MECHANISMS OF CYCLIC AMP-DEPENDENT MODULATION IN PACEMAKER CHANNELS

by

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B. Sc. Molecular Biology and Biochemistry, Simon Fraser University, 2005

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ABSTRACT

Pacemaker (HCN) channels are hyperpolarization-activated and their activity is enhanced by cyclic AMP (cAMP), but whether cAMP-gating is coupled to voltage-gating remains unresolved. With long hyperpolarizations, HCN channels form a secondary open state after the initial opening; this briefly sustains the activation of open channels after a return to a resting voltage. CAMP effects on the formation and decay of the secondary open state remain unclear. We studied mouse HCN4 channels in excised oocyte membrane patches, and found cAMP increases sustained activation. We also studied a charge-reversing S4 mutation (K381E) and found it causes surprisingly little change in voltage-dependence. Notably, K381E dramatically increases the cAMP-dependence of sustained activation. Our results suggest cAMP and K381E synergistically enhance the formation of a secondary open state and that this state decays during deactivation through a voltage- and cAMP-dependent step. These results suggest a physical coupling of voltage-sensing and cAMP-modulatory machinery in HCN channels.

Keywords: Ion channel; pacemaker; voltage; cyclic nucleotide
DEDICATION

For My Family.
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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction to Ion Channels

Ion channels are the foundation of electrical excitability in living organisms. Their ability to conduct ions at phenomenal rates with exquisite selectivity in a remarkably regulated fashion allows them to generate and propagate electrical signals within individual cells and throughout entire organisms. Ion channels are gated in a number of ways: voltage-gated channels open in response to changes in membrane potential, ligand-gated channels in response to direct binding of chemicals, and mechanically-gated channels in response to membrane stretch or perturbation. In addition, ion channels are selective for different ions and can be cation- or anion-selective.

Despite the continually growing diversity of ion channels, their fundamental properties are the same. These elegant macromolecular switches are integral membrane proteins containing gated pores that, when open, permit the flow of ions down their electrochemical gradients. Ion channels are thus able to retrieve the energy stored in ionic gradients maintained between cells and the extracellular environment. Similar to members of an orchestra responding to a conductor and creating symphonies, ion channels respond to external stimulus and create organized electrical signals known as action potentials. These signals self-propagate and result in the excitation of neighbouring channels and cells. They ultimately control contraction of fibres in cardiac or skeletal muscle and the communication of sensory information throughout the nervous system.
This thesis focuses on a family of non-selective voltage-gated cation channels that open in response to membrane hyperpolarization and are responsible for membrane depolarizations that generate rhythmic activity in the heart and brain.

1.2 Introduction to HCN Channels

Hyperpolarization-activated cyclic nucleotide-modulated (HCN, “pacemaker”) channels are responsible for triggering rhythmic electrical activity in the heart and nervous system and their voltage-gated activity is enhanced by the binding of second messenger cyclic nucleotides. HCN current (I_h (hyperpolarization), I_{HCN}, I_f, I_q), was first identified nearly three decades ago as a “funny” or “queer” current (because it activated upon hyperpolarization rather than depolarization) in cardiac sinoatrial node (SAN) cells (1, 2), and was later shown to be directly modulated by cyclic-AMP (cAMP) (3). Further research identified a family of genes encoding weakly selective cation channels (4, 5), structurally related to the voltage-gated superfamily of potassium channels (6), that produce I_h. Initially referred to as HAC (Hyperpolarization-Activated Cation channels) or BCNG (Brain Cyclic Nucleotide-Gated channels), the gene family underlying I_h is now consensually referred to as HCN channels. Four mammalian isoforms (HCN1-HCN4) with key physiological functions have been identified.

1.3 Physiological Roles of HCN Channels

HCN channels are found in autorhythmic and non-rhythmic cells of the heart, brain and nervous system. Because of their non-selective nature, HCN channels have a reversal potential of -20 to -40mV under physiological conditions. Thus, at hyperpolarized voltages (negative to ~50 mV), HCN channels conduct an inward cation
current, largely sodium, that results in a membrane depolarization (7-9) that contributes to a number of functions, including generation of spontaneous activity and maintenance of resting membrane potential.

1.3.1 Roles in autorhythmic myocytes

In the heart, \( I_h \) has two major physiological roles: 1) to generate the spontaneous activity that results in rhythmic cardiac contractions and 2) to regulate the timing of these contractions in response to autonomic stimulus.

In autorhythmic myocytes, action potentials fire when the membrane potential is depolarized to the threshold (~40mV) required for voltage-gated calcium channel (\( \text{Ca}_v \)) activation. When open, \( \text{Ca}_v \) channels conduct an inward calcium flux that results in rapid membrane depolarization. Then, at the resulting depolarized membrane voltages, voltage-gated potassium (\( \text{K}_v \)) channels activate, and conduct an outward potassium current that first repolarizes and then hyperpolarizes the membrane. HCN channels typically activate at potentials more hyperpolarized than -60mV and conduct an inward cation current that, in addition to currents carried by additional \( \text{Ca}_v \) channels, including \( \text{Cav1.2} \) and 1.3, depolarizes the membrane to the threshold for the next action potential (8). Thus, HCN channels help maintain spontaneous pacing in the heart.

In addition, HCN channels mediate autonomic system modulation of heart rate by responding to changes in intracellular cAMP caused by B-adrenergic (sympathetic) or muscarinic (parasympathetic) stimulation (10). Intracellular cAMP directly regulates channel activity (3) by binding to an intracellular cyclic nucleotide-binding domain (CNBD) (4), which generally increases channel activity (11, 12). By responding to levels
of cAMP, HCN channels thus regulate the time between action potential firings and thus
determine heart rate.

1.3.2 Roles in autorhythmic nervous system cells

In the nervous system, I_h has a variety of roles, including autorhythmic activity in
basal ganglia and thalamus. Basal ganglia circuitry contributes to movement and motor
behaviour, and requires synchronous electrical activity in order to maintain proper
function; this synchronicity is attributed to the pacemaking properties of I_h (13). The
thalamus regulates consciousness and the sleep-wake cycle; sleep is characterized by
billions of synchronous low-frequency electrical oscillations, these are blocked by
various neurotransmitter systems during wakefulness. I_h contributes to the sleep-related
synchronous rhythm of thalamocortical neurons (14).

1.3.3 Roles in non-pacing cells

In addition to roles in cardiac and neuronal pacemakers, HCN channels have
multiple roles non-pacing cells, including maintaining resting membrane potential,
preventing over-hyperpolarizations and contributing to dendritic integration and synaptic
transmission. Membrane hyperpolarizations caused by potassium leak or gated cation
current activates HCN channels; their inward cation current results in a self-deactivating
depolarization that prevents further deviation from resting membrane potential (9). I_h is
also important for the integration of dendritic excitatory post-synaptic potentials (EPSPs);
it contributes to equal processing of distal and proximal EPSPs (8). Furthermore, I_h
contributes to the regulation of synaptic transmission: it has been shown to mediate
cAMP-dependent neurotransmitter-facilitated transmission at neuromuscular junctions (15) and also permits adaptation in photoreceptors in response to bright light stimulus (9).

1.4 Expression Patterns of HCN Genes

In situ hybridizations (9, 16), RT-PCR and immunohistochemistry (13), and northern blots (4) have shown HCN genes are expressed primarily within the heart, brain, nervous system and sensory organs.

HCN1 is expressed in basal ganglia (13), hippocampus, thalamus, brain stem (4), olfactory bulb (16), retinal photoreceptors and at low levels in SAN (9, 17). HCN2 is expressed in SAN, dorsal root ganglia (16, 18), basal ganglia (13), olfactory bulb, thalamus, cerebral cortex and cerebellum (4, 16). HCN3 is expressed at low levels in various regions of the brain (16, 19). HCN4 is the predominant cardiac HCN isoform: it is expressed at high levels in SAN and throughout ventricular and atrial tissue (9, 18, 20, 21).

