCN2 derivatives using VCF. I chose to place the reporter on the N-terminal region of S4, as this region may move during HCN channel gating. I chose to focus on the deactivation pathway for several reasons. First, this pathway is overall less studied than the activation pathway, despite evidence that the pathways are not simply the reverse of one another (Wicks et al., 2009). Second, I can examine the effects of mode shift on
deactivation, as I can isolate channels initiating deactivation from the O₁ or Oᵢ states by regulating the length of the activation step in a VCF recording. Third, I can examine the effects of cAMP binding (see section 1.1.4). In contrast, the effects of mode shift on activation cannot be studied because channels in the Cᵢ state have never been isolated; the effect of cAMP binding on activation cannot be studied in intact oocytes as endogenous cAMP is rarely bound to channels in the initial period of HCN channel activation (Wang et al., 2002).

In this study I characterize the speed of a voltage-dependent VSD movement that precedes channel closure. The speed of this VSD movement did not limit the rate of the deactivation pathway at strong depolarizations, and showed stronger voltage-dependence than pore closure. Both of these results support the proposals of the cyclic allostery model. The speed of this VSD movement was independent of cAMP binding in the strong voltage regime that was tested. The speed of this VSD movement was independent of mode shift at both strong depolarizations and weak depolarizations where S4 outward movement is presumed to be the rate-limiting step in deactivation. I thus propose the existence of an additional VSD movement step in the HCN channel pathway that occurs before pore closure that is independent of mode shift.

3.2. Materials and Methods

3.2.1. Channel composition

All channels in this chapter were subcloned into the high-expression vector pGEM-HE and expressed in *Xenopus laevis* oocytes as homomers. “HCN2” or “HCN2 WT” and “HCN2 R591E” are described in Chapter 2.

“HCN2ₐ” denotes an HCN2 derivative channel designed for VCF studies. HCN2ₐ possesses the Rneg3C mutation (HCN2 R300C, homologous to HCN1 R247C) in the N-terminal region of S4 to allow for covalent binding of the fluorophore. This channel also has a HCN1-based pore sequence that is identical to the pore from the ‘HCN1-R’ channel used previously for cysteine-based accessibility studies (Bell et al., 2004). HCN2ₐ thus has the N-terminal region through S3 from mHCN2 (residues mHCN2 1-
289), the S4-S6 helices from mHCN1 (mHCN1 residues 237-387), and the C-terminal region from mHCN2 (residues mHCN2 441-863). The mHCN1 region of HCN2F has four of five PD cysteines mutated: HCN1 C289I, C318S, C347S, C374T (homologous to HCN2 C351I, C371S, C400S, C427T) to prevent binding of the fluorophore to the pore. The HCN2F pore retains a cysteine at residue HCN1 303 (homologous to HCN2 356) as the channel does not express when this cysteine is mutated (Bell et al., 2004). This cysteine is not reactive to labeling agents (Bell et al., 2004). HCN2F was assembled from the HCN2 WT gene and a synthetic DNA fragment from Integrated DNA Technologies. “HCN2F R591E” is identical to HCN2F with the exception of the mHCN2 R591E mutation. “HCN2F ΔCNB” is identical to HCN2F with the exception of a stop codon at residue mHCN2 526 at the end of the C-linker to truncate the CNB fold and the extreme-C region. Both HCN2F R591E and HCN2F ΔCNB were constructed by subcloning based on HCN2 R591E and HCN2 ΔCNB (from Chapter 2). The coding sequences of all derivatives were verified using dideoxy sequencing (Eurofins Operon).

3.2.2. Electrophysiology

3.2.2.1. Oocytes

Oocytes were obtained and injected with RNA as described in Chapter 2. All data sets in this chapter were collected from the oocytes of at least two frogs for each channel type or condition.

3.2.2.2. Equipment and protocols

TEVC was performed as described in Chapter 2 and VCF was set up and performed as previously described (Van Slyke et al., 2010) using equipment in the laboratory of my collaborator Dr. Tom Claydon. The bath for VCF recordings was identical to that used for TEVC (described in Chapter 2). VCF reports fluorescence and current transients simultaneously for a given protocol. Each VCF recording was repeated 6-10 times (6-10 ‘runs’ as per the Clampfit 10.2 software), and the final transients represents the average from all runs. The first and last runs were always nearly superimposable, as determined by visual inspection. The photomultiplier tube gain setting was kept at a value of 700 – 1000 V/unit.
Fluorescence data was sampled at 4000 Hz and Bessel filtered at 250 Hz to improve signal to noise ratio in Clampfit 10.0 before analysis. Fluorescence traces routinely had a positive sloping baseline, meaning a higher final amplitude than starting amplitude, despite each protocol starting and ending at -40 mV. Each protocol with voltage changes to generate fluorescence and current deflections was thus followed by a baseline control protocol. This baseline control protocol was identical in length to the original but was held at a constant voltage of -40mV.

3.2.2.3. Labeling procedure

The fluorophore used for this chapter was the sulphydryl-reactive fluorescent tag tetramethylrhodamine-5-maleimide (TMRM; Invitrogen T6027). Oocytes were labeled using 5 μM TMRM in a “depolarizing solution” (in mM: 98 KCl, 1 MgCl₂, 2 CaCl₂, and 5 HEPES, pH 7.4). Oocytes were left in the labeling solution for 30-45 min at 10°C-16°C in the dark. Oocytes were only used for experiments for up to four hours after the end of their labeling incubation period.

3.2.2.4. Analysis

Fluorescence transients were analyzed as follows. The entire fluorescence trace of the associated baseline control recording (described above, filtered at 250 Hz using Clampfit 10.2) was fit to the straight line equation $F = mt + b$, where $F$ is fluorescence (measured in volts), $m$ is the slope, $t$ is the time within the recording, and $b$ is fluorescence at zero time. Baseline control recordings (taken before or after the protocol of interest) typically produced an $m$ value of approximately 10 mV/s. The fluorescence transient deflection of interest was filtered before fitting. Deactivation transients in entirety were then fitted to a single exponential equation with a sloping baseline ($F(t) = A \exp(-t/\tau) + mt + C$), where $A$ is the maximum amplitude of the fluorescence deflection, $\tau$ is the deactivation time constant, and $C$ is an arbitrary vertical offset. Fitting the fluorescence transients from the -130 mV activation pulse often required a double exponential fit with a sloping baseline ($F(t) = A_{\text{early}} \exp[-t/\tau_{\text{early}}] + A_{\text{late}} \exp[-t/\tau_{\text{late}}] + mt + C$, where $\tau_{\text{early}} < \tau_{\text{late}}$). Even when fitting to a double exponential equation, no fluorescence transient included an initial lag segment. For all fluorescence fits the $m$ value of the equation was fixed to be that of the associated baseline control recording.
Double exponential fits of activation current transients were completed as described in Chapter 2, with the exception of the calculation of $f_{\text{early}}$. The $f_{\text{early}}$ value was determined by the equation $f_{\text{early}} = A_{\text{early}} / \{A_{\text{early}} + A_{\text{late}}\}$. This procedure was also applied to deactivation current transients. A tau-weighted ($\tau_w$) value was computed. The $\tau_w$ value is a time constant that is a weighted average of the $\tau_{\text{early}}$ and $\tau_{\text{late}}$ components ($\tau_w = f_{\text{early}}(\tau_{\text{early}}) + (1-f_{\text{early}})(\tau_{\text{late}})$), where $f_{\text{early}}$ is the proportion of the transient described by $\tau_{\text{early}}$.

Steady state G-V curves were generated as described in Chapter 2. F-V curves were generated using the mean fluorescence value of a 10-ms window at the beginning of the -120 mV tail epoch immediately following the activation epoch in each sweep. Voltage sweeps from -40 mV to -150 mV were typically tested. Using Sigmaplot 10.0, activation voltages ($V$) and tail fluorescence values ($F$) were fit to the four parameter sigmoid Boltzmann equation $F = y0 + a / (1 + \exp[-(V - V_{1/2}) / s])$. The floating parameters were $a$ (positive, maximum time-dependent HCN channel fluorescence amplitude), $s$ (positive, reciprocal slope), $V_{1/2}$ (negative, midpoint activation voltage), and $y0$ (positive, baseline fluorescence). For presenting normalized G-V and F-V curves, the fitted curve was extended to extremely hyperpolarized and depolarized voltages (-215 mV and +15 mV, respectively). The curve value at +15 mV was used as a baseline and the curve was then normalized using the maximum curve value at -215 mV. The associated F-V or G-V data points were normalized using the same amplitude values as the fitted curve. In F-V or G-V figures, the curve is only extended from -195 mV to -5 mV.

For generating figures of transients, the number of data points within the fluorescence transient was reduced in Clampfit 10.2 for visual clarity so that data points occurred approximately every 600 $\mu$s. Then (unless otherwise noted in the figure caption) a smoothing technique was applied in Sigmaplot 10.0 software to reduce the fluorescence transient noise: each data point was replaced with the mean value of the data point along with the 10 preceding and 10 following data points. Thus each data point became a representation of the mean value of itself and 6000 $\mu$s on either end, or a total interval of 12.6 ms. Smoothing was not applied to current transients.
Mean values are reported ± standard deviation (SD) with number of determinations indicated by n. Comparisons of mean values were assessed by a two-sided t-test with a significance threshold of p = 0.05.

3.3. Results

The results section of this chapter is divided into five sections. The first discusses the characterization of HCN2 derivative channels for use with VCF (section 3.3.1), and the next three discuss results directly related to answering Question #3 (section 3.3.2), Question #4 (section 3.3.3) and Question #5 (section 3.3.4) of my thesis. A final section (section 3.3.5) discusses my interpretation of the structures in the HCN channel deactivation pathway based on the answers to Questions #3, #4, and #5.

3.3.1. Characterisation of HCN2 derivatives for VCF experiments

3.3.1.1. Rationale

I created a set of HCN2 derivatives with autoinhibition present or relieved (as in Chapter 2), with additional modifications to enable VCF studies of deactivation. As in Chapter 2, I sought to study the speed of VSD movements of a trio of HCN2 derivative channels: a channel with an intact CNB fold, a channel with an intact CNB fold and the R591E mutation to prevent cAMP binding, and a channel with a truncated CNB fold. This trio of HCN channels would allow me to examine VSD movements during deactivation in a variety of conditions. The comparison of channels with an apo or holo CNB fold will suggest how the conformational changes upon cAMP binding alter VSD movements. The comparison of truncated and apo channels will suggest how VSD movements differ in autoinhibited and autoinhibition-free channels. Finally, the comparison of truncated and holo channels will suggest how VSD movements differ in channels with and without OST. HCN2 WT and HCN2 R591E channels have been shown to undergo mode shift (Männikkö et al., 2005), thus the effects of mode shift on VSD movements during deactivation could be examined using more than one of these channels. Additionally, the VSD movements of all three channels can be studied across different depolarizations, to determine their voltage dependence. Thus this trio of channels will allow me to address
Question #3, #4, and #5 of my thesis, which all concern the speed of VSD movements and how the speed of these VSD movements is regulated.

I opted to bind the fluorophore to the residue Rneg3. The previous VCF studies on spHCN1 channels achieved the largest fluorescence deflections when the cysteine was introduced at position Rneg3 (Bruening-Wright et al., 2007). I thus introduced the cysteine mutation for covalently binding TMRM at the equivalent Rneg3 residue for all three HCN2 derivative channels. My focus in this chapter will be primarily on labelled channels, but unlabelled channels will be briefly addressed (see section 3.3.1.5). In addition to the Rneg3C mutation to permit labeling, the VCF channels also have an HCN1 pore region with four endogenous cysteines removed (see methods) that has been used previously and minimizes labeling to other extracellular regions of the channel (Bell et al., 2004) (Figure 3.1). I thus designated the HCN2 derivative channel with the Rneg3C mutation, and additional pore mutations to permit fluorescence experiments, as “HCN2-F”. The trio of channels used in this chapter is thus HCN2-F, HCN2-F R591E (which is unable to bind cAMP), and HCN2-F ΔCNB (which has no extreme-C region or CNB fold).
Figure 3.1. Schematic of HCN2_F channel composition

Note: **TOP:** Box schematic showing select channel subregions. PH = pore helix between S5 and S6. Position of Rneg3C mutation shown by pink line, position of cysteine mutations in the HCN1-pore shown by light blue lines, and position of invariant cysteine in S5 shown by dark blue line. See section 3.2.1 for rationale for mutations. Residues numbers are for HCN2_F, not HCN1. **BOTTOM:** Amino acid sequences of the S4 region of spHCN1, mHCN1, mHCN2, and HCN2_F. Pink C represents Rneg3C residue. Purple arrows designate residues in the neg/pos notation used throughout this thesis. Red line shows residues mutated to cysteines in previous VCF studies (Bruening-Wright and Larsson, 2007; Bruening-Wright et al., 2007): filled diamonds show positions that tracked mode shift movements, unfilled diamonds show positions that tracked gating movements. Green lines show mHCN1 residues tested by (Bell et al. 2004) for external MTSET accessibility. Green asterisk ‘**’ shows residues modified by external MTSET upon both hyperpolarized and depolarized voltages. Orange lines show mHCN1 residues tested by (Vemana et al., 2004) for external MTSET accessibility: Orange asterisk shows residue modified by external MTSET upon both hyperpolarized and depolarized voltages, consistent with (Bell et al., 2004). Orange ampersand ‘&’ shows residues modified by external MTSET upon depolarized voltages, in contrast with (Bell et al., 2004).

3.3.1.2. Labeled HCN2_F-based channels have behaviour predicted by the autoinhibition model

TEVC showed oocytes injected with RNA for any of the three HCN2_F-based channels and labeled with TMRM generated HCN-like currents (Figures 3.2 – 3.4). Both the activation and deactivation transients of all three channels started with an initial lag period, and the decay following the lag was well fitted to a double exponential equation (see methods). The two components were characterized as \( t_{\text{early}} \) and \( t_{\text{late}} \) as in Chapter 2. A \( \tau_{\text{weighted}} \) (\( \tau_w \), see methods) value was computed that represented the weighted
average of both components of current transient. We predict that $\tau_w$ would approximate the $\tau$ value of a poor single exponential fit. Though $\tau_w$ cannot be assigned to either component of a double exponential fit, it is a parameter that provided a general representation of the current transient: channels with larger $\tau_w$ values had slower deactivation.

![Figure 3.2](image-url)  
**Figure 3.2.** Representative TEVC recording of oocytes expressing HCN2f and labeled with TMRM  
**Note:**  
A: Voltage protocol, with a holding potential of -40 mV, activation steps every 10 mV from -40 mV to -170 mV for 3 s, and a deactivation step at +20 mV for 4 s. The deactivation step is followed by steps at 0 mV, +40 mV, 0 mV, for 500 ms and -40 mV for 1 s, to test channel leak. B: Current transients from the voltage protocol in A. Sweep with -130 mV activation highlighted in purple for clarity.
Figure 3.3. Representative TEVC recording of oocytes expressing HCN2<sub>F</sub> R591E and labeled with TMRM
Note: HCN2<sub>F</sub> R591E current transients from the voltage protocol in Figure 3.2. Sweep with -130 mV activation highlighted in green for clarity.

Figure 3.4. Representative TEVC recording of oocytes expressing HCN2<sub>F</sub> ΔCNB and labeled with TMRM
Note: HCN2<sub>F</sub> ΔCNB current transients from the voltage protocol in Figure 3.2. Sweep with -130 mV activation highlighted in pink for clarity.
In addition to characterizing the current transients produced by labeled oocytes, I also examined whether labeled channels adhered to present gating models. For HCN2<sub>F</sub> and HCN2<sub>F</sub> R591E, the data used is from VCF recordings. For HCN2<sub>F</sub> ΔCNB the data is from TEVC recordings of labeled oocytes. As this chapter is focused on the deactivation pathway, I tested the applicability of the autoinhibition and OST models to the three labeled channels. See Table 3.1 for the thermodynamic parameters of all HCN2 derivatives used in this chapter. See Table 3.2 for the activation kinetic parameters of all HCN2 derivatives, and Table 3.3 for the deactivation kinetic parameters of all HCN2 derivatives.

Table 3.1. Thermodynamic parameters for HCN2 derivatives expressed in unlabeled or labeled oocytes

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<tr>
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<th>G-V</th>
<th>F-V</th>
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<tbody>
<tr>
<td></td>
<td>V&lt;sub&gt;1/2[corr]&lt;/sub&gt; (mV)</td>
<td>s (mV)</td>
</tr>
<tr>
<td>Labeled HCN2&lt;sub&gt;F&lt;/sub&gt;</td>
<td>-107.0 ± 8.3</td>
<td>9.3 ± 2.0</td>
</tr>
<tr>
<td>Labeled HCN2&lt;sub&gt;F&lt;/sub&gt; R591E</td>
<td>-119.1 ± 4.9</td>
<td>12.4 ± 2.9</td>
</tr>
<tr>
<td>Labeled HCN2&lt;sub&gt;F&lt;/sub&gt; ΔCNB</td>
<td>-97.1 ± 6.9</td>
<td>17.8 ± 2.7</td>
</tr>
<tr>
<td>Unlabeled HCN2&lt;sub&gt;F&lt;/sub&gt;</td>
<td>-100.4 ± 6.0</td>
<td>13.6 ± 3.4</td>
</tr>
<tr>
<td>Unlabeled HCN2&lt;sub&gt;F&lt;/sub&gt; R591E</td>
<td>-110.3 ± 6.5</td>
<td>15.5 ± 4.1</td>
</tr>
<tr>
<td>Unlabeled HCN2&lt;sub&gt;F&lt;/sub&gt; ΔCNB</td>
<td>-97.4 ± 5.9</td>
<td>16.5 ± 1.5</td>
</tr>
<tr>
<td>Unlabeled HCN2 WT</td>
<td>-83.3 ± 5.4</td>
<td>14.5 ± 2.5</td>
</tr>
<tr>
<td>Unlabeled HCN2 R591E</td>
<td>-100.5 ± 3.5</td>
<td>11.5 ± 2.3</td>
</tr>
<tr>
<td>Unlabeled HCN2 ΔCNB</td>
<td>-82.9 ± 7.4</td>
<td>19.6 ± 1.4</td>
</tr>
</tbody>
</table>

Note: Data for labeled G-V curves taken from only VCF recordings for HCN2<sub>F</sub> and HCN2<sub>F</sub> R591E. No VCF recordings were successfully completed with labeled HCN2<sub>F</sub> ΔCNB channels and thus G-V data was only from TEVC recordings.
<table>
<thead>
<tr>
<th></th>
<th>Lag, (d) (ms)</th>
<th>(\tau_{\text{early}}) (ms)</th>
<th>(\tau_{\text{late}}) (ms)</th>
<th>(f_{\text{early}})</th>
<th>(\tau_w) (ms)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeled HCN2(_F)</td>
<td>32 ± 16</td>
<td>277 ± 95</td>
<td>620 ± 340</td>
<td>0.42 ± 0.27</td>
<td>520 ± 150</td>
<td>9</td>
</tr>
<tr>
<td>Labeled HCN2(_F) R591E</td>
<td>58 ± 52</td>
<td>340 ± 110</td>
<td>1700 ± 1200</td>
<td>0.46 ± 0.13</td>
<td>1100 ± 740</td>
<td>17</td>
</tr>
<tr>
<td>Labeled HCN2(_F) ΔCNB</td>
<td>150 ± 100</td>
<td>353 ± 61</td>
<td>1590 ± 330</td>
<td>0.50 ± 0.11</td>
<td>960 ± 190</td>
<td>11</td>
</tr>
<tr>
<td>Unlabeled HCN2(_F)</td>
<td>50 ± 23</td>
<td>230 ± 80</td>
<td>1370 ± 240</td>
<td>0.497 ± 0.056</td>
<td>800 ± 160</td>
<td>23</td>
</tr>
<tr>
<td>Unlabeled HCN2(_F) R591E</td>
<td>63 ± 38</td>
<td>179 ± 55</td>
<td>1220 ± 370</td>
<td>0.571 ± 0.069</td>
<td>610 ± 180</td>
<td>14</td>
</tr>
<tr>
<td>Unlabeled HCN2(_F) ΔCNB</td>
<td>67 ± 47</td>
<td>352 ± 62</td>
<td>1360 ± 260</td>
<td>0.54 ± 0.10</td>
<td>800 ± 110</td>
<td>14</td>
</tr>
<tr>
<td>Unlabeled HCN2 WT</td>
<td>117 ± 51</td>
<td>149 ± 29</td>
<td>980 ± 270</td>
<td>0.762 ± 0.068</td>
<td>350 ± 100</td>
<td>49</td>
</tr>
<tr>
<td>Unlabeled HCN2 R591E</td>
<td>146 ± 28</td>
<td>137 ± 28</td>
<td>330 ± 120</td>
<td>0.62 ± 0.19</td>
<td>203 ± 32</td>
<td>14</td>
</tr>
<tr>
<td>Unlabeled HCN2 ΔCNB</td>
<td>170 ± 140</td>
<td>318 ± 65</td>
<td>1500 ± 1200</td>
<td>0.796 ± 0.062</td>
<td>510 ± 200</td>
<td>6</td>
</tr>
</tbody>
</table>

Note: Activation length was 3 s for all recordings.
Table 3.3. Kinetic parameters of the +20 mV deactivation transient for unlabeled and labeled channels

<table>
<thead>
<tr>
<th></th>
<th>Lag, d (ms)</th>
<th>$\tau_{\text{early}}$ (ms)</th>
<th>$\tau_{\text{late}}$ (ms)</th>
<th>$f_{\text{early}}$</th>
<th>$\tau_w$ (ms)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeled HCN2$_F$</td>
<td>64 ± 22</td>
<td>227 ± 23</td>
<td>1580 ± 210</td>
<td>0.62 ± 0.10</td>
<td>750 ± 200</td>
<td>10</td>
</tr>
<tr>
<td>Labeled HCN2$_F$ R591E</td>
<td>61 ± 31</td>
<td>221 ± 30</td>
<td>1460 ± 280</td>
<td>0.681 ± 0.080</td>
<td>620 ± 150</td>
<td>20</td>
</tr>
<tr>
<td>Labeled HCN2$_F$ ΔCNB</td>
<td>95 ± 47</td>
<td>375 ± 59</td>
<td>1450 ± 160</td>
<td>0.51 ± 0.13</td>
<td>886 ± 72</td>
<td>5</td>
</tr>
<tr>
<td>Unlabeled HCN2$_F$</td>
<td>72 ± 52</td>
<td>157 ± 34</td>
<td>2930 ± 910</td>
<td>0.20 ± 0.10</td>
<td>2420 ± 870</td>
<td>13</td>
</tr>
<tr>
<td>Unlabeled HCN2$_F$ R591E</td>
<td>41 ± 35</td>
<td>162 ± 50</td>
<td>1140 ± 200</td>
<td>0.32 ± 0.20</td>
<td>820 ± 210</td>
<td>13</td>
</tr>
<tr>
<td>Unlabeled HCN2$_F$ ΔCNB</td>
<td>70 ± 46</td>
<td>430 ± 110</td>
<td>1550 ± 180</td>
<td>0.65 ± 0.10</td>
<td>820 ± 160</td>
<td>10</td>
</tr>
<tr>
<td>Unlabeled HCN2 WT</td>
<td>225 ± 70</td>
<td>148 ± 28</td>
<td>720 ± 320</td>
<td>0.895 ± 0.070</td>
<td>207 ± 64</td>
<td>41</td>
</tr>
<tr>
<td>Unlabeled HCN2 R591E</td>
<td>117 ± 17</td>
<td>64 ± 19</td>
<td>290 ± 110</td>
<td>0.83 ± 0.14</td>
<td>111 ± 63</td>
<td>14</td>
</tr>
<tr>
<td>Unlabeled HCN2 ΔCNB</td>
<td>130 ± 17</td>
<td>110.1 ± 6.1</td>
<td>540 ± 140</td>
<td>0.844 ± 0.023</td>
<td>176 ± 21</td>
<td>6</td>
</tr>
</tbody>
</table>

Note: Activation step was to -130 mV for 3 s for all recordings. Deactivation step was for 4 -10 s for all recordings; longer deactivation steps were used for slower deactivating channels to permit full current decay.

First, I examined the ability of the apo CNB fold to impart inhibition on HCN2$_F$-based channels. For the steady-state G-V relationship, labeled HCN2$_F$ R591E channels had a hyperpolarized $V_{1/2}$ value (-119.1 ± 4.9 mV, n=5) relative to labeled HCN2$_F$ ΔCNB channels (-97.1 ± 6.9 mV, n=10) (p< 0.0001) (Figure 3.5). For deactivation labeled HCN2$_F$ R591E channels also have a faster $\tau_w$ value (620 ± 150 ms, n=20) relative to labeled HCN2$_F$ ΔCNB channels (886 ± 72 ms, n = 5) (p<0.0006) for current transients at a +20 mV depolarization. Both these results support the prediction that an apo CNB fold destabilizes the open state thermodynamically and kinetically, and that removal of the CNB fold can remove this inhibition.
Figure 3.5. Autoinhibition correctly predicts that labeled HCN2_F R591E channels would have a significantly more hyperpolarized V_{1/2} than HCN2_F ΔCNB channels.

Note: Representative conductance–voltage relations of labeled HCN2_F ΔCNB channels (pink) and labeled HCN2_F R591E (green) as described in methods (section 3.2.2.4).

Second, I examined the ability of cAMP to stabilize the open state of the channel. For the steady-state G-V relationship, labeled HCN2_F R591E channels had a hyperpolarized V_{1/2} value relative to HCN2_F channels (-107.0 ± 8.3 mV, n = 4) (p < 0.03), consistent with cAMP potentiation. Binding of cAMP also slowed deactivation current transients in labeled HCN2F channels (750 ± 200 ms, n = 10) compared to HCN2_F R591E channels at a +20 mV depolarization (p < 0.05). This suggests the endogenous cAMP in the oocyte can stabilize the open state in labeled HCN2_F channels both thermodynamically and kinetically.

Third, I examined whether OST was present in labeled HCN2_F channels. The deactivation τ_w of HCN2_F current transients was not significantly larger than the deactivation τ_w of HCN2_F ΔCNB (p < 0.16) at a +20 mV depolarization. However τ_{early} represents the majority component for both channels, and the deactivation τ_{early} of HCN2_F current transients was significantly larger than the deactivation τ_w of HCN2_F ΔCNB (p < 0.0001). OST was initially described with deactivation transients fit by a single exponential, which makes it difficult to describe OST in transients with two components. However the slower τ_{early} of HCN2_F relative to HCN2_F ΔCNB suggests there...
might be residual OST in labeled HCN2F channels. Characterizing VCF recordings of HCN2F and HCN2F R591E

I generated VCF recordings using oocytes expressing HCN2F or HCN2F R591E and labeled with TMRM. Recordings for both channels had HCN-like currents and fluorescence deflections in response to step changes in the applied voltage (Figures 3.6 and 3.7). Recordings from both channels were able to achieve fluorescence deflections with amplitudes up to 0.25 V. The deflections in the HCN2F and HCN2F R591E fluorescence transients suggest that there is a conformational change occurring in the environment around the fluorescent reporter at position neg3. The fact that the start of the deflection coincides with a change in the voltage protocol suggests this conformational change involves the movement of at least one charged residue. Given the position of the reporter on S4, I thus assume the reporter is tracking a conformational change within the VSD.
Figure 3.6. Representative VCF recording of HCN2

Note: TOP: Voltage protocol, with a holding potential of -40 mV, an activation step to -130 mV for 3 s, and a deactivation step to +20 mV for 4 s. The deactivation step is followed by leak measurement steps (-40 mV, 0 mV, and +40 mV), and a return to the -40 mV holding potential for 800 ms. MIDDLE: Current transient associated with above voltage protocol. BOTTOM: Fluorescence transient (purple) associated with above voltage protocol. Smoothing was not applied to the fluorescent transient.
Figure 3.7. Representative VCF recording of HCN2\textsubscript{\textit{F}} R591E
Note: Same voltage protocol as in Figure 3.6. Current transient shown in black and fluorescence transient shown in green. Smoothing was not applied to the fluorescent transient.

VCF recordings were attempted (over three days) on the HCN2\textsubscript{\textit{F}} ΔCNB channel, but fluorescence deflections of substantial amplitude were not achieved before the conclusion of this thesis. See section 4.2.3 for predictions regarding the speed of VSD movements in HCN2\textsubscript{\textit{F}} ΔCNB channels.

I initially characterized steady-state fluorescence values to determine the midpoint voltage of activation \((V_{1/2})\) and the steepness of the voltage dependence \((s)\) for HCN2\textsubscript{\textit{F}} and HCN2\textsubscript{\textit{F}} R591E channels. In this chapter [fluor] and [curr] designations will be used when discussing behaviours of the fluorescence trace and current trace, respectively.
For HCN2\textsubscript{F} channels the $V_{1/2}\[\text{fluor}\]$ was $-99.9 \pm 5.7$ mV, ($n = 4$), while the $V_{1/2}\[\text{curr}\]$ was not significantly different ($-107.0 \pm 8.3$ mV, $n = 4$) ($p > 0.2$) (Figure 3.8). A noteworthy feature of the HCN2\textsubscript{F} F-V curve was its qualitative shallowness relative to its partner G-V curve. The F-V curves had a reciprocal slope of $(17.4 \pm 7.2$ mV, $n = 4$), while the G-V curves had a reciprocal slope of $(9.3 \pm 2.0$ mV, $n = 4$) ($p > 0.073$). In three out of four recordings the $s_{\[\text{fluor}\]}$ was between 4 and 18 mV larger than its $s_{\[\text{curr}\]}$, and in one case the $s_{\[\text{fluor}\]}$ was 2.5 mV smaller than the $s_{\[\text{curr}\]}$. When qualitatively examining the F-V and G-V curves, the curves from the same recording crossed. This is unsurprising given the relative slopes of the two curves. Overall, the $V_{1/2}\[\text{fluor}\]$ was depolarized to its corresponding $V_{1/2}\[\text{curr}\]$ by $(7.1 \pm 8.2$ mV, $n = 4$). In three out of four recordings the $V_{1/2}\[\text{fluor}\]$ was between 3 and 18 mV more depolarized than its $V_{1/2}\[\text{curr}\]$, and in one case the $V_{1/2}\[\text{fluor}\]$ was 1 mV more hyperpolarized than the $V_{1/2}\[\text{curr}\]$. This suggests that the typical behaviour of HCN2\textsubscript{F} channel G-V and F-V curves is for the F-V curve to have a more depolarized $V_{1/2}$ and a more shallow reciprocal slope.