1.5 Structure and Function of HCN Channel Domains

1.5.1 Topology

HCN channels are members of a structurally related family of integral membrane proteins, the voltage-gated superfamily of potassium channels (6, 22). They are believed to share the topology of several potassium channels whose structures have been solved (23-27). These channels function as tetramers, with four subunits arranged around a gated conducting pore. Each HCN subunit is comprised of three domains (Fig. 1): an intracellular amino terminal (NH2-) region, a six-helix (S1-S6) transmembrane region containing the voltage-sensing helix (S4) and pore forming helices (S5-S6), and an
intracellular carboxy terminal (COOH-) region containing the CNBD. Each of these three domains is responsible for distinct channel functions.

1.5.2 Domain functions

The three domains of HCN channels (NH2-, S1-S6, and COOH-) have each been associated with a variety of functions.

NH2- domains have been shown to interact with one another and contribute to tetramer assembly and trafficking (28, 29), but this region does not have a major role in voltage-gating, kinetics or cAMP-modulation (30-34).

S1-S6 contains both the voltage-sensing (S1-S4) and pore (S5, pore loop, S6) regions. This domain is therefore the principal determinant of voltage-dependence (35, 36) and has also been shown to regulate kinetics (32, 34, 37). The positively charged S4 helix is the primary voltage-sensor in HCN channels (38), as in K+ channels (39). The S5 and S6 helices line the aqueous ion conducting pore, and the selectivity filter is formed by a re-entrant “pore-loop” between S5 and S6 (12, 22). The selectivity filter contains the conserved GYG motif that is known to be responsible for the exquisite selectivity of potassium channels (40). The reduced selectivity of HCN channels relative to potassium channels probably involves differences in the flexibility of the inner pore region (41). An intracellular activation gate that opens in response to hyperpolarization-activation is thought to be formed by the crossing of the carboxyl ends of the S6 helices (42, 43).

The COOH- region contains two smaller subdomains: the C-linker and the CNBD. The C-linker is comprised of six alpha-helices and connects the carboxyl end of S6 to the CNBD (44). The 120 amino acid CNBD is homologous to the cAMP binding domains of cAMP and cGMP protein kinases, catabolite-activating protein and cyclic-
nucleotide gated (CNG) channels (4, 45). The COOH- region has been shown to exert an intrinsic inhibition on channel activity that is relieved upon cAMP binding (34), and also contributes to regulating channel kinetics (33).

1.5.3 Sequence identity and phylogeny

To date, 58 non-redundant HCN sequences have been identified across a wide spectrum of species, from urochordates to mammals (46). Four mammalian isoforms have been cloned; these are closely related (Fig. 2), having an overall amino acid sequence similarity of 50-60% and a similarity of 80-90% in their core region (S1 through the CNBD) (45). Outside their own family, HCN channels share the highest sequence similarity with other potassium channels containing a CNBD, such as CNG channels, plant inward rectifiers and the ether-a-go-go-(EAG)-related channels. However, the hydrophobic core of HCN channels is also similar (~14%) to voltage-gated potassium channels, notably to the quintessential Kv channel, Shaker (45). Interestingly, the CNBD of HCN channels is more similar to that of protein kinases than are those of other channels containing a CNBD. It has been suggested that the family of HCN genes arose through three different duplication events a before the divergence of the fish lineage, about 450 million years ago (46). Their common ancestor may have arisen from a fusion between a potassium channel and an ancient CNBD before the evolutionary separation of plants and animals (47). Furthermore, it has also been suggested that HCN channels may be an evolutionary link between voltage-gated potassium and CNG channels, having properties intermediate between the two (47).
1.6 Biophysical Properties of HCN Channels

The voltage-gated and kinetic properties of HCN channels, and the effects of cAMP\(^1\) on these properties, have been examined in detail. While HCN channels exhibit qualitatively similar behaviours, a number of studies (9, 21, 31, 33, 48, 49) report quantitative differences in kinetics, half-maximal voltage activation (\(V_{1/2}\)) values and cAMP-modulatory effects (for a review see 50); these findings are summarized in Table 1. It should be noted that the quantitative biophysical values that describe channel behaviours are highly dependent on the expression system, electrophysiological technique and ionic concentration and composition of solutions used. Therefore, a direct numerical comparison does not make sense unless identical techniques and experimental conditions are used. Thus, only data obtained using the inside-out excised macropatch technique are included in Table 1, and even so, non-identical experimental conditions will contribute to slight numerical disagreements. Generally, HCN1 channels exhibit rapid kinetics (opening and closing in tens of milliseconds) and their behaviour is only weakly enhanced by cAMP. HCN2 channels have moderately slow kinetics (hundreds of milliseconds) and their behaviour is strongly enhanced by cAMP. HCN3 channels also have moderately slow kinetics but are unique in being virtually unresponsive to cAMP. Finally, HCN4 channels have very slow kinetics (seconds) and their behaviour is strongly enhanced by cAMP.

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\(^1\) cGMP also modulates HCN channels, but with far less efficiency (Biel et al., 2002). For this reason, and because cAMP is the more relevant physiological ligand, this thesis will focus only on effects of cAMP.
1.7 Mechanisms of Voltage-Sensing, Gating and CAMP-Modulation

1.7.1 Voltage-sensing mechanisms

As in $K_v$ channels, the highly conserved and positively charged S4 helix acts as the primary voltage sensor in HCN channels. S4 contains nine conserved basic residues (35) and moves in response to changes in membrane potential (38). Mutagenesis experiments have indicated that certain basic S4 residues are determinants of voltage gating efficiency: neutralizing or reversing their charges pushes $V_{1/2}$ values towards more negative voltages (36). Cysteine accessibility studies have conclusively shown that S4 traverses the membrane from an outward to an inward facing position in response to hyperpolarization (38). Several models describing S4 movement have arisen (for a review see 51). Most notably, the traditional “helical screw” model (52, 53) suggests S4 rotates and traverses perpendicular to the membrane plane in response to voltage; the “paddle” model (Jiang et al., 2003) suggests S4 and S3 form a “paddle” that traverses the membrane; finally, a “transporter” model (54) proposes that S4 charges lay in either an intracellular or an extracellular aqueous crevice, and that tilt and rotation of S4 are responsible for charges moving from one crevice to another. While some controversy remains over its precise movements, there is no doubt that S4 is the voltage sensor in HCN channels and it moves from an outward- to inward-facing position in response to membrane hyperpolarization, and that this movement is coupled to gate-opening.

1.7.2 Gating mechanisms

Many aspects of gating in HCN channels are understood. In particular, activation is known to involve multiple closed and open states, the physical gate is intracellular and formed by the carboxyl ends of the S6 helices, gating is coupled to voltage-sensing
through the S4-S5 linker, and HCN channels can form a secondary open state after the initial opening step. However, it remains controversial whether the initial gate-opening event occurs in a voltage dependent or independent manner.

Both activation and deactivation $I_h$ currents are more easily fitted with a sigmoidal rather than a single exponential function, indicating that gating involves multiple closed and open states. These states can differ in the positions of their S4 helices, the status of the gate and in the conformations of their C-linker and CNBD (30, 32, 49).

The physical activation gate in HCN channels is known to be intracellular; when applied to the intracellular side of previously opened HCN channels, the $I_h$ blocker ZD7288 (55) can be trapped in the intracellular mouth of the pore by gate closure (42). Cysteine mutagenesis and cross-linking experiments (43, 56) suggest the gate itself is formed by the S6 helices, which sit in an “inverted tee-pee” fashion near the intracellular face of the membrane (22, 26).

The crystal structure of Kv1.2 (57) shows that S4 is connected to the S6 gate by the S4-S5 linker, an amphipathic helix that hugs the intracellular membrane and interacts extensively with the inner S6 helix. In hERG channels, the S4-S5 linker has been shown to interact directly with S6 to stabilize a closed state (58). This linker has also been shown to mediate coupling between the physical movement of S4 and gate opening in HCN channels; specific interactions between the S4-S5 linker and residues near the gate-forming region of S6 have been shown to stabilize both closed (59, 60) and open (61) states.

It remains unresolved whether the initial gate-opening event is voltage-dependent or voltage-independent. While one model suggests that voltage directly opens channels in
a Hodgkin-Huxley manner (62-65), growing evidence suggests that a voltage-independent closed-open transition occurs after S4 movement (32, 49, 66). Support for a voltage-independent opening step includes the discoveries that voltage-activated kinetics in HCN2 channels are saturated at very negative voltages (32), and that cAMP increases maximum open probability at strongly hyperpolarized voltages where voltage activation is saturated (49, 66). Moreover, while the S4-S6 region has been shown to be a determinant of voltage-independent kinetics (32), the regions governing the energetics of voltage-independent opening remain unknown.

Once the initial closed-open transition has occurred, and given sufficiently long hyperpolarizations, HCN channels are able to form a secondary open state; this is observable under voltage-clamp conditions as a brief delay, or "sustained activation", before the onset of decay of previously hyperpolarized current after a return to a resting membrane potential (67). The transition to this secondary state is known to be dependent on the length of the activating pulse, but the effects of cAMP on the formation and stability of this state are not known.

1.7.3 CAMP-modulatory mechanisms

cAMP binding to the intracellular CNBD of HCN channels modulates several aspects of channel behaviour: it speeds opening, positively shifts $V_{1/2}$ values, increases maximal open probability and slows closing (3, 4, 12, 32, 45). These effects result from the relief of an intrinsic inhibition by the CNBD in the absence of cyclic nucleotide; deleting the CNBD of HCN channels causes a positive shift in $V_{1/2}$ values that mimics and slightly surpasses the action of cAMP (Wainger et al., 2001). The solved crystal structure of the HCN COOH-region (44) shows these regions tetramerize in the presence
of cAMP and associate through specific inter-and intra-subunit interactions. The physical mechanisms of inhibition and relief are thought to involve changes to the interactions existing between C-linker regions and CNBDs upon ligand binding (31, 49).

Experimental support for this notion includes the findings that an intact C-linker is required for cAMP gating (60) and salt bridges involving the C-linker are involved in cAMP-gating: breaking them through mutagenesis mimics cAMP binding (49). While it is generally thought that cAMP modulatory and voltage-sensing machinery work independently of each other in HCN channels, there is no conclusive evidence showing this to be true.

1.8 An Overview of Electrophysiology

1.8.1 The cell as an electrical circuit

Since ion channels are responsible for the electrical behaviour of cells, it is useful to view them in terms of their electrical, rather than biological, properties. Cells are simple electrical circuits with two parallel branches: one branch contains a capacitor and the other contains a battery and a conductor in series. The lipid bilayer acts as a capacitor; it separates charges and maintains a voltage difference (the “membrane potential”) across its boundary. Ion channels act as conductors; they provide a path for current flow. Finally, the ionic gradients maintained between cells and the extracellular environment act as batteries; they are an electromotive force (68).

Electrophysiological techniques like voltage- and patch-clamp enable experimenters to control the membrane potential and composition of intra- and extracellular solutions and record the channel activity (current) that results from any number of test conditions.
1.8.2 The voltage-clamp technique

The voltage clamp technique was developed in 1949 by Cole (69), Marmont (70) and Hodgkin, Huxley and Katz (71, 72) and has revolutionized our understanding of ion channels. To “voltage-clamp” is to control the voltage difference, or membrane potential, across a lipid bilayer. Using voltage-clamp, the experimenter is able to hold the membrane at different potentials, which effectively eliminates the capacitive current that results from charge rearrangement and allows the careful measurement of the ionic current currents carried by channels. Classical voltage-clamp methods normally use two intracellular electrodes, one to record voltage and another to deliver current to maintain a set voltage. A third grounding electrode sits in the extracellular bath and measures ionic current (68). Classical voltage clamping has since evolved into the patch-clamp technique that is commonly used today.

1.8.3 The patch-clamp technique

Patch-clamp is a variation of the voltage-clamp method and was developed by Neher and Sakmann (73-75). To patch clamp, an experimenter fuses a narrow polished glass pipette with an intact cell membrane to form a stable high resistance “giga-seal” (a seal with an electrical resistance in the giga-ohm range). Ionic currents can then be recorded in the “on cell” configuration. Alternatively, the seal can be ruptured to allow the pipette and intracellular solutions to equilibrate; the experimenter can then record the current from an entire population of channels in the “whole cell” configuration. Additionally, the membrane patch can be pulled away from the rest of the membrane to form an “inside-out” or “outside-out” excised patch. In the inside-out configuration (Fig. 3), the cytoplasmic side of the membrane faces into the bath solution, whereas in the
outside-out configuration the extracellular side faces into the bath (68). The patch-clamp technique has a number of advantages over classical voltage-clamp, namely, it allows the experimenter to control the composition of intra- and extracellular solutions and to study single channel molecules. Hence, patch clamping remains one of the principal techniques used in the study of ion channels.

1.9 Summary of Outstanding Questions, Research Aims and Results

1.9.1 Outstanding questions

The literature review presented above highlights four outstanding critical questions in the field of HCN channels. First, does the initial gate-opening event in HCN channels occur in direct response to voltage or through a voltage-independent transition? Second, if the initial gate-opening step is indeed voltage-independent, what are the physical determinants that limit the extent of voltage-independent opening in the absence of cAMP? Third, what are the effects of cAMP on the formation and decay of a secondary open state previously reported to exist in HCN channels? Finally, do voltage-sensing and cAMP-modulatory machinery work independently in HCN channels, or are they functionally coupled in a significant way? Resolving these unanswered questions will help provide a physical model for HCN channel gating and a structural basis for the regulation of gating by voltage-sensor movement and cAMP binding. Importantly, identifying the effects of cAMP on the stability of specific channel states could lead towards the development of pharmaceutical ligands able to regulate HCN pacemaking activity at the molecular level.
1.9.2 Research aims and hypothesis

The aims of this research were to 1) quantitatively characterize the yet unreported voltage- and cAMP-gating properties of mouse HCN4 channels relative to other isoforms, 2a) determine whether a voltage-independent transition is responsible for the initial gating step in HCN4 channels and if so, 2b) identify the physical determinants that regulate this transition, 3) determine whether cAMP modulates sustained activation by affecting the formation of a previously reported secondary open state, and 4) to explore the possibility of a functional coupling between voltage-sensing and cAMP-gating machinery.

We hypothesized that 1) based on sequence similarity, the voltage- and cAMP-dependence of HCN4 would most closely resemble that of HCN2, 2a) a voltage-independent step is responsible for the initial opening of HCN4 channels and 2b) the efficiency of this step involves a cAMP sensitive region, 3) cAMP enhances sustained activation by stabilizing an open channel state and 4) that voltage-sensing and cAMP-modulatory machinery do not work independently in HCN channels.

1.9.3 Results summary

We found that 1) HCN4 channels resemble HCN2 channels with respect to voltage- and cAMP-gating, 2a) a voltage-independent step governs the initial closed to open transition in HCN4 channels, 2b) the efficiency of this voltage-independent transition involves the COOH region, 3) cAMP increases sustained activation by stabilizing and enhancing the formation of a secondary, rather than initial, open state, and 4) the stability of a secondary open state is cooperatively determined by voltage-sensing
and cAMP-modulating machinery, indicating these regions do not work independently in HCN channels.
CHAPTER 2: EXPERIMENTAL PROCEDURES