For HCN2\textsubscript{F} R591E channels the $V_{1/2}\[\text{fluor}\]$ was $-115.3 \pm 4.8$ mV, ($n = 5$) and was not significantly more depolarized relative to the $V_{1/2}\[\text{curr}\]$ ($-119.1 \pm 4.9$ mV, $n = 5$) ($p > 0.25$) (Figure 3.9). The HCN2\textsubscript{F} R591E F-V curve produced a significantly more shallow reciprocal slope $(17.7 \pm 4.1$ mV, $n = 5)$ than the reciprocal slope of the G-V curve $(12.4 \pm 2.9$ mV, $n = 5)$ ($p < 0.05$). In four out of five recordings the $s_{\[\text{fluor}\]}$ was between 7 and 12 mV larger than its $s_{\[\text{curr}\]}$, and in one case the $s_{\[\text{fluor}\]}$ was 6 mV smaller than the $s_{\[\text{curr}\]}$. Again, the F-V and G-V curves crossed upon visual inspection. This suggests the typical behaviour of a more larger $s_{\[\text{fluor}\]}$ than $s_{\[\text{curr}\]}$ is consistent for recordings of both of the HCN2\textsubscript{F}-based channels.
Figure 3.8. Representative F-V and G-V curves of HCN2<sub>F</sub>

Note: Representative conductance–voltage relations (black) and fluorescence-voltage relations (purple) for a single VCF recording, as described in methods (section 3.2.2.4). See Table 3.1 for averages.
3.3.1.3. Controls

**Behaviour of labeled HCN2\(_F\)-based channels deviated from the behaviour of unlabeled HCN2 WT channels**

TEVC showed labeled oocytes injected with HCN2\(_F\) generated HCN-like currents. However, the transients were notably different from HCN2 WT transients (described in Chapter 2, for full HCN2 WT traces see Figure 2.1 and Figure 2.4). For example, labeled HCN2\(_F\) channels had a significantly more hyperpolarized \(V_{1/2\{curr\}}\) (-107.0 ± 8.3 mV, \(n = 4\)) than unlabeled HCN2 WT channels (\(p < 0.0001\)). The HCN2\(_F\) current transient \(\tau_w\) at a +20 mV depolarization was significantly larger than that of unlabeled HCN2 WT (\(p < 0.0001\)). Labeled HCN2\(_F\) R591E channels had a \(V_{1/2\{curr\}}\) (-119.1 ± 4.9 mV, \(n = 5\)) that was significantly more hyperpolarized than the \(V_{1/2\{curr\}}\) of unlabeled HCN2 R591E channels (-100.5 ± 3.5 mV, \(n = 13\)) (\(p < 0.0001\)). Similarly, labeled HCN2\(_F\) ΔCNB channels had a \(V_{1/2\{curr\}}\) (-97.1 ± 6.9 mV, \(n = 10\)) that was significantly more hyperpolarized than unlabeled HCN2 ΔCNB channels (-82.9 ± 7.4 mV, \(n = 6\)) (\(p < 0.002\)). For both HCN2\(_F\) R591E and HCN2\(_F\) ΔCNB channels, the deactivation \(\tau_w\) of the current transient of the
HCN2\textsubscript{F} derivative was significantly slower than the corresponding HCN2 version (p < 0.0001 for both comparisons). The mutations introduced to HCN2 WT to generate HCN2\textsubscript{F}-based channels for VCF experiments and the labeling process thus significantly slows channel deactivation kinetics and generates a hyperpolarizing shift in the $V_{1/2}^{\text{curr}}$.

**Labeling HCN2\textsubscript{F}-based channels with TMRM changed channel behaviour**

Labeling oocytes with TMRM changed at least one parameter of channel behaviour in the current transients in all three HCN2\textsubscript{F}-based channels. Labeled HCN2\textsubscript{F} channels had a significantly more hyperpolarized $V_{1/2}^{\text{curr}}$ (-108.9 ± 6.2 mV, n = 14) than unlabeled HCN2\textsubscript{F} channels (-100.4 ± 6.0 mV, n = 20) (p < 0.02). Labeled HCN2\textsubscript{F} R591E channels had a significantly more hyperpolarized $V_{1/2}^{\text{curr}}$ [-119.1 ± 4.9 mV (n=5)] than unlabeled HCN2\textsubscript{F} R591E channels (-110.3 ± 6.5 mV, n = 20) (p < 0.01). Labeled HCN2\textsubscript{F} channels also have a significantly smaller deactivation $\tau_w$ for the current transient than unlabeled HCN2\textsubscript{F} channels (p < 0.0001)(Figure 3.10). Labeling did not shift the $V_{1/2}$ of HCN2\textsubscript{F} ΔCNB channel current transients in TEVC recordings (p > 0.9156). However, labeling did significantly slow activation $\tau_{\text{early}}$ values of HCN2\textsubscript{F} ΔCNB channels from (800 ± 110 ms, n = 14) to (960 ± 190 ms, n = 11) (p < 0.01) at a +20 mV depolarization. These changes in channel gating behaviour after oocyte labeling suggest the TMRM label is binding to the HCN2\textsubscript{F}–based channels directly. The consequences of TMRM labeling on HCN channel behaviour are discussed in section 3.4.2.1.
Figure 3.10. **Labeling HCN2\textsubscript{F} channels with TMRM speeds deactivation**

Note: Superimposition of normalized deactivation transients from representative HCN2\textsubscript{F} unlabeled (grey) and HCN2\textsubscript{F} labeled (purple) recordings at a +20 mV depolarization.

**Fluorescence deflections only occurred when VCF is done on oocytes expressing HCN2F-based channels and labelled with TMRM**

The TMRM label may be covalently binding to extracellular cysteines on endogenous proteins of the oocyte in addition to HCN channels (Dascal, 1987). I thus performed VCF on labeled but uninjected eggs. These recordings showed typical levels of leak current at a -40 mV holding potential (approximately -100 nA) and an increase in the absolute level of leak current upon a hyperpolarizing step in the voltage protocol. However the current levels then stayed approximately constant over the length of the hyperpolarizing pulse, indicating no HCN channels were opening. The fluorescence trace was of typical amplitude but did not have any deflections (Figure 3.11A). This suggests the label covalently binds to cysteines on the extracellular side of the oocyte in other endogenous proteins, but does not report any voltage-dependent changes in these proteins. Thus I assume that any fluorescence deflections I see during VCF experiments with labeled HCN channels expressed on the oocyte can be attributed to the VSD region of HCN channels.

Though the HCN channels on the oocyte had an available cysteine to covalently bind with TMRM, there are potentially other fluorescent compounds on the oocyte
membrane that could contribute to the registered fluorescence signal. I thus completed a VCF recording on an unlabeled HCN2-R591E channel. The recording showed HCN-like currents but a near-zero (< 0.5 V) amplitude of the fluorescence trace and no deflections (Figure 3.11B). The baseline control protocol (see methods) also had a fluorescence trace with a near-zero amplitude, and a very shallow slope that generated an $m$ value of approximately 10 mV/s. This $m$ was similar to the $m$ values of recordings from labeled oocytes. Thus the applied TMRM label is not responsible for the baseline drift but is responsible for the majority of the amplitude of the fluorescence trace and any fluorescence deflections produced by HCN channels.
3.3.1.4. Characterization of HCN2F unlabeled channels

I also briefly examined unlabeled HCN2F-based channels to determine if they have behaviour predicted by present gating models (Figures 3.12-3.14). A notable feature of unlabeled HCN2F channels was that they had a very slow deactivation transient with a $\tau_w$ of $(2420 \pm 870 \text{ ms}, n = 13)$ and required up to 10 s to deactivate with a $+20 \text{ mV}$ depolarization.
Figure 3.12. Representative TEVC recording of unlabeled HCN2_F channels
Note: Current transients of unlabeled HCN2_F from the voltage protocol in Figure 3.2. Sweep with -130 mV activation highlighted in purple for clarity.

Figure 3.13. Representative TEVC recording of unlabeled HCN2_F R591E channels
Note: Current transients of unlabeled HCN2_F R591E from the voltage protocol in Figure 3.2. Sweep with -130 mV activation highlighted in green for clarity.
**Figure 3.14. Representative TEVC recording of unlabeled HCN2\(_F\) ΔCNB channels**

Note: Current transients of unlabeled HCN2\(_F\) ΔCNB from the voltage protocol in Figure 3.2. Sweep with -130 mV activation highlighted in pink for clarity.

Unlabeled HCN2\(_F\)-based channels have behaviour predicted by the autoinhibition model, OST, and the quickening conformation

The autoinhibition model predicts the relationship between the \(V_{1/2[\text{curr}]}\) values of all three of the unlabeled HCN2\(_F\)-based channels (Figure 3.15). Unlabeled HCN2\(_F\) R591E apo channels had a \(V_{1/2[\text{curr}]}\) that was significantly more hyperpolarized than the \(V_{1/2[\text{curr}]}\) value of its HCN2\(_F\) holo counterpart \((p < 0.0001)\). The \(V_{1/2[\text{curr}]}\) of unlabeled HCN2\(_F\) ΔCNB channels is not significantly more depolarized than that of HCN2\(_F\) channels \((p > 0.20)\). These similar \(V_{1/2[\text{curr}]}\) values are consistent with the autoinhibition model, and indicate HCN2\(_F\) has sufficient autoinhibition removal by the binding of cAMP to be comparable to a fully autoinhibition-free channel. The \(V_{1/2[\text{curr}]}\) of unlabeled HCN2\(_F\) ΔCNB channels was also significantly more depolarized than that of HCN2\(_F\) R591E channels, again supporting removal of autoinhibition by the CNB fold truncation \((p < 0.0001)\). Thus autoinhibition correctly predicted the relationship between the \(V_{1/2[\text{curr}]}\) values of all three of the HCN2\(_F\) channels.
Figure 3.15. $V_{1/2}$ values of unlabelled HCN2<sub>F</sub>-based channels are predicted by the autoinhibition model

Note: Representative conductance–voltage relations of unlabelled HCN2<sub>F</sub> channels (purple) and unlabelled HCN2<sub>F</sub> R591E channels (green) and unlabelled HCN2<sub>F</sub> ΔCNB channels (pink), as described in methods (3.2.2.4).

The OST model predicts the relationship between the deactivation speeds of HCN2<sub>F</sub> and HCN2<sub>F</sub> ΔCNB channels. The HCN2<sub>F</sub> ΔCNB channel has a deactivation $\tau_w$ of (790 ± 180 ms, n = 13), and thus much faster deactivating than the HCN2<sub>F</sub> channel ($p < 0.0001$). This result is consistent with the OST model, as both channels have equal autoinhibition relief (indicated by their similar $V_{1/2[\text{curr}]}$ values) and thus the holo channel has a degree of sustained activation not predicted by autoinhibition at a +20 mV depolarization. HCN2<sub>F</sub> ΔCNB channels cannot be influenced by the OST mechanism due to the CNB fold truncation. Thus in addition to HCN4-derived channels (Wicks et al., 2011, 2009) and HCN2 WT channels (Chapter 2), OST can also predict the deactivation kinetics of HCN2 derivatives with a pore domain derived from HCN1.

The quick activation model proposed in Chapter 2 predicts the speeds of initial activation of all three HCN2<sub>F</sub>-based channels. The model suggests that HCN2<sub>F</sub> and HCN2<sub>F</sub> R591E channels would both possess the quickening conformation where the intact CNB fold speeds channel activation, while HCN2<sub>F</sub> ΔCNB channels would not. The activation $\tau_{\text{early}}$ value of HCN2<sub>F</sub> ΔCNB was (352 ± 62 ms, n = 14) and thus significantly larger than both the activation $\tau_{\text{early}}$ values of HCN2<sub>F</sub> (230 ± 80 ms, n = 23) ($p < 0.0004$)
and HCN2\textsubscript{F} R591E (179 ± 55 ms, n = 12) (p < 0.0001). Thus as in Chapter 2, an autoinhibition-free truncated HCN2\textsubscript{F} channel has slower activation than both intact holo and intact apo HCN2\textsubscript{F} channels. This lends further support to my conclusion of a quickening conformation existing in HCN channels with an intact CNB fold that promotes a fast transition from the closed to open unliganded states. I can now also suggest that the quickening conformation occurs when either an HCN1-like pore or an HCN2 pore is present.

3.3.2. The neg3 reporter tracks a VSD movement that precedes pore closure at a +20 mV depolarization

VCF current transients during deactivation were well-fitted by a double exponential equation omitting an initial lag (see methods). A multicomponent current trace is not unexpected, as there are multiple deactivation pathways; channels may open after 1, 2, 3, or 4 S4s move inwards (see section 3.3.3). VCF fluorescence transients during deactivation did not include any lag and only included a single component (Figure 3.16). VCF fluorescence transients also had sloping baseline, as the final level of fluorescence emission was always higher than the initial level even when recording stayed at a constant voltage (see methods). VCF fluorescence transients during deactivation were thus well-fitted to a single exponential equation with a sloping baseline (see methods). The $\tau_w$ value allowed comparison of the current transient with the fluorescence transient using a single number ($\tau_{w[curr]}$ for current decay, $\tau_{w[fluor]}$ for fluorescence decay) (see Table 3.4).
Figure 3.16.  
**HCN2** fluorescence transients are fit to a single exponential equation with a sloping baseline

Note:  
Fluorescence transient (purple) during the +20 mV deactivation of the recording shown in Figure 3.6. Transient was filtered at 250 Hz but did not undergo smoothing (see methods) or adjustments for sloping baseline. Single exponential fit with a sloping baseline shown (black dashed line) in relation to the fluorescence trace.