2.1 Molecular Biology

2.1.1 Subcloning and DNA purification

A pGH oocyte expression vector (76) containing a full-length mouse HCN4 gene (5; Swiss-Prot accession number O70507) was provided by Dr. B. Santoro at the Center for Behavior and Neurobiology at Columbia University (New York, USA). Upon sequencing, the gene was found to contain two point mutations, a charge-reversing mutation at a conserved basic residue in the S4 voltage-sensing helix (K381E) and a second mutation within the C-linker region (E567G) (Fig. 1). These mutations presumably occurred through random mutation during an amplification or cloning procedure. First, to increase expression levels, the wild-type double-mutant construct was truncated after the CNBD (Δ723-1201, Fig. 1) using PCR and primers designed to incorporate an Xba-I cut site into the post-CNBD sequence. The resulting PCR fragment was then digested with Blp-I and Xba-I and subcloned into a similarly digested vector to generate a truncated HCN4 gene with two point mutations, mHCN4Δx K381E E567G. In order to revert the two point mutations to wild-type sequence, two site-specific reversions (E381K and G567E) were performed independently using mutagenic oligonucleotide primers and a nested PCR strategy to generate wild-type fragments that were subcloned into the existing truncated gene to replace those containing a point mutation. Specifically, the E567G reversion was generated using two forward (5′CACCAACCCCTGATCGTC TTAATG3′ and 5′CAAGGCAAGATGGTTGATGAGGAAGCATCC TG3′) and two
reverse (5' CAGGATGCTTTCCATCAAAACATCTTGCCTTGG3' and 5' CTAGATGCTTTCCATCAAAACATCTTGCCTTGG3') primers designed to revert the mutant base pair to wildtype sequence. The two resulting PCR products were ligated and extended to generate a 1367bp fragment that was digested with Blp-1 and HindIII. The mutant truncated vector (described above) was similarly digested; vector and inset were then ligated using a rapid DNA ligation kit (Roche, Germany). The resulting construct (mHCN4ΔxK381E) was used as a template to generate a truncated wild-type construct, and because of the interesting nature of the K381E point mutation, was also retained for further study. The E381K reversion was made using a similar strategy; using forward primers 5' CACCACACCCTGATCGTCTTAATG3' and 5' CCGTTTCACTAAGATCCTCAGCCTCAGCCTCGGC3' and reverse primers 5' GCAGGAGGCTGAGGATCTTTAGTGAAACGGACAGTCTC3' and 5' CTAGAGACTCCATTCGG3'. The resulting PCR products were ligated, extended and then digested with Blp-1 and Xba-1. The truncated mHCN4ΔxK381E gene (described above) was similarly digested; vector and insert were then ligated to generate the wild-type truncated gene, mHCN4Δx.

Domain swapped HCN2 chimeras containing the S1-S6 region of HCN4 were generated using an extreme COOH-truncated HCN2 channel vector(pGHmHCN2Ax) and inserts from mHCN4Δx or mHCN4ΔxK381E using standard subcloning techniques (77). Briefly, mHCN4Δx or mHCN4ΔxK381E S1-S6 inserts and an mHCN2Δx vector were generated using double restriction digests with Nhe-1 and Blp-1 and then ligated using a Rapid DNA Ligation kit (Roche, Germany) to form two chimeric constructs, 242Δx and 242ΔxK381E.
All constructs were transfected into competent DH5-α or Stbl2 *Escherichia coli* bacterial strains. After reaching the stationary growth phase, cells were collected by centrifugation, lysed and plasmid DNA was purified using a maxi-prep plasmid DNA purification kit (Marligen Biosciences, USA). Plasmid DNA purity and concentration was determined using gel electrophoresis and UV spectroscopy, and verified by complete sequencing of coding regions (Macrogen, Korea). DNA was stored in 0.5mL microfuge tubes at -20°C.

2.1.2 RNA transcription and purification

Plasmid DNA was linearized using single restriction enzyme digests, verified for a single band using gel electrophoresis, and purified using standard phenol:chloroform:isoamyl alcohol (25:24:1, Sigma-Aldrich, USA) and ethanol precipitation (77). mRNA was transcribed from linearized DNA using a T7 RNA polymerase (mMessage Machine, Ambion, USA) and purified using phenol:chloroform:isoamyl alcohol and isopropanol precipitation. Quality and quantity of RNA were assessed using gel electrophoresis and UV spectroscopy. RNA in DEPC treated sterile water was stored in 0.5mL microfuge tubes at -80°C.

2.2 Electrophysiology

2.2.1 Animal care

Mature female *Xenopus laevis* frogs were housed at 16-18°C in clear polycarbonate 20L tanks containing a minimum of 10L of dechlorinated saline water. Frogs were rested a minimum of four weeks between surgeries and euthanized by tricaine
overdose after four surgeries. All animal-related protocols received necessary approvals under Simon Fraser University policies R20.03.

2.2.2 Oocyte harvest and preparation

Frogs were anesthetized before oocyte harvest using a 0.8g/L ethyl 3-aminobenzoate methanesulfonic acid (tricaine, MS-222) solution (Sigma-Aldrich, USA). Loss of consciousness was determined by lack of muscle tone in limbs and loss of responsiveness to touch. Oocytes were harvested using a surgical ovarian biopsy (78). Briefly, oocytes were removed from the ovary after skin and muscle incisures, which were subsequently closed with multiple sutures. Oocytes were defolliculated immediately after harvest by 3 hours incubation in 1mg/mL collagenase (Sigma-Aldrich, USA) in ND96 buffer (Chemicon International, USA) on a bench top rotator, followed by three 20-minute washes in modified Barth’s saline (Chemicon International, USA) at room temperature. Oocytes were stored in a 16°C incubator in modified Barth’s saline for 0-1 days until RNA microinjection.

2.2.3 Oocyte RNA microinjection

Healthy oocytes were visually selected for injection using a stereo microscope. 15-40 ng of RNA was injected (79, 80) into 0 or 1 day-old oocytes using a Drummond digital microdispenser mounted on a manual micromanipulator. After injection, oocytes were stored in 0.5mL of Leibovitz’s L-15 media (Invitrogen, USA) at 16°C for up to 7 days.
2.2.4 Solutions

Extracellular (pipette) solution contained 5mM NaCl, 92mM KCl, 10mM HEPES acid, 1mM MgCl₂, 3mM CaCl₂; the pH was adjusted to 7.4 with KOH. Intracellular (bath) solution contained 5mM NaCl, 92mM KCl, 10mM HEPES acid, 1mM MgCl₂, 3mM EGTA acid; the pH was adjusted to 7.4 with KOH. Bath solution containing saturating cAMP consisted of intracellular solution supplemented with 10 μM cAMP (Sigma-Aldrich, USA). Solutions were stored at 4°C, and equilibrated to room temperature (19°C-23°C) before use.

2.2.5 Current recordings

Currents were recorded from excised macropatches in the inside-out patch-clamp configuration (73) using an Axopatch 200B amplifier (Axon Instruments, Inc.). Pipettes were pulled from KINMAX-51® glass capillary tubes (Kimble Glass, Inc.) and had a resistance of 0.5-5MΩ after fire polishing. Ag-AgCl grounding and headstage wires were chlorided by 20 minutes incubation in chlorine bleach whenever pipette currents drifted under seal-test conditions. The grounding wire was connected to the bath solution with a KCl agarose bridge. Data were filtered at 10kHz using the Axopatch 200B low-pass Bessel filter and sampled at 2kHz. Voltage protocols were specified and data obtained using pClamp 9.0 software (Axon Instruments, USA). Activation currents were elicited from excised patches with a series of 2 or 3 second hyperpolarizing voltage steps (-70 to -160mV) from a holding potential of -40mV. Deactivation currents were elicited using maximally activating hyperpolarizing pulses of varying lengths (50ms-3s) and then returning to a depolarized holding voltage (-40mV or +40mV). Solutions were applied
using a manually controlled gravity-flow capillary system. All current recordings were obtained at room temperature (19°C -23°C).

2.3 Data Analysis

Data were analyzed using pClamp 9.0 (Axon Instruments, USA) and SigmaPlot 10.0 (Systat Software, Inc.) software. To generate steady-state activation curves, mean peak tail conductance (G) values were calculated from the peak tail current values, leak subtracted, normalized to the maximal conductance (G_{max}) and plotted as a function of the activating voltage. The resultant activation curves were fit with a Boltzmann function [G/G_{max} = 1/(1+exp(V_{1/2}-V)/slope)] where V_{1/2} is the midpoint activation voltage and V is the activation voltage in mV, using SigmaPlot software. Half-times of activation (T_{1/2, act}) values were obtained from steady state activation current traces. Briefly, after the mean peak and mean baseline currents were obtained using pClamp 9.0 software, the mid-point amplitude was manually calculated and the corresponding T_{1/2} value located using sliding cursors in pClamp 9.0. Time constants of deactivation (\tau_{deact}) were obtained using pClamp9.0 software to fit fully deactivated tail current traces with a single exponential function after an initial delay (30). \tau_{deact} ratios were obtained by calculating the ratio of \tau_{deact} in the absence of cAMP to \tau_{deact} in the presence of cAMP (\tau_{deact, CAMP}/\tau_{deact, basal}).