A superimposition of the fluorescence transient and current transient from the same VCF recording shows that the fluorescence decay precedes the current decay in both HCN2\textsubscript{F} and HCN2\textsubscript{F} R591E channels (Figures 3.17 and 3.18). During a +20 mV depolarization the HCN2\textsubscript{F} channels fluorescence transients had a \( \tau_{\text{fluor}} \) of \((153 \pm 47\) ms, \( n = 10 \)) and a significantly larger \( \tau_{\text{curr}} \) of \((750 \pm 200\) ms, \( n = 10 \)) (\( p < 0.0001 \)). The HCN2\textsubscript{F} R591E channels had a deactivation \( \tau_{\text{fluor}} \) of \((143 \pm 33\) ms, \( n = 20 \)) and a significantly larger \( \tau_{\text{curr}} \) of \((620 \pm 150\) ms, \( n = 20 \)) at a +20mV depolarization (\( p < 0.0001 \)). This suggests that the tracked VSD movement during deactivation at a +20 mV depolarization precedes pore closure.
Figure 3.17. The HCN2\textsubscript{F} reporter tracks a VSD movement during deactivation that precedes pore closure

Note: Superimposition of normalized current (black) and fluorescence (purple) +20 mV deactivation transients of the VCF recording shown in Figure 3.6. Subtraction of fluorescence transient sloping baseline occurred before normalization (see methods) with an $m$ value of 9.52 mV/s and a decay amplitude of 0.25 V. Smoothing was also applied to fluorescent transient (see methods).
Figure 3.18. The HCN2\textsubscript{F} R591E tracks a VSD movement during deactivation that precedes pore closure

Note: Superimposition of normalized current (black) and fluorescence (green) +20 mV deactivation transients of the HCN2\textsubscript{F} R591E VCF recording shown in Figure 3.7. Subtraction of fluorescence transient sloping baseline occurred before normalization (see methods) with an \textit{m} value of 9.07 mV/s and a decay amplitude of 0.18 V. Smoothing was also applied to fluorescent transient (see methods).

Since the entire fluorescence decay was fit to a single exponential without any lag, I propose the tracked VSD movement is early in the deactivation pathway. I thus assign the tracked VSD movement to the \textit{O}\textsubscript{S4in}-\textit{O}\textsubscript{S4out} step of the \textit{O}\textsubscript{S4in}-\textit{O}\textsubscript{S4out}-\textit{C}\textsubscript{S4out} deactivation pathway of the cyclic allostery model (see Figure 1.2).

The $\tau_{w[\text{curr}]}$ value of the deactivation current transient is much larger than the $\tau_{[\text{fluor}]}$ for the fluorescence transient, indicating the VSD movement is not rate-limiting at a +20 mV depolarization. Though I cannot assign the current transient $\tau_w$ value to one step, as it is a weighted average of multiple components, I can still compare it to the speed of the VSD movement step. The mean of the current transient $\tau_{w[\text{curr}]}$ was more than 4-fold slower than the mean of the fluorescence transient $\tau_{[\text{fluor}]}$ for both HCN2\textsubscript{F} and HCN2\textsubscript{F} R591E channels. This four-fold difference suggests that the fluorescence transient will have completed the majority of its decay before the current transient approaches its $\tau_{w[\text{curr}]}$ value. Thus, I suggest that for the VSD movement tracked by the HCN2\textsubscript{F} R591E
and HCN2_F neg3 reporter at +20 mV, the speed of this step does not substantially limit the rate of the overall deactivation pathway. I thus conclude for Question #3 of my thesis that, for both HCN2_F-based channels studied, the VSD movement step is no longer rate-limiting for the deactivation pathway at a +20 mV depolarization.

### 3.3.3. The tracked VSD movement step has stronger voltage dependence than the pore closure step

As I predict the conformational change tracked by the neg3 reporter occurs within the VSD, I imagine its speed may be voltage-dependent. I thus created a VCF voltage protocol with a consistent -130 mV activation pulse and deactivation voltages ranging from -80 mV to +40 mV. I initially studied HCN2_F R591E, as cAMP binding to HCN2_F channels is known to slow deactivation speeds but the degree of cAMP binding within each oocyte is difficult to quantify. By using HCN2_F R591E, I can assume that all channels are in the apo state at the start of the deactivation pathway.

The speed of the fluorescence transient is strongly voltage-dependent while the speed of the current transient is weakly voltage-dependent. Both the fluorescence and current transients decayed faster as the deactivation voltage became more positive. The HCN2_F R591E fluorescence transient became approximately 7-fold faster across the 120 mV range (Figure 3.19 and 3.20D). At its slowest, upon the -80 mV depolarization, this VSD movement occurred with a $\tau_{\text{fluor}}$ of $(750 \pm 100 \text{ ms}, n = 8)$; at its fastest upon the +40 mV depolarization it occurred with a significantly smaller $\tau_{\text{fluor}}$ of $(109 \pm 26 \text{ ms}, n = 14)$ ($p < 0.0001$). In contrast, all current time constant parameters ($\tau_{\text{late}}$, $\tau_{\text{w}}$, and $\tau_{\text{early}}$) show much weaker voltage-dependence (Figure 3.19 and 3.20A-C). The current $\tau_{\text{w}}$ and $\tau_{\text{early}}$ decreased approximately 2-fold from -80 mV to +40 mV, while the $\tau_{\text{late}}$ remained within 1400 – 1700 ms over the entire 120 mV range. The steps following the tracked VSD movement (including the pore closure step) thus have weaker voltage dependence than the tracked VSD movement step for HCN2_F R591E.
Table 3.4. Kinetic parameters of deactivation for VCF recordings at strong (+20 mV) and weak (-60 mV) depolarizations

<table>
<thead>
<tr>
<th></th>
<th>HCN2_F +20 mV</th>
<th>HCN2_F R591E +20 mV</th>
<th>HCN2_F R591E -60 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>τ_{fluoro} (ms)</td>
<td>153 ± 47</td>
<td>143 ± 33</td>
<td>625 ± 91</td>
</tr>
<tr>
<td>Lag, d (ms)</td>
<td>64 ± 22</td>
<td>61 ± 31</td>
<td>31 ± 11</td>
</tr>
<tr>
<td>τ_{early[ curr]} (ms)</td>
<td>227 ± 23</td>
<td>221 ± 30</td>
<td>323 ± 79</td>
</tr>
<tr>
<td>τ_{late[ curr]} (ms)</td>
<td>1580 ± 210</td>
<td>1460 ± 280</td>
<td>1680 ± 210</td>
</tr>
<tr>
<td>f_{early}</td>
<td>0.62 ± 0.10</td>
<td>0.681 ± 0.080</td>
<td>0.388 ± 0.032</td>
</tr>
<tr>
<td>τ_{w[ curr]} (ms)</td>
<td>750 ± 200</td>
<td>620 ± 150</td>
<td>1150 ± 190</td>
</tr>
</tbody>
</table>
Figure 3.19. Voltage dependence of HCN2\(_F\) R591E channel fluorescence and current transients during deactivation

Note: A: HCN2\(_F\) R591E time constant values for fluorescence (green) and current (\(\tau_{\text{early}}\) = grey triangles, \(\tau_{w}\) = black, \(\tau_{\text{late}}\) = grey squares) transients for range of deactivation voltages. Current time constants are omitted at -40 mV as this voltage is the approximate reversal potential for HCN channels and the currents possessed very limited amplitudes. All recordings included a 3 s activation pulse to -130 mV and then a 4 s deactivation pulse. N values range from 8 - 20 recordings. B: Average lag for the same recordings in A. C: Average \(f_{\text{early}}\) for the same recordings in A.
Figure 3.20. **Stronger depolarizations during deactivation sped HCN2<sub>F</sub> R591E fluorescence transients to a greater degree than corresponding current transients**

Note:  
A: Superimposition of normalized current transients from same oocyte of all tested deactivation voltages (-80 mV to +40 mV). The -40 mV sweep is omitted (see explanation in Figure 3.19). +20 mV transient highlighted in red, -60 mV transient highlighted in blue. Voltage protocol shown in inset, with a 3 s activation epoch and a 4 s deactivation epoch with sweeps every 20 mV from -80 mV to +40 mV.  
B: Same superimposition as in A except shown on a logarithmic scale.  
C: Same as panel A except only the +20 mV transient (highlighted in red) and -60mV transient (highlighted in blue) shown.  
D: Superimposition of normalized fluorescence transients from same oocyte as A of +20 mV (highlighted in red) and -60mV (highlighted in blue) depolarizations.
Though the time constants of the HCN2$_F$ R591E current transient did not change drastically over the 120 mV range of depolarizations, the relative fraction of the early component changed significantly (Figure 3.19C). From a -80 mV to +40 depolarization, $f_{\text{early}}$ significantly increased from $(0.313 \pm 0.029, n = 8)$ to $(0.711 \pm 0.089, n = 14)$ ($p < 0.0001$). This increase in $f_{\text{early}}$ caused a shift in the dominant component from $\tau_{\text{late}}$ to $\tau_{\text{early}}$ at approximately -40 mV. Thus current transients in the weak and strong voltage regimes are inherently different as they possess different dominant components.

The tetrameric nature and weak VSD-PD temporal association of HCN channels may explain the change in $f_{\text{early}}$ with deactivation voltage. There may be two deactivation pathways which differ in the number of S4’s that move outwards before the pore closure step. The early component with faster deactivation might reflect channels with all four S4’s moving outward before pore closure, favoured by strong depolarizations; the late component with slower deactivation might reflect channels with fewer than four S4’s moving outward before pore closure, favoured by weak depolarizations. Stronger depolarizations make drive more channels into the deactivation pathway that produces the early component. Changes in the primary channel deactivation pathway could theoretically occur based on the deactivation voltage, even if all channels received the same activation voltage.

The cyclic allostery model predicts the rate-limiting step of the HCN channel deactivation pathway will be the VSD movement step at weak depolarizations and the pore closure step at strong depolarizations. This prediction was based on the fact that HCN2 channels reach a maximum activation speed at strong hyperpolarizations (Chen et al., 2007). It is also intuitive that a VSD movement step would be voltage-dependent and thus become very fast at certain voltages. However this is the first time the difference in the voltage dependence of the VSD movement step and pore closure step has been measured directly in HCN2 channels. The fluorescence transient $\tau_{\text{fluoro}}$ value during deactivation sped much more over the 120 mV range compared to any of the current transient kinetic parameters. I thus conclude for Question #4 that the speed of the VSD movement step has a stronger voltage dependence than the pore movement step. I therefore assign the $O_{S4\text{in}}$-$O_{S4\text{out}}$ step to be rate-limiting at weak depolarizations
and the O$_{S4in}$-C$_{S4in}$ step to be rate-limiting at strong depolarizations in the cyclic allostery model of the HCN channel deactivation pathway.

The HCN2$_F$ R591E current and fluorescence deactivation transients are more closely associated in time at -60 mV (Figure 3.21) compared to +20 mV (Figure 3.18). This is a consequence of the difference in voltage dependence of the speeds of the fluorescence and current deactivation transients. This suggests the temporal association between the VSD step and pore closure step may be tighter at weak depolarizations and looser at stronger depolarizations.

**Figure 3.21.** HCN2$_F$ R591E fluorescence transients precede current transients only slightly at weak depolarizations

Note: Superimposition of the fluorescence (green) and current (black) deactivation transients during a -60 mV depolarization from the same recording of an HCN2$_F$ R591E channel.

To quantify the temporal association between the fluorescence and current transients I computed a ratio between values of $\tau_{[fluor]}$ and the $\tau_{w[curr]}$ which I term the “voltage sensor to gate rate ratio” (VGRR) (VGRR= $\tau_{[fluor]}/\tau_{w[curr]}$) (Figure 3.22). Each VGRR was determined from the $\tau_{[fluor]}$ and $\tau_{w[curr]}$ from one VCF recording, not from mean values taken from a pool of recordings. The average VGRR value (mean ± standard deviation) was then determined from this population of paired values. The VGRR will always be a value less than 1 in cases where the fluorescence decay is faster than current decay. The VGRR suggests at the kinetics of the VSD and PD temporal
association: in this thesis I define a channel with a pore movement that occurs quickly after the tracked VSD movement as having a tight temporal association, and a channel with a lag between the tracked VSD movement and PD movement as having weak temporal association. A VGRR value of 1 would correspond to a perfectly tight temporal association between the VSD movement step and the pore closure step; the pore movement would occur instantaneously after the tracked VSD movement. For the +40 mV depolarization, the fluorescence transient speed is more than 4-fold faster than the current transient speed, and a VGRR of \((0.194 \pm 0.078, n = 13)\) indicates a fairly weak temporal association. For the -80 mV depolarization, the fluorescence transient speed is only 1.8-fold faster than the current \(\tau_w\) speed, and a VGRR of \((0.56 \pm 0.11, n = 8)\) indicates a tighter temporal association. The VGRR of the -80 mV depolarization is significantly larger than that of the +40 mV depolarization \((p < 0.0001)\). Thus not only is the tracked VSD movement itself voltage-dependent, but the temporal association between the tracked VSD movement step and the pore closure step is voltage-dependent, with stronger depolarizations leading to smaller VGRR values.

As discussed in section 1.2.1, HCN channels lack the obligatory coupling between S4 and pore movement that is apparent in Hodgkin-Huxley type channels; Bruening-Wright suggests two S4s must move inwards to open the pore (Bruening-Wright et al., 2007). The fluorescence decay encompasses movements of all S4s, regardless of how many S4s are required to move outwards before pore closure. The fact that the fluorescence transient is well fitted to a single exponential equation suggests all the S4s move independently of one another. If all four of the S4s need to move outward before pore closure (as in the Hodgkin-Huxley model), then a substantial amount of fluorescence decay would occur before current decay began. During a VCF recording, early in the epoch most of the channels would only have one or two S4s in the outwards position and thus fluorescence decay would occur but very little current decay. However, a current transient that superimposes well with the fluorescence transient (indicating a high VGRR value), may suggest that the pore can close after outward movement of only one or two S4s in the tetramer. On a recording, this would be represented by a current decay that would begin not long after fluorescence decay. The VGRR data and superimpositions favour the following interpretation for HCN2\(_f\) R591E, where I have data at both weak and strong depolarizations. At +20 mV, the VGRR value
is low as the fluorescence transient is noticeably faster than the current transient. This suggests that multiple S4s may need to move outwards to allow pore closure. However, at -60 mV, the VGRR is higher: the two transients superimpose more clearly, though fluorescence still slightly precedes current. This suggests fewer S4s may be required to move outward to allow for pore closure, so the current decay starts quickly after fluorescence decay. This raises the possibility that the number of S4s required to move outwards to allow for pore closure, is voltage-dependent, with more S4s required to move outward before pore closure at stronger depolarizations.

The change in VGRR across deactivation voltages suggests that the pore closure step is rate-limiting at strong depolarizations whereas the tracked VSD movement step is rate-limiting at weak depolarizations. As shown in section 3.3.2, for a +20 mV depolarization the tracked VSD movement step is not rate-limiting for the deactivation pathway. However, for weaker depolarizations such as -60 mV, the higher VGRR value suggests pore closure occurs more quickly following the tracked VSD movement step. Thus the HCN channel deactivation pathway may have different rate-limiting steps depending on the deactivation voltage. As such, it is important to consider both weak

Figure 3.22. VGRR is voltage-dependent
Note: VGRR values of HCN2 R591E for a range of deactivation voltages. VGRR = \( \frac{\tau_{\text{fluor}}}{\tau_{\text{curr}}} \), with the time constants shown in Figure 3.19. VGRR values were computed using fluorescence and current time constants from the same recording, and the mean VGRR value was then computed from the VGRR of each recording.
(e.g.: -60 mV) and strong (e.g.: +20 mV) voltage regimes to ensure voltages where either the VSD movement step or pore closure step is rate-limiting are both represented when studying the HCN channel deactivation pathway.