CAMP-dependent increases in maximal conductance were quantified by calculating G_{max, basal}/G_{max, CAMP} ratios from peak tail current values obtained in the absence and presence of cAMP after a 2-3 s maximally activating pulse to -160 mV.
2.4 Statistical Analysis

All numerical values presented are mean ± S.D., n refers to the number of patches. Statistical significance was determined using either paired or unpaired student’s t-tests; the P-value threshold for statistical significance was P < 0.05, unless otherwise stated.
CHAPTER 3: RESULTS

We investigated and quantified the voltage-gating properties and cAMP-modulatory effects of mouse HCN4 channels and characterized the effects of a voltage-sensor point mutation using heterologous expression in *Xenopus* oocytes and patch clamp of excised inside-out patches.

3.1 An S4 Mutation Has No Effect on HCN4 Voltage-Activation

We first quantified voltage- and cAMP-dependence in two versions of the mouse HCN4 gene: a wildtype version and one containing a K381E mutation within S4. Some HCN isoforms can be difficult to express heterologously at levels sufficient for measuring biophysical properties using patch-clamp (for example, see 48), we found this also to be the case for mouse HCN4. We found deleting the extreme COOH-terminus (previously reported to have essentially no effect on gating (31, 34)) to increase channel expression over wildtype levels. We therefore used HCN4 constructs with the extreme COOH-terminus deleted (HCN4Δx and HCN4ΔxK381E, Fig. 1 and Fig. 4A) for this study.

Based on sequence identity (Fig. 2), we predicted that of the mouse isoforms, the voltage-gated behaviour of HCN4 would most closely resemble that of HCN2. Indeed, we find that while HCN4Δx kinetics are qualitatively slower than those of other HCN isoforms (see Ch.1 and Fig. 5B), the half maximal-activation ($V_{1/2}$) values (Table 1) are similar to those reported for HCN2. Surprisingly, we also find that a charge reversing mutation (K381E), to a conserved basic residue in the voltage-sensing S4 helix, has little
effect on voltage-activation (Fig. 4B-C, Table 1); \( V_{1/2} \) and Boltzmann (G-V) slope values for HCN4AxK381E channels are \(-123\pm9\) mV \((n=5)\) and \(6.9\pm1.4\) \((n=5)\), compared with \(-124\pm8\) mV \((n=3)\) and \(8.8\pm0.5\) \((n=3)\) in HCN4Ax. These mean values do not differ significantly between the two isoforms \((P>0.96\) for \( V_{1/2} \) and \(>0.067\) for G-V slope, respectively) suggesting that the overall voltage-gating properties remain intact in HCN4AxK381E, despite a charge reversing mutation at a conserved position in the voltage-sensor.

We measured channel activation for both HCN4 constructs in multiple excised inside-out patches using a series of hyperpolarized voltage steps from \(-70\) mV to \(-160\) mV and then returning the voltage to a depolarized holding voltage of \(-40\) mV to elicit deactivation tail currents. We obtained conductance-voltage (G-V) relationships (Fig. 5A) by plotting normalized peak tail conductances \((G/G_{\text{max}}\text{, see methods})\) as a function of the activating voltage. We then fitted the resultant curves with a Boltzmann function \[ G/G_{\text{max}} = 1/(1+\exp(V_{l/2}-V)/\text{slope}) \] in order to obtain \( V_{l/2} \) values. To quantify activation kinetics in the two constructs, we first determined time constants of activation \((\tau_{\text{act}})\) by fitting the inward activation current traces with a single exponential function following an initial delay \((30, 49, 81)\). However, due to the varying and pronounced sigmoidicity of activation currents observed in different constructs and under different test conditions, we found that the time course of activation was better described by half-times \((T_{1/2, \text{act}})\). We recorded \( T_{1/2, \text{act}} \) values as the time point corresponding to the current midpoint between the instantaneous leak current at the onset of an activation pulse and the mean steady state endpoint (see methods). \( T_{1/2, \text{act}} \)-voltage curves for HCN4Ax and HCN4AxK381E are shown in Fig. 5B. We then quantified deactivation kinetics by obtaining deactivation
time constants ($\tau_{\text{deact}}$) from fitting tail currents with a single exponential function

\[ I = I_0 \exp(-t/\tau) + I_{\text{leak}} \]

after an initial delay (21, 30, 49). $\tau_{\text{deact}}$ values for HCN4Ax and HCN4AxK381E are summarized in Table 2. Unexpectedly, in addition to having little effect on voltage-dependence, K381E appears to stabilize an open state in the absence of cAMP. Despite having a similar $V_{1/2}$ value to HCN4Ax, HCN4AxK381E activation kinetics are significantly increased at -160 mV ($P<0.0036$, Fig. 5B-C) and deactivation kinetics are significantly slowed at -40 mV ($P<0.0016$, Fig. 5D) relative to HCN4Ax. Taken together, these data indicate that, despite being a conserved basic residue within S4, K381 contributes to open state stability rather than having a major voltage-sensing role.

3.2 CAMP Increases Maximum Voltage-Dependent Open Probability in HCN4 Channels

CAMP has been previously reported to increase the maximum voltage-saturated current level, reflecting an increased maximum conductance ($G_{\text{max}}$), in HCN2 channel macropatches (49, 66). This effect has not been reported for any other HCN isoform. Because of the high sequence identity between the cyclic nucleotide-binding regions of HCN2 and HCN4 (Fig. 2A), we wondered if HCN4 would also exhibit a CAMP-dependent increase in $G_{\text{max}}$. We tested CAMP-effects on $G_{\text{max}}$ by maximally activating channels in both the presence and absence of saturating (10 μM) CAMP, and then returning to a depolarized holding voltage of -40 mV to elicit tail currents. Maximal conductance values in the absence of CAMP ($G_{\text{max, basal}}$) were calculated from mean peak tail current values and normalized to maximal conductance values in the presence of CAMP ($G_{\text{max, basal}} / G_{\text{max, CAMP}}$). We found CAMP does significantly increase $G_{\text{max}}$ in HCN4...
channels (Fig. 6C): $G_{\text{max, basal}}/G_{\text{max, cA}}$ values are 0.87±0.09 (n=3) for HCN4Δx, and 0.88±0.08 (n=4) for HCN4ΔxK381E (Table 3), compared with 0.76±0.05 in HCN2 channels (66). As expected from high sequence similarity with HCN2, we also find that cAMP binding positively shifts $V_{1/2}$ values in both HCN4ΔxK381E and HCN4Δx (Fig. 6A-B, Table 1). This effect has been documented for other HCN isoforms (Table 1).

That $G_{\text{max, basal}}/G_{\text{max, cA}}$ values are significantly less than 1 ($P < 0.0311$ for HCN4Δx and < 0.0268 for HCN4ΔxK381E) means that cAMP is able to induce a current level unobtainable by voltage alone. This means that a voltage-independent gating step controls the initial closed to open transition in HCN4 channels, and that this step is modulated by cAMP binding. The energetics of this transition do not completely favour the open state in both HCN2 and HCN4 channels in the absence of cAMP; this results in an increased $G_{\text{max}}$ upon cAMP binding, as the closed-open equilibrium is shifted towards the open state.

### 3.3 CAMP Enhances Sustained Activation in HCN4 Channels

We then examined the effects of cAMP binding on activation and deactivation kinetics by measuring $T_{1/2, \text{act}}$ and $\tau_{\text{deact}}$ values in the presence of saturating cAMP. We find that, similar to HCN2 (21, 37), cAMP binding qualitatively decreases $T_{1/2, \text{act}}$ values (Fig. 6C) in HCN4Δx, indicating cAMP binding enhances opening kinetics.

We were particularly interested in cAMP effects on the sustained activation that manifests as a sigmoidicity of (or delay before) tail current decay (62, 67). CAMP has been previously observed to slow deactivation in HCN2 channels (32). We hypothesized that cAMP might also slow deactivation in HCN4 channels, and this might result in an
increased sustained activation. We tested this idea by maximally activating channels and then deactivating them by returning to a depolarized holding voltage (-40 mV), in both the absence and presence of saturating cAMP. We quantified cAMP effects on sustained activation by calculating $\tau_{\text{deact}}$ values for tail current decay in the absence and presence of cAMP and calculating their ratio under these two conditions ($\tau_{\text{deact, cAMP}} / \tau_{\text{deact, basal}}$; "$\tau_{\text{deact}}$ ratio", see methods) for paired current traces. We found that cAMP increases sustained activation in HCN4Ax (Fig. 7): at -40 mV the $\tau_{\text{deact}}$ ratio is $1.6 \pm 0.4$ (n=4, Table 2), indicating channel closure is slower in the presence of cAMP. Since cAMP significantly increases $\tau_{\text{deact}}$ values at -40 mV ($P < 0.0214$), cAMP binding must stabilize an open state, but whether this involves the initial or a secondary open state cannot be determined from this set of experiments. However, a surprising enhancement of cAMP-dependent sustained activation in our HCN4AxK381E construct led to an additional series of experiments that revealed more details about the open state stabilization that leads to sustained activation.

3.4 K381E Enhances CAMP-Effects on Sustained Activation

When we examined cAMP-effects on sustained activation in HCN4AxK381E channels, we found a striking result: K381E dramatically enhances cAMP-dependent sustained activation compared with non-mutant channels (Fig.8A-B). We calculated $\tau_{\text{deact}}$ ratios for HCN4AxK381E as described above, with the incorporation of deactivation pulses long enough (45 s) to allow completion of tail current decay. Compared with the $\tau_{\text{deact}}$ ratio of $1.6 \pm 0.4$ (n=4) we report here for HCN4Ax, the $\tau_{\text{deact}}$ ratio for HCN4AxK381E is $8.9 \pm 0.6$ (n=4) (Table 2). That cAMP results in a significantly increased $\tau_{\text{deact}}$ ratio ($P < 0.0000013$) in K381E mutants relative to non-mutants, means
that K381E cooperatively contributes to the open state stability that leads to cAMP-dependent sustained activation. Thus, in addition to enhancing opening under basal conditions, K381E synergistically enhances a cAMP-dependent behaviour; this indicates a direct coupling of voltage-sensing and cAMP-modulatory machinery in HCN channels.

3.5 **K381E Effects on CAMP-Dependent Sustained Activation are Pulse Length- and Voltage-Dependent**

We predicted the cAMP-dependent K381E effects in HCN4 channels might result from the enhanced formation and increased stabilization of a secondary, rather than initial, open state. A secondary open state that forms only after the initial gate-opening step has been reported to cause a slight sustained activation in HCN channels (62, 67). If our hypothesis is true, then cAMP-dependent sustained activation in HCN4ΔxK381E channels should be pulse-length dependent: with sufficiently short hyperpolarizations, the secondary open state will not have time to form. We tested this idea by activating HCN4ΔxK381E channels with a range of pulse lengths (3 s, 500 ms and 50 ms). We found cAMP-dependent sustained activation is indeed dependent on length of hyperpolarizing pulse (Fig. 9A); moreover, it is abolished entirely with sufficiently short hyperpolarizations. We also found that the cAMP-independent slowing of deactivation kinetics caused by K381E alone (see Fig. 5D) is also dependent on activating pulse length and similarly abolished with short pulses (Fig. 9B). This suggests that both the cAMP-independent and the dramatic cAMP-dependent effects of K381E on deactivation must result from the enhanced stability of a secondary open state that is unable to form with very short hyperpolarizations.
We then asked if the cAMP-dependent gating steps modified by K381E are also voltage-dependent. We tested this by activating HCN4ΔxK381E channels and subsequently deactivating them with a strongly depolarized holding voltage (+40mV) in both the presence and absence of saturating cAMP (Fig. 10). We calculated τ_{deact} ratios as described and compared them with those obtained at -40 mV; these results are shown in Table 2. CAMP-dependent sustained activation decreases as the holding voltage is increasingly depolarized; τ_{deact} ratios are substantially decreased at +40 mV (3.2±0.5, n=2) relative to -40 mV (8.9±0.6, n=4). Thus, the stable secondary open state must decay during deactivation through a step that is both voltage- and cAMP-dependent.

3.6 S1-S6 Contains the Physical Determinants of CAMP-Dependent Sustained Activation

We wondered whether the HCN4 transmembrane region (S1-S6) containing the K381E mutation is sufficient for the effects on cAMP-dependent sustained activation. To find out, we substituted the HCN4 voltage sensing and pore region (S1-S6), either with or without K381E, into an HCN2 channel to form the constructs 242Δx and 242ΔxK381E (Fig. 11, inset), respectively. This substitution results in functional channels that display normal HCN-like voltage activation (Fig. 11A) and cAMP-response (Fig. 11B-C). Both channels have a V_{1/2} value similar to that we report for HCN4 (Table 1). cAMP binding results in a depolarized V_{1/2} shift (Fig 11A-B, Table 1). The K381E mutation in the context of an HCN2 channel preserves the cAMP-independent decrease in activation kinetics (Fig. 12A) and increase in τ_{deact} (Fig. 12B) seen in HCN4. Importantly, we found this substitution also preserves the cAMP-dependent effects of K381E (Fig. 13A-B, Table 2): 242ΔxK381E channels have a τ_{deact} ratio of 7.4±1.7 (n=4), compared with
3.1±1.2 (n=4) in 242Δx. Since the \( \tau_{\text{deact}} \) ratios significantly differ between the mutant and non-mutant chimeric constructs (\( P < 0.0055 \)), and the \( \tau_{\text{deact}} \) ratio in the mutant chimera is similar to that of the mutant HCN4 construct, the HCN4 S1-S6 region with the K381E mutation must contain the necessary physical determinants for the synergistic enhancement of cAMP-dependent sustained activation.

Notably, we also find that the cAMP-dependent \( G_{\text{max}} \) increase observed in HCN2 and HCN4 is present in the chimeric constructs (Fig. 11C, Table 3). We quantified cAMP-effects on \( G_{\text{max}} \) as previously described, and found that the \( G_{\text{max,basal}}/G_{\text{max,cA}} \) value for 242Δx (0.75±0.05, n=4) is virtually identical to that reported for HCN2 (0.76±0.05, Shin et al., 2004), and is different from that we report for HCN4 (0.87±0.09, n=3). This indicates that the –COOH region contributes to determining the extent of basal opening in HCN channels in the absence of cAMP. Interestingly, the voltage independent gating step is increased in 242ΔxK381E (\( G_{\text{max,basal}}/G_{\text{max,cA}} = 0.85±0.06, n=4 \)) relative to 242Δx. That these values significantly differ (\( P < 0.0439 \)) provides further evidence that K381E stabilizes an open state and shifts the closed-open equilibrium towards the latter. Given the high sequence similarity between the HCN2 and HCN4 core -COOH regions (C-linker through the CNBD, Fig. 2A), it will be intriguing to pursue the specific determinants responsible for isoform-specific energetics of voltage-independent gating.

The results presented above show that cAMP enhances maximal open probability in HCN4 channels, and that this mechanism involves the COOH region. CAMP also enhances sustained activation in HCN4 channels, this occurs through the stabilization of a secondary open state that forms after the initial gate-opening step. Furthermore, this secondary state decays, during deactivation, through a voltage- and cAMP dependent
gating step. Importantly, the effect of cAMP on sustained activation is synergistically enhanced by a K381E mutation. Thus, the conserved basic S4 residue K381 appears to cooperatively contribute to open state stability, rather than having a major voltage-sensing role. These results suggest that voltage-sensing and cAMP-modulatory machinery are functionally coupled in HCN channels.
CHAPTER 4: DISCUSSION

Here we have quantified the voltage-gating and cAMP-modulatory properties of mouse HCN4 channels relative to other isoforms, examined in detail cAMP effects on sustained activation, and characterized the surprising effects of a voltage-sensor mutation that modulates a cAMP-dependent channel behaviour.