3.3.4. Neither cAMP binding nor mode shift slow the tracked VCF movement during HCN channel deactivation

Cyclic AMP binding slows deactivation but not the tracked VSD movement during deactivation. The HCN2F R591E deactivation $\tau_{w\text{[curr]}}$ at a +20 mV depolarization was 1.2-fold faster than that of HCN2F ($p < 0.05$) (Figure 3.23B-C). However, the HCN2F R591E $\tau_{\text{fluoro}}$ value at this depolarization was very similar to that of HCN2F ($p > 0.50$) (Figure 3.23A). This result suggests that the conformational changes that occur in the CSD upon cAMP binding either do not transduce to the VSD or they transduce to a region of the VSD not tracked by the N-terminal S4 reporter. I thus conclude for Question #5 that the binding of cAMP does not slow the VSD movement tracked by the neg3 reporter at a depolarization of +20 mV for HCN2F R591E channels.

Comparing the F-V curves of HCN2F channels (Figure 3.8) and HCN2F R591E channels (Figure 3.9) shows that cAMP binding can depolarize the $V_{1/2}$ of the fluorescence transient to stabilize the open channel state. The $V_{1/2\text{[fluoro]}}$ of HCN2F channels was significantly more depolarized than the $V_{1/2\text{[fluor]}}$ of HCN2F R591E channels ($p < 0.003$). This is consistent with the predictions of the autoinhibition model, and the $V_{1/2\text{[curr]}}$ of labeled HCN2F channels was also significantly depolarized relative to the $V_{1/2\text{[curr]}}$ of labelled HCN2F R591E channels. The shift in the $V_{1/2\text{[fluoro]}}$ upon cAMP binding is important evidence that the tracked VSD movement is coupled to a gating pathway. It also serves as another example of a mechanism (cAMP potentiation) not shifting the thermodynamic and kinetic properties of a channel in parallel.
Figure 3.23. Cyclic AMP binding slows deactivation but not the tracked VSD movement step during deactivation

Note: A: Superimposition of normalized fluorescence transients from representative HCN2_{F} (purple) and HCN2_{F} R591E (green) VCF recordings. Same transient and time constants as in Figure 3.6 for HCN2_{F}. B: Superimposition of normalized current transients from representative HCN2_{F} (purple) and HCN2_{F} R591E (green) VCF recordings on a linear time scale. Same transient and time constants as in Figure 3.6 for HCN2_{F}. C: Same as B, except superimposition shown on a logarithmic time scale.

In preparation for testing whether mode shift can effect VSD movement, I confirmed that I could track mode shift in both HCN2 WT (holo) and HCN2 R591E (apo) channels using TEVC and the published protocol (Elinder et al., 2006). Superimposition of deactivation transients that occurred after increasingly long activation pulses show progressively longer lag periods and progressively slower deactivations (Figures 3.24 and 3.25). This was expected based on the previous mode shift study (Elinder et al., 2006). When extending the activation pulse length no longer slowed the deactivation transient, I assumed the channels had reached the mode ii open state.
Figure 3.24. Representative TEVC recordings of the mode shift of HCN2 WT and HCN2 R591E channels

Note: A: TEVC voltage protocol to measure mode shift. Protocol starts with at least a 1 s pulse at the holding potential of -40 mV. Protocol includes 25 sweeps with increasingly long activation lengths. Starting s to -130 mV is 10 ms and each sweep has an activation length that is an additional 10 ms longer. Final sweep has an activation pulse of 250 ms. Deactivation step is to +20 mV for 1.5 s. Protocol then returns to holding potential of -40 mV for 1.5s (not shown). Only 750 ms – 1750 ms range of protocol shown. B: Current transients for HCN2 WT with the voltage protocol in A. Sweep with 200 ms activation step highlighted in green for clarity. C: Same as B, for HCN2 R591E channels.
Figure 3.25. **Mode shift prolongs deactivation of HCN2 WT and HCN2 R591E channels**

Note: **A:** Superimposition of normalized currents of HCN2 WT in the +20 mV deactivation in voltage protocol shown in Figure 3.24. Only every second sweep is shown, and sweeps with 10-40ms activation pulses are omitted due to low amplitude. Green sweep underwent 200ms activation pulse. The same superimposition is shown on a linear (top) and on a logarithmic (bottom) time scale. Axis break is at 400 ms. **B:** Same as **A,** except for HCN2 R591E channels. Axis break is at 200 ms. Deactivation step is 1.5 s but only 0 – 0.7 s is shown.

The HCN2\(_F\) R591E channel takes much longer to complete mode shift than HCN2 R591E. For the mode shift investigation in VCF I again opted to study the apo channel as both cAMP binding and the transition to the mode ii open state during
activation can result in similarly slowed deactivation transients. Using an apo channel eliminates cAMP binding during activation as a confounding factor when tracking mode shift using prolonged activation pulses. Both labeled and unlabeled HCN2\textsubscript{F} R591E channels had a significantly slower activation $\tau_w$ ($p < 0.0001$ for both) and deactivation $\tau_w$ ($p < 0.0001$ for both) than HCN2 R591E channels. TEVC experiments showed that unlabeled HCN2\textsubscript{F} R591E channels required substantially longer activation pulses to induce mode shift than were needed to induce mode shift in HCN2 R591E channels (Figures 3.26 and 3.27). HCN2\textsubscript{F} R591E had not fully completed mode shift even after activation pulses reach at least 2300 ms. Thus after any given activation pulse length, HCN2\textsubscript{F} R591E would have less channels in the more stable $O_i$ state relative to HCN2 R591E, and thus HCN2\textsubscript{F} R591E should deactivate faster. Yet HCN2\textsubscript{F} R591E deactivates more than 4-fold slower than HCN2 R591E, suggesting the HCN2\textsubscript{F} background allows for a more stable open state compared to the HCN2 WT background. Considering also the extremely slow deactivation of the unlabeled HCN2\textsubscript{F} channel, there may be a structural feature of the HCN2\textsubscript{F} background, such as the loss of an S4 basic residue due to the Rneg3C mutation, which inherently slows deactivation of all HCN2\textsubscript{F} channels.
Figure 3.26. Representative TEVC recording of mode shift of HCN2<sub>F</sub>R591E channels

Note: TEVC Voltage protocol (top) and associated current transients (bottom) of an unlabeled HCN2<sub>F</sub>R591E channel undergoing mode shift. Protocol has 12 sweeps total. Protocol starts with at least 500 ms at the holding potential of -40 mV. Starting activation length to -130 mV is 100 ms and each sweep has an activation length that is an additional 200 ms longer. Longest activation sweep is 2300 ms long. Deactivation step is to +20 mV for 6 s followed by a return to the holding potential of -40 mV for 1.25 s. Sweep with 1500 ms activation at -130 mV is highlighted in green for clarity.
Figure 3.27.  HCN2<sub>F</sub> R591E requires longer activation pulses to undergo mode shift than HCN2 WT and HCN2 R591E channels

Note:  
A:  Superimposition of normalized deactivation transients at a +20 mV depolarization from recording shown in Figure 3.26.  Superimposition shown on a linear time scale with axis break at 2 s. Sweep with 100ms activation step omitted due to low transient amplitude.  
B:  Same as A, except superimposition shown on a logarithmic time scale with no axis break.

Mode shift does not slow the fluorescence transients of HCN2<sub>F</sub> R591E.  VCF experiments on HCN2<sub>F</sub> R591E were completed using a mode shift protocol with activation lengths from 500 ms to 6 s (Figures 3.28 and 3.29).  The deactivation $\tau_{w[\text{curr}]}$ for the sweep after a 6 s activation was more than 2.5-fold slower than the deactivation $\tau_{w[\text{curr}]}$ of the sweep with a 500 ms activation.  However, the deactivation fluorescence transients had a similar speed regardless of the length of the activation sweep.  Quantitatively, the deactivation $\tau_{[\text{fluoro}]}$ values were approximately 150 ms across all the measured activation lengths (Figure 3.30).
Figure 3.28. Representative VCF recording of HCN2\textsubscript{F} R591E channels undergoing mode shift

Note: Representative mode shift recording for HCN2\textsubscript{F} R591E with voltage protocol (top) current transient (middle) and fluorescence transient (bottom). Voltage protocol starts with at least 1.25 s at the holding potential of -40 mV. Shortest activation pulse is to -130 mV for 500 ms, and each subsequent sweep has an activation length extended by an additional 500 ms for 12 sweeps until a maximum activation pulse of 6000 ms. Deactivation step is to +20 mV for 4 s. Deactivation pulse is followed by a step to -40 mV for 1 s, 0 mV and +40 mV for 500 ms each, and then -40 mV for 1.25 s. Fluorescence trace is not smoothed. Sweep with a 3000 ms activation highlighted in green for clarity.
Figure 3.29. Mode shift prolongs deactivation of HCN2<sub>F</sub> R591E channels

Note: Superimposition of normalized deactivation transients at a +20 mV depolarization on a linear time scale (left) and logarithmic time scale (right) of the HCN2<sub>F</sub> R591E recording shown in Figure 3.28. Sweep with 3000 ms activation pulse is highlighted in green for clarity. Axis break in left superimposition is at 1.5 s.
Figure 3.30. Mode shift slows current transients of HCN2\textsubscript{F} R591E channels but not fluorescence transients at a +20 mV deactivation

Note: A: Time constants of HCN2\textsubscript{F} R591E channels for fluorescence (green) and current (\(\tau_{\text{early}}\) = grey triangles, \(\tau_{\text{w}}\) = black, \(\tau_{\text{late}}\) = grey squares) transients for a range of activation lengths. All recordings included an -130 mV activation step and then a 4 s deactivation pulse at +20 mV. N is 3 recordings. B: Lag of each of the three recordings in panel A. C: \(f_{\text{early}}\) for the three recordings in panel A.
Since weak and strong depolarizations may drive channels into different kinetic pathways, channels may react differently to mode shift at various deactivation voltages. I thus tested the effect of mode shift using a weak depolarization of -60 mV for deactivation. Mode shift still did not slow the speed of the HCN2_F R591E fluorescence transients at -60 mV. With depolarization of -60 mV, the fluorescent deactivation transients were all slower than their corresponding traces with the same activation length and a depolarization of +20 mV. The -60 mV fluorescence transients were on average more than 3-fold slower across all activation pulse lengths. This voltage-dependence of $\tau_{[fluoro]}$ expands the result from 3.3.3, where weak depolarization slowed the tracked VS movement with only a 3 s activation pulse. But similar to results for a +20 mV deactivation voltage, the deactivation $\tau_{[fluor]}$ values at a -60 mV deactivation voltage were approximately constant, after a 500 ms activation step or a 6000 ms activation step (Figure 3.31). The current deactivation time constants still became progressively larger with longer activation lengths. This suggests the deactivation speed of the tracked deactivation VSD movement is independent of mode shift regardless of the deactivation voltage. I thus conclude for Question #5 that the speed of the VSD movement tracked by the neg3 reporter during deactivation does not decrease when HCN2_F R591E channels undergo mode shift.
Figure 3.31. Mode shift slows current transients of HCN2<sup>F</sup> R591E channels but not fluorescence transients at a -60 mV deactivation

Note: A: Time constants of HCN2<sup>F</sup> R591E channels for fluorescence (green) and current (\(\tau_{\text{early}}\) = grey triangles, \(\tau_{\text{w}}\) = black, \(\tau_{\text{late}}\) = grey squares) transients for a range of activation lengths. All recordings included an activation step and then a 4s deactivation pulse at -60 mV. N is three recordings, except for the activation lengths of 1s, 3s, and 5s, where there are four recordings. B: Lag of each of the four recordings in A. Recording with only 1s, 3s, and 5s activation shown with grey circles. C: \(f_{\text{early}}\), for the four recordings in A.
Bruening-Wright identified a VSD movement using reporters on residues neg9 to neg11, and this movement occurs after pore opening (Bruening-Wright and Larsson, 2007). This suggests that there is a conformational change in the VSD during mode shift and thus deactivation pathways initiating from the O₁ or O₂ state should have different starting conformations. It is thus surprising that deactivation does not slow with mode shift, as this suggests the speeds the VSD movement steps initiating from the O₁ state and O₂ state have different starting conformations but coincident speeds. However, I find it unlikely that mode shift would result in slower deactivation without slowing any VSD movement, particularly in the weak voltage regime where VSD movement is predicted to be rate-limiting. If mode shift does not slow the rate-limiting VSD step, how can it slow deactivation? I therefore propose that there exist two VSD movement steps in the HCN channel deactivation pathway. Both of these VSD movement steps would have strong voltage dependence. One, tracked by Rneg3C on the HCN2-F derivative channels, does not slow with mode shift. A second does slow with mode shift. As the tracked VSD movement had no appreciable lag, I propose the tracked VSD movement occurs first in the deactivation pathway. My proposed deactivation pathway for apo HCN channels in mode i and mode ii is shown in Figure 3.32. I have named the new state in a three-step deactivation pathway O₅₄in2.
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Figure 3.32. Mode shift slows one of two VSD movement steps in a three-step HCN channel deactivation pathway

Note: Schematic showing the states of the HCN channel deactivation pathway at weak (e.g.: -60 mV) and strong (e.g.: +20 mV) depolarizations for HCN channels in mode i or mode ii. The neg3 reporter tracked a movement that is mode shift independent with a $t_{\text{fluor}}$ of approximately 500 ms. This results suggest a new state in the pathway ($O_{\text{S4in2}}$). The $O_{\text{S4in1}}$-$O_{\text{S4in2}}$ transition does not slow with mode shift. The second VSD movement step, $O_{\text{S4in2}}$-$O_{\text{S4out}}$, is not tracked by the neg3 reporter but I propose it is mode shift-dependent at weak depolarizations where a VSD movement is presumed to be rate-limiting. The speed of this second VSD movement must be slower than the first VSD movement in both mode i and mode ii channels. Additionally the second VSD movement step must be slower for mode ii channels compared to mode i channels. RLS = rate-limiting step.
Returning to the comparison of cAMP-bound and cAMP-free channels, I do not yet have data at weak depolarizations for HCN2F. However cAMP is known to significantly slow deactivation at a weak depolarization of -40 mV (Wicks et al., 2009), where the VSD movement is presumably still rate-limiting. Cyclic AMP must thus slow VSD movement at weak depolarizations in order to slow the deactivation pathway. Yet the fluorescence transients of HCN2F and HCN2F R591E suggest the tracked VSD step is not slowed by cAMP binding at strong voltages. If there is only one VSD movement step, as proposed by Chen (Chen et al., 2007), this is incompatible with the known role of cAMP slowing deactivation at weak voltages. Therefore in order for cAMP to slow a VSD movement step at weak depolarizations, as it must to slow deactivation, there must be a second VSD movement step. Thus the slowing of deactivation by both mode shift and cAMP at weak voltages support the idea of a second VSD movement step in addition to the tracked VSD movement step. My proposed deactivation pathway for the mode ii HCN channel in the apo and holo state is shown in Figure 3.33.
Apo channels at weak depolarizations:

\[
O_{ii,S4in} \xrightarrow{\text{slow}} O_{ii,S4in} \xrightarrow{\text{not tracked}} O_{ii,S4in} \xrightarrow{\text{RLS}} O_{ii,S4in}
\]

Holo channels at weak depolarizations:

\[
O_{ii,S4in} \xrightarrow{\text{very slow?}} O_{ii,S4in} \xrightarrow{\text{not tracked}} O_{ii,S4in} \xrightarrow{\text{RLS}} O_{ii,S4in}
\]

B

Apo channels at strong depolarizations:

\[
O_{ii,S4in} \xrightarrow{\text{slow}} O_{ii,S4in} \xrightarrow{\text{not tracked}} O_{ii,S4in} \xrightarrow{\text{RLS}} O_{ii,S4in}
\]

Holo channels at strong depolarizations:

\[
O_{ii,S4in} \xrightarrow{\text{very slow}} O_{ii,S4in} \xrightarrow{\text{not tracked}} O_{ii,S4in} \xrightarrow{\text{RLS}} O_{ii,S4in}
\]

Figure 3.33. Cyclic AMP binding does not slow the tracked VSD movement during deactivation but must slow the second VSD movement step at weak depolarizations

Note: Schematic showing the states of the HCN channel deactivation pathway at weak (e.g.: -60 mV) and strong (e.g.: +20 mV) depolarizations for apo and holo HCN channels in mode ii. The neg3 reporter tracked a movement that is cAMP-independent, and this suggests a new state in the pathway ($O_{S4in2}$) as cAMP must slow a VSD movement step to slow deactivation at least at weak depolarizations where VSD movement is presumed to be rate-limiting. RLS = rate-limiting step.