4.1 A Voltage-Independent Gating Step is Mediated by the COOH Region

As is expected from high sequence similarity, of the four mouse isoforms, we show here that HCN4 most closely resembles HCN2 with respect to voltage-dependence and cAMP-modulatory effects. CAMP modulates kinetics and positively shifts $V_{1/2}$ values in HCN4. Notably, cAMP also results in an increased maximum voltage-inducible open probability. Since conductance values saturate at very negative voltages, but are further increased by cAMP binding, channel opening must occur through a voltage-independent gating step that is modulated by cAMP. If this gating step were voltage-dependent, then conductance would reach a voltage-dependent maximum and not increase any further with cAMP. That cAMP-effects on Gmax are readily observable in HCN2 and HCN4 suggests these two isoforms are inhibited under basal conditions, and experience a large cAMP effect due to relief of inhibition upon ligand binding. Domain-swapped chimeras suggest the COOH-region is a determinant of the extent of inhibition under basal conditions; chimeric HCN4 derivatives containing the COOH- region of HCN2 more closely resemble HCN2 than HCN4 with respect to basal inhibition.
Previous observations suggest that the COOH-region inhibits channel opening in the absence of cAMP (34) and that isoform-specific variations in cAMP-sensitivity result from differences in COOH regions (34). The results presented here agree that cAMP binding stabilizes the closed to open equilibrium and allows more complete activation, and suggest that the basal closed to open equilibrium is determined by the identity of the COOH region.

Chen et al. (32) recently reported that a voltage-independent step limits the speed of opening in HCN2 channels at negative voltages; opening kinetics become saturated with increasingly hyperpolarized activation voltages. However, the results of that study differed somewhat from those presented here; Chen et al. suggest that the kinetics limiting voltage-independent opening are determined primarily by the transmembrane domain while our results implicate the COOH-region in voltage-independent gating.

What may first appear to be a contradiction is actually not: Chen et al. focused solely on the kinetics of the voltage-independent gating step in the absence of cAMP, and found that S1-S6 determines whether the kinetics are slow or fast. Here, we present data suggesting the energetics, rather than kinetics, of this voltage independent transition are predicted by the COOH region. We thus agree that a voltage-independent step is responsible for the initial closed to open transition in HCN channels, and conclude that while the kinetics of this transition may be determined by the transmembrane domain, the equilibrium between the closed and open states is determined primarily by the identity of the COOH region.

The presence of a voltage independent gating step in HCN4 channels has immense physiological significance. In the heart, where HCN4 channels are the
predominant isoform and act as biological pacemakers, a voltage-independent gating step would provide a ceiling, or upper limit, on channel activity— even under intense electrical stimulus— in the absence of sympathetic stimulation. This mechanism could serve to prevent dangerous arrhythmias that might result from over-excitability. Likely, given the high similarity between isoforms, the specific residues and mechanisms responsible for determining the kinetics and the energetics of voltage-independent gating in HCN channels will soon be identified.

4.2 CAMP Stabilizes a Secondary Open State

Elinder et al. (67) and Mannikko et al. (62) previously identified a secondary open state in HCN channels that forms through a time-dependent transition after the initial gate opening step. We report here that cAMP binding enhances this transition and increases sustained activation in both non-mutant and K38E channels. In all four of our constructs, $\tau_{\text{deact}}$ values increase in the presence of cAMP. CAMP effects on sustained activation must stabilize a secondary, rather than initial, open state because when the formation of a secondary open state is prevented (by using very short hyperpolarizing activation pulses), resultant tail currents are essentially non-sigmoidal, even in the presence of cAMP. Since $\tau_{\text{deact}}$ ratios are both cAMP- and voltage-dependent, the stable secondary state must decay through a step that is sensitive to both.

To account for the cAMP- and voltage-dependence of sustained activation we propose a gating scheme that incorporates elements of two previously proposed models (32, 62, 67). Chen et al. (2007) proposed an allosteric gating model in which both voltage and cAMP allosterically modulate a voltage-independent gating step. Mannikko et al. (2005) proposed a model in which channels can exist in two modes, where the so-called
“mode 2” is easier to open and more stable than “mode 1”. Our circular five-state model (Fig. 14) includes two closed and three open states, all of which can be bound by cAMP. C and O denote the status of the gate, closed or open. R and A denote the position of S4: resting (outward-facing) or activated (inward-facing). According to this model, channels open through a voltage-independent step that is allosterically coupled to S4 movement (32). Thus, while voltage-independent gate opening can occur at depolarized voltages (CR – OR); this is supported by a previously reported instantaneous current (Iₘₚₜ) in HCN channels (82, 83), opening becomes much more favoured when S4 is in the activated configuration (CA – OA1). As is reported here, the voltage-independent equilibrium between CA and OA1 is determined by the COOH-region. Once gate opening occurs, and with sufficiently long hyperpolarizations, channels are able to transition to a secondary open state (OA1 - OA2); this step is enhanced by cAMP. Under hyperpolarizing conditions then, the energetically favoured pathway is CR – CA – OA1 – OA2. Upon subsequent depolarization, channel closure occurs through OA2 – OR – CR; because at depolarized voltages OA1 will be unstable, (S4 will rapidly return to its resting configuration). At very positive voltages, the rate of closing is determined primarily by the rate of the OR – CR transition, because the voltage-dependent step OA2 - OR will be rapid. At less positive voltages, where voltage-dependent steps are rate limiting, the closing rate will be determined mainly by OA2 – OR. These transitions through multiple open states accounts for sustained activation, or the sigmoidicity of tail currents. We show here that under hyperpolarizing conditions, OA2 is stabilized by cAMP binding; this modifies opening by shifting the CA-OA1 transition towards the latter, thereby increasing the extent of voltage-independent opening. Upon subsequent
depolarization to voltages where voltage-dependent steps are limiting, this cAMP-dependent stabilization of OA2 slows the OA2 – OR transition, resulting in enhanced sustained activation.

What is the physical mechanism underlying cAMP-dependent stabilization of a secondary open state? By studying a mutant channel with an enhanced cAMP-dependent sustained activation phenotype, we found that, unexpectedly, the mechanism underlying cAMP-dependent stabilization of the secondary open state cooperatively involves the voltage-sensor.

4.3 Voltage-Sensing and CAMP-Modulating Machinery are Coupled

We found that a K381E mutation in the S4 helix of HCN4 channels synergistically enhances cAMP-dependent stabilization of a secondary open state. It has been previously shown that conserved positive charges in S4 contribute to the voltage-sensing capacity of HCN channels, and that they physically traverse the membrane in response to changes in membrane potential (35, 36, 38, 84). Thus, a conserved positive residue in S4, whose primary role might be assumed one of voltage-sensing, instead contributes to open state stability and modulates a cAMP-dependent behaviour. Since a voltage-sensor mutation alters a cAMP-dependent behaviour while leaving voltage-gating properties essentially untouched, a voltage-sensitive region must somehow interact with a cAMP-sensitive region, and the mechanisms governing voltage-sensing and cAMP-modulatory effects must not work independently of one another.

Interestingly, mutations of the fifth basic residue (K381 in mHCN4, K303 in mHCN2 and K250 in spHCN) in S4 have been studied by other groups (35, 36, 38, 84),
however none reported the effects we have shown here. There are several reasons why this novel role for K381 remained undiscovered until now. Firstly, the previously studied mutations were not made in HCN4. Vaca et al (2000) studied a K303Q mutation in HCN2 channels, and found that it did not produce functional channels. While they did not address it directly, this lack of function could result from disrupted folding or trafficking, or from channels rendered unable to open due to the mutation. The study of Vaca et al. certainly does not eliminate an important gating role for K303. Chen et al. (2000) also studied a K303Q mutation in HCN2, and found the channels did express and activated more slowly and at potentials far more negative ($\Delta V_{1/2} = -40mV$) than wildtype channels. This might mean that a K to Q charge neutralization is not sufficient to produce the effects we observe with a K to E charge reversal. It is also possible that another physical element within the HCN4 S1-S6 is required for this effect; we have shown here that substitution of this region into an HCN2 channel does produce the K381E effects we observe in HCN4 channels. Vemana et al. (2004) studied a K250C mutation in spHCN channels and found it did not produce functional channels, again implying an important functional role for this residue. Mannikko et al. (2002) made a K335C mutation in HCN1, and found that the channels expressed and activated at voltages negative to those required for wildtype. Again, this suggests charge neutralization is not sufficient, or that another element within S1-S4 is required for the novel effects we report here.