3.3.5. Structural interpretation of results

My results allow me to generate a novel structural schematic of the conformational changes that occur during the HCN channel deactivation pathway. As described above, I propose that the HCN channel deactivation pathway requires three
steps: two distinct VSD movement steps, and a pore closure step with weak voltage-dependence. Figure 3.34 represents the structural changes that may occur in an apo mode i HCN channel in a three-step deactivation pathway.

I suggest a possible conformational change tracked by the neg3 reporter is movement of the S2 and S3 helices. I find this proposal reasonable for three reasons. First, both these helices have acidic residues, and thus may change conformation upon a depolarization. Second, mutating these S2 and S3 residues to a uncharged amino acid causes loss of channel function, indicating their relative importance in HCN channel voltage-gating (Chen et al., 2000). Finally, VSD movements have also been tracked in Shaker channels using a reporter on the S1-S2 linker, and this reporter tracked a movement that was up to three times faster than Shaker gating current (Cha and Bezanilla, 1997). This indicated a movement around the N-terminal region of S2 may be occurring before a movement of S4 in Shaker channels (Cha and Bezanilla, 1997). I propose I am able to track a movement of the S2 and S3 helices because the S3-S4 linker moves with them to alter the environment around the extracellular neg3 reporter. I find the proposal that S3-S4 linker movement changes fluorescence emission reasonable as the S3-S4 linker contains two glutamic acid residues and one aspartic acid residue, both of which have been shown to quench TMRM emission (Cha and Bezanilla, 1998).

The movement of the S2 and S3 helices may move the VSD into a conformation where the S4 is now competent to undergo a conformational change. Once the S4 is in the competent structure, it can change conformation in the second VSD movement step which is the S4 outward movement as traditionally conceived. As the fluorophore only tracked one movement around the N-terminal region of S4, this suggests the second VSD movement step does not change the conformation of the N-terminal region of S4. I thus show the VSD step as a movement primarily of the C-terminal portion of S4. This type of movement has previously been proposed by Bell (swinging tail model, see Figure 1.8). I have also shown an outward movement of S4 in the same step, as suggested by other accessibility studies (Vemana et al., 2004), but one that does not drastically change the environment around the reporter. After both VSD movement steps, pore closure occurs.
Apo:

\[ O_{i,S4in} \xrightarrow{\tau \sim 500 \text{ ms}} O_{i,S4in2} \xrightarrow{} O_{i,S4out} \xrightarrow{} C_{S4out} \]

Figure 3.34. The tracked VSD movement step changes the environment around the N-terminal region of S4, while a second VSD movement step does not.

Note: Structural interpretation of the movements of the apo mode i HCN channel during deactivation at a weak depolarization (such as -60 mV). Only relevant channel structures shown (full channel structure shown in Figure 1.1); CSD is shown in the first channel state and then removed in later states for clarity. The orange circle represents the environment around the fluorophore, where conformational changes may result in changes of fluorescence emission. In the initial tracked VSD movement step, the S2 helix moves inwards, and shifts the S3-S4 linker into the orange circle; this results in an increase in fluorescence. In the second VSD movement step, both an outward movement and C-terminal swing occur in S4; neither or these movements result in substantial changes in the structures in the orange circle, which is why it is not tracked by the neg3 reporter. The final step involves pore closure and again does not disturb the structures in the orange circle.

Apo mode ii channels have only one difference relative to apo mode i channels throughout the deactivation pathway (Figure 3.35). In the initial \( O_{S4in} \) state of apo mode ii channels, the S4 helix C-terminal region is initially positioned closer to S2 to allow for electrostatic interactions. This interaction is broken only in the second VSD movement step of \( O_{S4in2} \) to \( O_{S4out} \). This structural difference in the C-terminal region of S4 in mode ii channels does not affect the tracked VSD movement step of S2 and S3 movements, which is why the tracked VSD movement did not slow with mode shift. I propose that the \( O_{S4in2} \) to \( O_{S4out} \) step is slowed by mode shift. This slower VSD movement step allows for slower deactivation for mode ii channels relative to mode i channels. I find this proposal reasonable as there are acidic residues in S2 in the middle of the helix and in the C-
terminal region of the helix that may promote interactions with the C-terminal half of S4; there are no acidic residues in the N-terminal region of S2 to promote movement of the outer N-terminal region of S4 toward S2.

**Apo mode ii:**

\[
\text{O}_{ii,S4\text{in}} \xrightarrow{\tau \sim 500 \text{ ms}} \text{O}_{ii,S4\text{in}2} \xrightarrow{\text{slow}} \text{O}_{ii,S4\text{out}} \xrightarrow{\text{C}} \text{S4out}
\]

**Figure 3.35.** HCN channels initiating deactivation from mode i or mode ii have the same initial VSD movement step tracked by the reporter

Note: Structural interpretation of the movements of the apo mode ii HCN channel during deactivation at a weak depolarization (such as -60 mV). Same type of schematic as shown in Figure 3.34. Mode ii channels have C-terminal region of S4 that is bent towards S2 in the initial \(O_{S4\text{in}}\) state. This change does not affect structures in the orange circle, which is why it was not tracked by the neg3 reporter. The mode ii position of S4 is slower to undergo the second VSD movement step, which is why mode shift can slow deactivation. Second and third steps occur as in Figure 3.34.

Holo mode ii channels have similar structures to apo mode ii channels in the VSD during deactivation (Figure 3.36). The tracked VSD movement step involving S2 and S3 movement is the same, as cAMP binding does not slow the tracked VSD step. cAMP binding slows channel deactivation by at minimum slowing the \(O_{S4\text{in}2}\) to \(O_{S4\text{out}}\) step at weak depolarizations, and also perhaps slowing the pore closure step through promoting C-linker tetramerization. Holo mode ii channels may possess OST at strong depolarizations in the early kinetic component. They may additionally possess OST at weak depolarizations. I propose OST would slow the traditional S4 inward to outward step that the Rneg3C reporter does not track. Holo channels additionally include a final
step in the pathway (not shown in the schematic) where cAMP dissociates from the CNB fold.

**Holo mode ii:**

\[
\begin{align*}
O_{ii,S4in} & \xrightarrow[\tau \sim 500 \text{ ms}] {\text{tracked}} O_{ii,S4in2} \xrightarrow{\text{slower}} O_{ii,S4out} \xrightarrow{} C_{S4out}
\end{align*}
\]

**Figure 3.36.** Apo and holo HCN channels have the same initial VSD movement step tracked by the reporter

Note: Structural interpretation of the movements of the holo mode ii S4 channel during deactivation at a weak depolarization (such as -60 mV). Same type of schematic as shown in Figure 3.34. The presence of cAMP and OST slow the second VSD movement step, which is not tracked by the neg3 reporter because this step does not substantially change the environment in the orange circle. Second and third steps occur as in Figure 3.34. Holo channels require an additional step (not shown) where cAMP dissociates from the closed channel.

I also cannot rule out the possibility that the reporter is tracking an S1 movement, but I find this possibility less likely as I do not think an S1 movement would be voltage-dependent. The sequence proposed to constitute S1 does not have any charged residues (Santoro 1998).

My schematics thus describe a pathway where the S3-S4 linker moves during gating. This is in broad terms consistent with the paddle structure put forth by the MacKinnon research group (described in section 1.1.3.2). Though I cannot suggest the extent of S3 movements, direction of S3 movements, or whether S3 is divided into two segments, this result is consistent with a model where the S3-S4 linker plays a substantial role in voltage-gating.
3.4. Discussion

3.4.1. Comparison with previous VCF spHCN1 studies

There are only two previous VCF studies on HCN channels (Bruening-Wright and Larsson, 2007; Bruening-Wright et al., 2007). These studies addressed the G-V and F-V relationships of spHCN1 channels, and reported the activation kinetics of current and fluorescence transients for a range of activation voltages (-80 mV to -140 mV). They also tested fluorescence deflections from reporters on eight total S4 residues between the two studies. This study only uses a reporter bound to neg3, and thus I do not have as thorough an understanding of the environment surrounding the N-terminal region of S4 as that achieved for spHCN1. However, my range of voltages tested during deactivation was double the range of voltages tested for spHCN1 activation. In addition to expanding the fluorescent study of HCN channel VSD movements into the first mammalian subtype, this work also represents the first study primarily focused on HCN channel deactivation. This is also the first VCF study to examine HCN channels in the apo state and mode i state.

A perhaps surprising difference between my HCN2F results and the spHCN1 results when using a neg3 reporter was the direction of the fluorescence transient upon activation. In spHCN1 channels, channel activation at hyperpolarized voltages produced an increase in fluorescence emission, while in HCN2F channels, without or without the R591E mutation, channel activation at hyperpolarized voltages produced a decrease in fluorescence emission. There are several potential reasons for this discrepancy. Perhaps the neg3 reporter tracks a movement of the S3-S4 linker for both channels but the linker moves in opposite directions upon hyperpolarization in spHCN1 and HCN2F channels. Perhaps the neg3 reporter tracks a movement of the S3-S4 linker towards S4 for both channels, but the spHCN1 S3-S4 linker sequence has a residue that de-quenches fluorescence emission while the mHCN2 S3-S4 linker sequence has a residue that quenches fluorescence emission. Or, perhaps the neg3C reporter tracks two completely different movements in the two types of channels. One factor that might have contributed to the differences in fluorescence emission is the fact that the spHCN1
studies used the Alexa-488 C5-Maleimide fluorophore while this study used the TMRM fluorophore.

A second difference between the two types of channels is in regards to the kinetics of the VSD movement tracked by the neg3 reporter. Several factors make it difficult to compare the deactivation kinetics found in this study with those of the previous spHCN1 neg3 study. First, the spHCN1 study was focused on activation kinetics and only included raw traces of deactivation transients without analysis. Moreover, the authors used a +50 mV depolarization while the strongest depolarization in this study was +40 mV. The spHCN1 +50 mV current deactivation transients look qualitatively much faster than the +40 mV current deactivation transients from mHCN2: spHCN1 current decay is nearly complete after a 100 ms deactivation. This suggests that mHCN2 has relatively slower deactivation gating than spHCN1, which is supported by previous studies of spHCN and mHCN2 wildtype channels (Gauss et al., 1998; Chen et al., 2007). Additionally, the spHCN1 fluorescence decay during deactivation looks qualitatively faster than the mHCN2F fluorescence decay during deactivation. This limited comparison suggests that channels with slow kinetics will also have slow movements in the VSD, and thus may guide predictions for future VCF studies.

Although I did not study kinetics under the same parameters as Bruening-Wright with the neg3 reporter, both studies reported F-V and G-V relationships. The $V_{1/2[\text{curr}]}$ is similar between spHCN1 and HCN2F (spHCN1 $V_{1/2}$ was approximately -109 mV; HCN2F $V_{1/2}$ was approximately -107 mV). However the $V_{1/2[\text{fluor}]}$ is more hyperpolarized in HCN2F channels (spHCN1 $V_{1/2}$ was approximately -79 mV; HCN2F $V_{1/2}$ was approximately -99 mV). The difference between the $V_{1/2[\text{fluor}]}$ values but similarity between the $V_{1/2[\text{curr}]}$ values suggests a third difference between spHCN1 and HCN2F channels: the coupling between the VSD and PD for spHCN1 is weaker than the coupling between the VSD and the PD for HCN2F. This is because the $V_{1/2[\text{fluor}]}$ for spHCN1 occurs at a weaker hyperpolarization, but this does not translate to the $V_{1/2[\text{curr}]}$ occurring at a weaker hyperpolarization relative to HCN2F. HCN2F also has a noticeably shallower steepness of voltage dependence for fluorescence transients. Though the reciprocal slope values for the spHCN1 G-V and F-V curves for the channel with the neg3C reporter were not reported, they qualitatively appear very similar. This is in contrast to the results for both
HCN2F and HCN2F R591E, where the F-V curve appears right shifted to the G-V curve at weak hyperpolarizations, but the two curves eventually cross due to the shallow F-V slope.

Two key differences in the S4 sequence of these two channels are: the Kneg12 residue for mHCN2 versus the Eneg12 residue for spHCN1, and the Hpos18 residue for mHCN2 versus the Spos18 residue for spHCN1. The steeper voltage-dependence of the F-V curves of spHCN1 channels thus seems counterintuitive, as spHCN1 channels have less net positive charge in S4 than mHCN2. However the neg12 and pos18 residues are not necessarily HCN gating charges in either channel. Overall, the results of the mHCN2 and spHCN1 VCF studies were perhaps less consistent that would have been expected for homologous channels, and highlight the significance of having even a few differences in sequence in the VSD.

3.4.2. Limitations of this study

3.4.2.1. Labeled HCN2F channels behave differently than HCN2 WT channels

Labeled HCN2F channel behaviour deviates significantly from the behaviour of HCN2 WT. I summarize below these differences and how they might limit the applicability of the answers for Questions #3, #4, and #5 of my thesis.

Question #3: The behavioural differences from HCN2 WT means that the absolute reported speeds of VSD and PD movement may not be transferrable to physiological conditions.

Question #4: The mutations within the PD did not remove or introduce any charged residues, and thus I predict the voltage dependence of a channel with HCN1 PD would be similar to an HCN2F channel with the HCN1-like PD. However the Rneg3C mutation and labeling with neutral TMRM in the VSD means the voltage-dependence of VSD movements in a HCN2 WT channel might be different than that of the HCN2F channel, as HCN2 WT would include an additional basic residue.
Question #5 (cAMP): As the two HCN2_\text{F} channels had a WT HCN2 C-terminal region (with the exception of the well-characterized R591E mutation for the apo channel studies), I predict the effect of cAMP on the VSD would be similar in HCN2 WT channels to the HCN2_\text{F} channel results. In other words, were I able to study a HCN2 WT channel with VCF, I would predict the neg3 reporter would still track a VSD movement that is cAMP-independent.

Question #5 (mode shift): Additionally, as the HCN2_\text{F} R591E current transients slow with increased activation pulse lengths, mode shift still occurs in the HCN2_\text{F}-derivative channels. Thus I assume the neg3 reporter is not interfering with any S4-based mode shift movements. Therefore again, were I able to study a HCN2 WT channel with VCF, I would predict the neg3 reporter would also track a VSD movement that is mode shift independent.

3.4.2.2. VCF only tracks the N-terminal region of VSD helices

As described in the structural interpretation (section 3.3.5), it is difficult to make conclusions regarding the movements of the C-terminal region of S4, given the N-terminal position of the reporter. This is a limitation of all VCF experiments, as only the environment around a particular part of the channel is studied. My model suggests that there are substantial movements of S4 within the C-terminal region (a C-terminal swinging tail), but this region cannot be studied using VCF on intact oocytes with extracellular labeling. Patch clamp fluorometry is a technique similar to VCF but using excised membranes and has been successfully used to study cAMP regulation of the inner activation gate of HCN2 channels previously (Wu et al., 2012). Patch clamp fluorometry could be used in future work to study the speed of movements of the C-terminal region of S4 and compare them to the results from the N-terminal region. Patch clamp fluorometry still has its own set of limitations, as it would be subject to PIP_2-based rundown and loss of cellular factors that are important for the quick activation mechanism.

3.4.2.3. Speed of gating charge movement is unknown in the HCN2 subtype

A notable limitation of this study that was addressed in the original spHCN1 VCF studies was the comparison of the F-V relationship with gating currents. The Larsson
group was able to show that the Q-V relationship (with Q representing cumulative gating charge movement) was very similar to the F-V relationship. This suggests that the VSD movement tracked by their Rneg3C reporter is the same movement that occurs with the movement of gating charges. This strongly suggests that the spHCN1 neg3C reporter tracks a movement of S4. I also tracked a VSD movement, but without additionally studying the movements of gating charge associated with deactivation in HCN2 channels, I cannot know definitively that the fluorescence transients represent movement of S4. Gating current experiments have never been successfully tested for the HCN2 or HCN4 subtypes, and it is suggested they would not be feasible due to the slow kinetics of these subtypes (Bruening-Wright et al., 2007). If a research group does manage to track movements of HCN2 gating current, tracking fluorescence deflections and gating current simultaneously will allow me to confidently assign the tracked VSD movement to the S4 helix or the S1-S3 helices. I imagine there would be two components of gating charge movement, one (an early component) for the first step tracked by the neg3 reporter, and the second (a late component) for the second VSD movement step.

3.4.2.4. HCN2 channels in the holo mode i state were not studied

Although I studied apo mode i channels, apo mode ii channels, and holo mode ii channels, I was unable to isolate holo mode i channels. Reaching the holo mode i state would require a short activation pulse but a high proportion of channels bound to cAMP. When studying HCN channels using excised patches, very high concentrations of cAMP can be added to the bath before an experiment to shift channels into the closed holo state (Wainger et al., 2001). These channels would then shift into the mode i holo state upon opening. When using intact cells, however, such as the Xenopus oocytes used in this thesis, the cAMP levels in the cell are at lower physiological concentrations. Physiological concentrations of cAMP are not high enough to promote a large number of channels to enter the closed holo state ((Wang et al., 2002), see section 2.3.2). Examining HCN2_F channels in the holo mode i state would thus require using excised membranes, or treating oocytes with cAMP analogs such as 8-bromo-cAMP that increase cAMP concentrations in intact cells (Zolles et al., 2006). As neither mode shift nor cAMP binding slowed fluorescence transients during deactivation, I predict the fluorescence transients of holo mode i channels during deactivation would have
comparable speeds to apo mode i channels and holo mode ii channels at a given voltage.

3.4.2.5. The weak voltage regime was not investigated in HCN2_F channels

The deactivation $\tau_{[\text{fluor}]}$ and $\tau_{[\text{curr}]}$ values for HCN2_F R591E at -60 mV and -80 mV show the tracked VSD movement to be fairly rate-limiting. However, deactivation $\tau_{[\text{fluor}]}$ and $\tau_{[\text{curr}]}$ values for -90 mV or -100 mV depolarizations would further support this result. Data at these voltages would show us whether the fluorescence transient and current transient ever become fully coincident (with a VGRR approaching 1), indicating the VSD movement step is fully rate-limiting. For HCN2_F, I currently lack data for any voltage where the tracked VSD movement may be rate limiting, as I only have data for the +40 mV to -20 mV range (only +20 mV data shown). VCF recordings in the weak voltage regime for HCN2_F would provide further evidence for a second VSD movement step if cAMP did not slow the tracked VSD movement. Thus the deactivation model would benefit from further examination of the weak voltage regime using both the HCN2_F channels.

3.4.2.6. The $\tau_w$ value does not fully represent the current transient in the VGRR equation

The VGRR ratio compares the $\tau_{[\text{fluor}]}$ and $\tau_{[\text{curr}]}$ values to generate a parameter that measures the temporal association between the VSD and the PD. One issue with this VGRR ratio is the fact that the fluorescence and current transients were fit to different exponential equations. The $\tau_{\text{early}}$ and $\tau_{\text{late}}$ components of the current transients for both HCN2_F channels were approximately 50%, meaning I was unable to select one component as a representation of the majority of the current trace (as I did in Chapter 2, where the $\tau_{\text{early}}$ component approached 90%). The use of a single value ($\tau_{[\text{curr}]}$) to represent the entirety of the double exponential current trace is insufficient, but one of the simplest ways to compare the current transient with the single exponential decay of the fluorescence trace. The VGRR value is thus inherently limited as it does not fully represent all the characteristics ($d$, $\tau_{\text{early}}$, $\tau_{\text{late}}$, $f_{\text{early}}$) of the current transient. However, the VGRR ratio still shows, that to some degree with at least one parameter, the relative timescales of the VSD movement and pore movement is voltage-dependent.
Chapter 4. Conclusions and future directions

Contributions:

This chapter discusses preliminary results of HCN2₅-based channels from Chapter 3 (see Chapter 3 contribution section), and the additional channel JK1. Jennifer Kass subcloned the JK1 channel. I completed all VCF recordings and analysis for the JK1 channel (nine recordings total).
4.1. Conclusions

The overarching objective of my thesis was to clarify to what extent models (see section 1.2) correctly explain HCN channel kinetics for both the activation and deactivation pathways. This thesis aimed to answer five questions (see section 1.3). They are reproduced below, along with answers to the questions that were found in Chapters 2 and 3.

Question #1: Does autoinhibition relief by truncation speed opening kinetics to the same degree that it depolarizes the $V_{1/2}$?

Predicted Outcome #1: Yes. Binding of cAMP to relieve autoinhibition is known to both hyperpolarize $V_{1/2}$ values and slow activation kinetics, so I predicted that autoinhibition relief by truncation would depolarize $V_{1/2}$ values and speed activation kinetics in parallel.

Answer #1: No. Autoinhibition relief by truncation of the CNB fold depolarizes the channel $V_{1/2}$, but does not speed activation. Apo and holo channels with intact CNB folds were both found to have significantly faster activation kinetics than channels with a partial or full truncation of the CNB fold. I propose this behaviour is due to a “quickening conformation” in channels with an intact CNB fold. Channels in the quickening conformation have fast activation regardless of whether the channel is autoinhibited or autoinhibition free. Autoinhibition is thus sufficient to predict the $V_{1/2}$ values of HCN2 channels, but insufficient to predict corresponding activation speeds.

Figure 2.17 summarizes a new working model of the HCN channel activation pathway that incorporates the quickening conformation. In this model additional interactions in the C-linker (shown by the touching wavy blocks) occur in a truncated channel that slows the rate-limiting VSD movement step. These interactions cannot occur when a full CNB fold is present; thus the presence of the CNB fold places the channel in a quickening conformation that allows for faster activation. The CNB fold can be in the apo or holo state to disrupt the C-linker interactions, and hence our quickening conformation is cAMP-independent.
Question #2: As PIP$_2$ can potentiate channel $V_{1/2}$ values, does PIP$_2$ also speed channel activation?

Predicted Outcome #2: Yes. As I identified the quickening conformation through Question #1, it seemed reasonable that PIP$_2$ might contribute to the fast activation of channels with an intact CNB fold. PIP$_2$ is known to bind to the C-linker in spHCN1 channels (Flynn and Zagotta, 2011), and S4 inwards movement is dependent on C-linker conformation in my new model of HCN channel activation.

Answer #2: No. Chemical inhibition of PIP$_2$ in oocyte membranes did not change the activation speed of channels with a partially truncated or intact CNB fold. I thus propose the quickening conformation is a PIP$_2$-independent mechanism. The step modified by PIP$_2$ is thus the closed to open transition (the last step in Figure 2.17), which we found was not rate limiting for the activation pathway.

Question #3: Is there a voltage where speed of VSD movement during deactivation is no longer rate-limiting for the deactivation pathway, as predicted by the cyclic allostery model?

Predicted Outcome #3: Yes. As VSD movement steps are presumed to be voltage dependent, strong depolarizations would cause very fast and non-rate-limiting kinetics for a VSD movement step.

Answer #3: Yes. Tracking VSD movements directly by VCF and a fluorescent reporter on the N-terminal region of S4 showed that there is a VSD movement that is not rate-limiting for the deactivation pathway at a +20 mV depolarization. This result represents the first direct tracking and first reported speed of a VSD movement in an HCN2 channel.

Question #4: Does the speed of VSD movement during deactivation have stronger voltage-dependence than the speed of the pore closure step, as assumed by the cyclic allostery model?
Predicted Outcome #4: Yes. Primary structure suggests the pore region does not have many charged residues, and thus I predict the pore movement step to be voltage-independent.

Answer #4: Yes. VCF recordings showed the tracked VSD movement during deactivation can occur over 7-fold faster at a +40 mV depolarization than at a -80 mV depolarization. VCF recordings also showed a weak voltage-dependence of the time constants of the current transient, indicating a weak voltage-dependence of the pore closure step. This result is thus consistent with the prediction of the cyclic allostery model of the VSD movement step preceding the pore closure step and having a stronger voltage dependence. However these results are inconsistent with the prediction of the cyclic allostery model of a voltage-independent pore movement step. These results suggest the pore closure step may be weakly voltage-dependent.

Question #5: As the mode shift and cAMP binding can slow deactivation, does the speed of VSD movement during deactivation decrease when the channel undergoes mode shift or binds cAMP?

Predicted Outcome #5: Yes, at least at weak depolarizations where VSD movement would be rate limiting. The S4-S5 linker and C-linker have been shown to be in close proximity with one another (Kwan et al., 2012), which provides a plausible pathway for CNB fold conformational changes to result in VSD conformational changes. Mode shift is believed to arise from within the VSD, as S4 undergoes a lateral shift after pore opening (Bruening-Wright and Larsson, 2007).

Answer #5: No. The tracked VSD movement during deactivation was not dependent on the presence or absence of cAMP, or mode shift. The tracked VSD movement was independent of mode shift even at weak depolarizations where VSD movement is shown to be rate-limiting. However mode shift still slowed current transient of the VCF recording, indicating at least one step in the deactivation pathway is mode shift dependent. I thus propose the existence of an additional VSD movement step in the HCN channel deactivation pathway. This VSD movement step is the first in the pathway, involves movement around the N-terminal region of S4, and is voltage-dependent but cAMP and mode shift independent. The second VSD movement step is at a minimum
mode shift dependent at weak depolarizations, but may also be cAMP dependent. The second VSD movement step also does not involve movement around the N-terminal region of S4, and thus I propose involves a swinging of the C-terminal tail of S4. I propose both VSD steps are required to permit pore closure.

Figures 3.32-3.36 summarize my new working model of the HCN channel deactivation pathway that incorporates a new VSD movement step that is voltage-dependent but cAMP-independent and mode shift-independent. This new VSD movement step could arise from many different conformational changes, but I propose this step involves movement of the S2 and S3 peripheral helices, which is registered by the neg3 reporter on the N-terminal region of S4. The second VSD movement step may encompass both a C-terminal swing of S4 and an outwards movement, but neither can substantially alter the environment surrounding the fluorophore. I propose the second VSD step that was not tracked in this study is voltage-dependent, cAMP-dependent, and mode-shift dependent.

This thesis advances the field of HCN channel study by re-characterizing the activation and deactivation pathways. I propose two new working models, one to incorporate the quickening conformation mechanism into the activation pathway, and one to incorporate the second VSD movement step into the deactivation pathway. This thesis also represents the first time a VSD movement has been measured directly by fluorescence in mammalian HCN channels. Chapter 3 thus proves the VCF method can be used to study the speed of VSD conformational changes, and the temporal association between the VSD and the PD, in the kinetically slow but physiologically relevant mHCN2 subtype.

4.2. Future directions

The following section outlines future directions for both the Chapter 2 and Chapter 3 projects. Some of these future direction discussions include preliminary data that was not studied fully enough to include in the results sections. Progress on these future directions will be indicated. Others are purely theoretical and have not progressed to the experimental stage.
4.2.1. Identifying the structural elements responsible for the quickening conformation

The quickening conformation mechanism discovered in Chapter 2 may apply to various HCN subtypes due to the strong conservation of the key structures of the CNB fold and C-linker, in contrast with the poorly conserved extreme-C region which is inessential for the quickening conformation. However, the quantitative effect of the quickening conformation may be different among subtypes, as is the case with other CNB-fold-dependent mechanisms like autoinhibition and cAMP-dependent relief (Wainger et al., 2001; Vemana et al., 2004) and open-state trapping (Wicks et al., 2011).

Besides variations in subunit composition, variations in levels of cellular factors are a potential source of tissue-specific functional diversity in \( I_f \). This highlights the importance of the possibility that a cellular factor distinct from cAMP and PIP\(_2\) is necessary (but not sufficient) for preservation of the quickening conformation. Although the search for a cellular factor will require substantial investigation, measuring the speed of the rate-limiting QA step could provide a valuable experimental test for verifying the functional effect of any candidate factors.

Truncations of HCN4 have been reported in sinus bradycardia patients exhibiting abnormally low heart rate even at resting conditions (Schulze-Bahr et al., 2003; Schweizer et al., 2010). A full understanding of \( I_f \) kinetics in such patients can potentially benefit from better understanding of the relative orientation of cytoplasmic C-terminal regions in heteromeric HCN channels containing truncated subunits, within the context of an intact cell. Autoinhibition might be preserved or abolished depending on the presence of the N-terminal region of the CNB fold and on the number and placement of truncated subunits within the heterotetramer, but the most likely primary effect on S4 movement would be deceleration due to disruption of the quickening conformation. This would apply whether or not PIP\(_2\) potentiation was disrupted. And it would also apply even with basal concentrations of cAMP, where most HCN channels have their CNB folds unliganded. Thus, identifying the structural elements of the quickening conformation producing faster \( I_h \) activation may prove important in predicting and managing phenotypes of cardiopathies.
4.2.2. **Identifying the applicability of the quickening conformation in other HCN channel subtypes**

HCN2\(_F\) channels were not the only other channel type I found that was consistent with the quickening conformation. Though the work on these channels did not advance far enough to be addressed in the thesis, I additionally studied HCN2/HCN4 chimera channels from previous Young laboratory publications (Wicks et al., 2011, 2009). These channels include a K381E mutation in S4 and were originally used to study the OST mechanism (Wicks et al., 2009, 2011), and thus had not been studied in intact cells. Both HCN242 and HCN242 R591E had faster \(\tau_{\text{early}}\) values than the HCN24x channel with a fully truncated CNB fold. These data support the model of an intact CNB fold allowing for a conformation that promotes fast activation and thus smaller \(\tau_{\text{early}}\) values, regardless of whether or not the channel is capable of binding cAMP in a later stage of activation. I thus conclude that the quickening conformation applies also to channels with an HCN4 transmembrane domain, and even channels with a charge reversal mutation in S4.

Along with the presence of the quickening conformation in unlabeled HCN2\(_F\) channels, together these results suggest the quickening conformation may be a mechanism present in multiple HCN channel subtypes. Comparing the activation speeds of channels with intact and truncated CNB folds in additional subtypes will further clarify the extent of this kinetic mechanism.

4.2.3. **Characterizing the deactivation speed of VSD movements in HCN2\(_F\) ΔCNB**

One future direction for this project is further examining the role of the CNB fold in the VSD movement. I generated a HCN2\(_F\) ΔCNB channel and characterized it using TEVC, but was not able to generate a VCF recording with a substantial fluorescence deflection before thesis submission.

Comparing fluorescence transients of HCN2\(_F\) ΔCNB and HCN2\(_F\) R591E will clarify the role of the apo CNB fold in the tracked VSD movement. TEVC recordings showed that the presence of the apo CNB fold hyperpolarized the \(V_{1/2}\) and sped
deactivation in HCN2\textsubscript{F} channels. Would the presence of the apo CNB fold also speed the tracked VSD movement during deactivation? This experiment will help answer whether the tetramerization of the C-linker (not favoured in channels with an apo CNB fold) can result in slower deactivation movements in the VSD. The results in 3.3.4 show that relieving autoinhibition (via cAMP binding) did not change the speed of the tracked VSD movement. Thus I predict ΔCNB will have a fluorescence decay speed similar to that of HCN2\textsubscript{F} R591E and HCN2\textsubscript{F} (~150 ms at +20 mV). With similar VSD movement speeds in the three channels, this result would suggest that C-terminal region conformational changes may not result in conformational changes during the tracked VSD movement step at strong depolarizations, and the influence of the C-terminal region upon pore closure speeds is due to the C-terminal region regulating only the conformation of the lower S6 activation gate and second VSD movement step.

4.2.4. Characterizing activation kinetics of the VSD movement tracked by the neg3 reporter in HCN2\textsubscript{F}-based channels

While this study focused on the VSD movements during deactivation of HCN2\textsubscript{F} channels, I necessarily also collected data on the VSD movements during activation. Although the F-V and G-V curves were reported in chapter 3, this data alone does not show the kinetics of the tracked VSD movement. I have begun analyzing preliminary data on the kinetics of VSD movements during activation, but would require additional VCF recordings – particularly at the physiologically relevant activation voltage of -90 mV – to have a full data set.

The HCN2\textsubscript{F} reporter tracked a movement that occurred at a similar time to pore opening. The activation fluorescence and current transients superimposed very well in the early portion of the transients during a -130 mV activation pulse. This suggests a VSD activation movement and pore opening that are coincident (Figure 4.1). Preliminary examination of activation transients is thus consistent with the proposal that the reporter is tracking a conformational change in the VSD that is coupled to pore movement, but this temporal association can be much tighter during activation compared to deactivation.
Unlike deactivation fluorescence transients, the majority of activation fluorescence transients required a double exponential fit to accurately represent the raw trace (see methods). I do not think either component is tracking a mode shift movement, as neither are on the same time scale as our HCN2F R591E mode shift (shown in section 3.3.4). For the ten HCN2F activation transients at -130 mV I presently have, seven required double exponential fits with a sloping baseline while three were sufficiently described by a single fit with a sloping baseline. The fact that the ten VCF activation transients required two different fitting methods may suggest that the reporter is tracking two conformational changes in the VSD in the activation pathway in three oocytes and one conformational change in seven oocytes. This fact may also suggest that one component of the activation transient is cAMP-dependent, and in a minority of oocytes the level of cAMP were insufficient to initiate the later component. Heterogeneity among oocytes is a challenge for all studies in intact cells, as it is impossible to know the endogenous cAMP concentrations and exactly how many channels bind to cAMP in each oocyte.
Figure 4.1. The current and fluorescence transients in HCN2F channels are coincident in the early portion of activation

Note: Superimposition of normalized current (black) and fluorescence (purple) -130 mV activation transients of a single VCF recording of HCN2F channels. Recording is same as that shown in Figure 3.6. Unlike with deactivation fits, in this example the activation transient fluorescence trace required fitting by a double exponential equation.

Further study of the activation epoch of my VCF recordings for HCN2F and HCN2F ΔCNB will allow us to determine whether the quickening conformation results in faster VSD movements. As described in section 3.3.1.5, unlabelled HCN2F has smaller $\tau_{\text{early}}$ values than HCN2F ΔCNB, despite both having undergone autoinhibition relief and having similar $V_{1/2}$ values. If HCN2F ΔCNB has slower VSD movements, this would suggest that the quickening conformation of the CNB fold transduces to the VSD. Potentially, this could occur through an interaction between the C-linker and the S4-S5 linker. This experiment would help create a more defined structural model of the cAMP- and PIP$_2$-independent quick activation, by suggesting whether or not the quickening conformation includes structures within VSD.
4.2.5. Investigating the role of the C-terminal region of S4 and the S4-S5 linker in gating

The S4-S5 linker physically connects the VSD and PD. Mutations in the presumed S4-S5 linker region can disrupt channel deactivation (Macri and Accili, 2004), and even permit pore opening after other mutations had disrupted S4 movement (Chen et al., 2001). The Yellen group proposed a model of HCN channel activation where the S4-S5 linker and C-terminal end of S5 twist away from the pore. This movement would result in a tilt of the S6 helices that widens the intracellular opening of the pore (Kwan et al., 2012). A cryo-electron microscopy (cryo-EM) structure of hHCN1 in the closed state was very recently generated by the MacKinnon research group, and is the first structure of any HCN channel (Lee and MacKinnon, 2017). This structure shows that the S4 extends much further into the cytoplasm than the S4s of other Kv channels, and the hHCN1 S4-S5 linker is not α-helical but a very short loop. The authors propose that the elongated S4 interacts with the C-linker of an adjacent subunit, and when the S4 is depolarized it causes the C-linker to twist into a conformation that closes the pore. These results suggest that the movement of the C-terminal region of S4 /S4-S5 linker region could be another potential source of kinetic regulation of HCN channels: does the twisting movement from this region that opens the gate occur simultaneously with S4 inward movement, or in a later step?
Figure 4.2. Cryo-EM structure of hHCN1 channel

Note: 
A: Top down view of the homotetrameric hHCN1 channel. Each of the four subunits are labelled a different colour. B: Single hHCN1 subunit. Yellow = N-terminal region (group of 3 helices termed the HCN domain); dark blue = S1-S3; light blue = S4; red = PD; light green = C-linker; dark green = CNB fold; pink stick structure = cAMP. Both A and B structures were generated from PDB 5U6P, from (Lee and MacKinnon, 2017).
Figure 4.3. Human HCN1 C-terminal S4 and S4-S5 linker interact with the C-linker of an adjacent subunit

Note: A: Surface representation of hHCN1 channel. One subunit shown in shades of red, another in shades of blue, and two in grey. S4 – S6 of red subunit shown with secondary structure and stick sidechains. S4 = dark red; S5-S6 = raspberry. C-linker of blue subunit shown with secondary structure and stick sidechains in dark blue. cAMP in CNB fold shows as green sticks. B: Zoom-in of A. Both A and B structures were generated from PDB 5U6P, from (Lee and MacKinnon, 2017).
The C-terminal S4 / S4-S5 linker region and the C-linker in the CSD are within close proximity during gating and interactions between them are essential for proper gating (Kwan et al., 2012). An interaction between HCN2 R339 D443 stabilizes the closed state of wild-type channels in HCN2 channels (Decher et al., 2004). D443 is in the C-linker, and the hHCN1 cryo-EM structure suggests R339 is the inner most portion of S5 immediately following the S4-S5 linker. The Young laboratory has already generated and characterized with TEVC an HCN2/HCN4 chimera channel with the S4-S5 linker mutation HCN2 R339E.

A HCN2F-based channel with the additional R339E mutation would suggest the role of this C-terminal S4 / S4-S5 linker region in VSD and PD temporal association. Although I cannot generate a reporter bound to the region for VCF experiments due to the region’s position near the inner leaf of the membrane, I can still use VCF to indirectly study this region’s movements. I propose the native structure of the C-terminal S4 and the S4-S5 linker region is essential for proper temporal association between the VSD and PD. In the mutant R339E channel, I predict that temporal association between the VSD and pore will be weakened. I predict weakened temporal association in both the strong voltage regime (where the association is already fairly weak) and the weak voltage regime (where the association is fairly tight). In both cases, weakened temporal association would present itself as fluorescence and current transients that become less superimposable, and a smaller VGRR. Weakened temporal association introduced by an S4-S5 linker region mutation would support the model where the S4-S5 linker translates VSD gating movements to the PD, specifically the lower S6 gate. Studying the R339E mutant through VCF would provide new insights into linker kinetics in a way that is not typical for a helix residing so close to the intracellular leaflet of membrane.

4.2.6. Identifying VSD residues that contribute to the mode shift movement after pore opening

I have preliminary VCF data tracking VSD movements with a neg6C reporter on an HCN4-derivative channel. The spHCN1 VCF studies found three reporter positions that tracked a fluorescence deflection that occurred after pore opening: spHCN1 neg9, neg10, neg11 (see section 1.2.4 (Bruening-Wright and Larsson, 2007). The authors
suggest this deflection corresponds with a mode shift movement. I thus examined a channel already present in the Young laboratory called JK1. JK1 is a HCN1/2/4 chimera channel that includes the relevant structures of the HCN1-based pore from HCN2F, and an Rneg6C mutation in the S4 N-terminal region. JK1 does not have any other mutations in S4, such as Rneg3C.

Interestingly, the neg6 reporter position is the sole reporter position attempted in the previous spHCN1 VCF studies that was unsuccessful (Bruening-Wright et al., 2007). According to their report, their spHCN1 neg6C channel did not express well enough to produce a viable fluorescence signal. However, I found the JK1 channel produced typical HCN channel-like amplitudes during TEVC experiments of up to 2500 nA (Figure 4.2A). The unlabeled JK1 channel had a $V_{1/2}$ value of (-114.6 ± 3.1 mV, n = 13) (Figure 4.2B). I attempted VCF on this channel to determine whether the reporter tracks a VSD movement that precedes or follows pore closure.

![Figure 4.4. Representative TEVC data for JK1 channels](image)

**Figure 4.4. Representative TEVC data for JK1 channels**

*Note:* A: Representative TEVC recordings of unlabeled JK1. Same protocol as used in Figure 3.2. Sweep with -130 mV activation pulse highlighted in orange for clarity. B: Representative conductance–voltage relations for unlabeled JK1 (orange) and unlabeled HCN2F (grey) as described in methods (3.2.2.4).

The JK1 neg6 reporter tracks a VSD movement that occurs after pore opening and after pore closing. Though I only presently have preliminary data, the JK1 channels
produce measurable fluorescence deflections with VCF (Figure 4.3). The fluorescence
deflections were noticeably slower than the HCN2\textsubscript{F} and HCN2\textsubscript{F} R591E deflections in
both the activation and deactivation pulses. Superimposition of the fluorescence and
current transients during both the -130 mV activation pulse and the +20 mV deactivation
pulse show that the fluorescence transient is in fact slower than the current (Figure 4.4).
JK1 has a VGRR value of approximately 1.9 at a +20 mV depolarization, which is a
noticeably larger VGRR than the previous values and the only VGRR I have recorded
that is above 1.0. This suggests that the JK1 reporter position tracks a VSD movement
that occurs after the pore has opened during activation and after the pore has closed
during deactivation. With this channel, I can now investigate a step in the gating pathway
that is entirely unique from the step that was the predominant focus of this study. Though
I have not yet tested the timescale of mode shift in JK1, it is possible that the JK1
fluorescence transient is on a similar time scale as mode shift. If this is the case, instead
of only examining the effect of the mode shift movement on the speed of VSD steps
during deactivation, with this channel I can determine the speed of the mode shift
movement directly.
Figure 4.5. Representative VCF recording of JK1 channels
Note: Voltage protocol (top), current trace (middle), and fluorescence trace (bottom) of a representative JK1 VCF recording. Protocol includes a 3 s -130 mV activation step followed by a 500 ms -120 mV step, and a +20 mV deactivation pulse for 3 s. Holding potential is -40 mV.
Figure 4.6. JK1 fluorescence transients follow current transients during both activation and deactivation

Note: A: Superimposition of normalized current and fluorescence activation transients from the recording in Fig 4.4 during the -130 mV activation pulse. B: Superimposition of normalized current and fluorescence deactivation transients from recording in Figure 4.4 during the +20 mV deactivation pulse.

Although the current transients of the VCF recording can by themselves verify the presence or absence of mode shift, VCF provides the extra benefit of understanding how S4 movement changes with modified mode shift. For example, introducing extra acidic residues on S2 may enhance mode shift; if this resulted in faster movement of S4 towards S2 upon activation, this could be registered by smaller value of $\tau_{[fluor]}$. As the structural changes in the VSD during mode shift are presently unclear, identifying residues critical for mode shift would greatly advance the mode shift model.
References


Evans, A., R.D. Bagnall, J. Duflou, and C. Semsarian. 2013. Postmortem review and


Goldschen-Ohm, M.P., V.A. Klenchin, D.S. White, J.B. Cowgill, Q. Cui, R.H. Goldsmith,