What is the mechanism underlying K381E effects on cAMP-dependent sustained activation? While we do not have any direct physical evidence implicating the precise channel parts involved in a putative interaction between voltage- and cAMP-sensitive regions, it is difficult to imagine a physical mechanism that does not involve elements
located within proximity of S4 and within proximity of the CNDB. Based on this, and on evidence within published literature, it is interesting to speculate which components might interact to produce these effects.

A number of studies have reported interactions between the C-linker of the COOH-region, and the S4-S5 linker that physically connects S4 to the activation gate. In particular, Decher et al. (2004) reported that disrupting an electrostatic interaction between the S4-S5 and C-linkers prevents normal channel closure. In addition, Prole and Yellen (2006) showed that cross-linking these two regions prevents normal deactivation. Importantly, these two studies also suggest these specific interactions have a role in cAMP-gating: Decher et al. showed that an intact C-linker is required for normal cAMP-gating, and the cross-linking done by Prole et al. reduced the normal effects of cAMP-binding on channel activity. Taken together, these two studies show that 1) the S4-S5 and C-linkers come into close proximity during normal gating, and 2) these regions contribute to cAMP-gating. In addition to these inter-domain interactions, a third study by Craven and Zagotta (49) confirmed that specific inter- and intra- subunit interactions observed in the crystal structure (44) are important in normal cAMP-gating. Specifically, Craven and Zagotta showed that disruption of a salt bridge triad between neighbouring C-linkers and their CNBDs increases opening and mimics cAMP binding. This result also has two important implications: 1) specific interactions involving the C-linker and CNBD may mediate cAMP-modulatory effects, and 2) since breaking these salt bridges increases channel opening, the crystal structure likely shows the C-linker in its inactive conformation even though cAMP is bound (49). Together, these three studies suggest the following: First, S4-S5 comes into physical contact with the C-linker during gating,
second, these two regions are important for normal cAMP-gating and finally, the C-linker has at least two possible conformations. Based on these results and the results we have presented here, we propose a possible physical mechanism for a cooperative interaction between voltage-sensing and cAMP-modulating machinery that explains cAMP-dependent effects on HCN gating and the striking effects of K381E.

In our model (Fig. 15), the COOH region of voltage-activated channels can exist in one of three conformations: 1) a resistant (R) state in which the CNBD is ligand-unbound, 2) a willing (W) state in which the CNBD is ligand-bound but the C-linker remains in its inactive conformation or 3) a persistent (P) state in which the CNBD is ligand-bound and the C-linker is in its active conformation. In the R state, upon voltage-activation, the voltage-independent gating step favours the closed-activated state, but the exact closed-open equilibrium varies between isoforms. In the W state, the closed to open transition favours the open state. In the P state, which is able to readily form only when the CNBD is ligand bound and with sufficiently long hyperpolarizations, the C-linker is in its active conformation; this enables the C-linker to interact with the intracellular S4-S5 linker; this forms a lasting synergistic interaction that results in a super stable secondary state. In this stable state, the voltage-dependent movement of S4 would be physically resisted by a molecular tug-of-war, due to the stable C-linker-S4-S5 interaction. In this model, the dramatic effect of K381E on sustained activation could result from a simple twisting or reorientation of S4-S5, relative to its usual position, which renders the synergistic C-linker-S4-S5 interaction highly favourable, essentially locking channels in the P-state. Importantly, this model can account for all the observed effects of cAMP on channel behaviour: the time constant of activation is increased by
directly modulation of a voltage-independent gate-opening step, while the slowing of deactivation and increased maximal open probability are achieved by the enhanced stability of a secondary open state upon cAMP binding. This suggests that a single molecular mechanism involving voltate- and cAMP-sensing regions may be responsible for ligand-modulation of HCN channels.

The presence of these putative interactions could be easily tested with cross-linking experiments, designed, for example, to lock S4-S5 to the C-linker and form permanently sustained (P state) channels, or to lock the C-linker in an inactive state and render channels unable to form the P-state. Additionally, advances that allow researchers to measure gating currents in HCN channels, or monitor S4 movement using fluorescence, could provide definitive evidence for a channel state in which the movement of S4 is restricted by specific interaction between voltage-sensitive and cAMP-sensitive channel elements.

4.4 Significance of Coupling Between Voltage- and CAMP-Sensitive Regions

Evidence for a coupling between voltage-sensing and cAMP-modulatory machinery provides critical insights into gating mechanisms in HCN channels, and importantly, precisely how cAMP-binding exerts its effects on channel activity. Despite years of research, the exact mechanisms of cAMP binding remain elusive. Here we show that all the observed cAMP-effects on HCN gating can be accounted for by the presence of a highly stable secondary open state, and that the stability of this state is mediated by voltage- and cAMP-sensitive regions. We propose that the interactions mediating the stability of this secondary open state may involve the S4-S5 and C-linkers.
It has been suggested that the HCN channel core (S1-S6) represents an ancient voltage-gated module that has been modified with a number of different regulatory mechanisms during evolution (34, 47). In the case of HCN channels, this modification likely occurred through the fusion of genes encoding a soluble cyclic nucleotide-binding domain and a self-sufficient voltage-gated channel. Our results suggest that, rather allosterically regulating gating by two independent mechanisms, the mechanics underlying voltage- and cAMP-sensing became more complexly coupled at some point during evolution. It will be interesting to examine other voltage-and cAMP-sensitive channels to determine when in the course of ion channel evolution this coupling occurred.

Elucidating the mechanisms of cAMP-modulation in HCN channels also has profound medical relevance. Once researchers understand how cAMP binding exerts its effects on pacemaker activity, it will be possible to design analogs that target HCN channels in the heart and mimic or antagonize cAMP binding. Such therapeutic agents could treat cardiac arrhythmias and regulate heart rate at the molecular level.
CHAPTER 5: SUMMARY AND CONCLUSIONS

Cyclic AMP increases maximal open probability and enhances sustained activation in HCN4 channels. The intracellular C-terminal domain is a physical determinant of the former effect, and the latter effect manifests through the stabilization of a secondary open state that forms after the initial gate-opening step. cAMP-dependent sustained activation is cooperatively enhanced by K381E, suggesting this conserved S4 residue mediates communication with a cAMP-sensitive channel region and surprisingly, does not appear to have a significant voltage-sensing role.

To explain these results on a physical level, we propose a model in which the status of the COOH region (resistant, willing or persistent) dictates the respective stabilities of at least two open states. We suggest a secondary open state is stabilized by a synergistic interaction between voltage- and cAMP-sensitive regions and that this interaction may involve the S4-S5 and C-linkers.

These findings provide insights into the mechanisms and evolution of cAMP-modulation in HCN channels. In particular, rather than exerting their effects on gating in an independent allosteric manner, voltage- and cAMP-sensing in HCN channels appear coupled in a far more complex manner. Importantly, this work may lead towards the development of pharmaceutically important ligands able to target specific channel states and regulate pacemaker activity at the molecular level.

Future work involving mutagenesis, fluorescence studies and cross-linking experiments will no doubt reveal the specific residues involved in determining the
equilibrium of voltage-independent gating, how S4 movement is altered by the putative synergistic interactions between voltage- and cAMP-sensitive regions, and the precise regions involved in forming these interactions.
**APPENDICES**

Appendix 1: Figures

**Figure 1: Topology of an HCN subunit.** (A) Single HCN subunit with an intracellular NH2- region, six transmembrane helices and intracellular COOH-region containing the C-linker and cyclic-nucleotide binding domain (CNBD). Sissors indicate location of COOH truncation and asterisks indicate location of K381E and G567E point mutations. Voltage-sensing S4 helix shown in red. Four monomers form a functional channel. (B) Intracellular cartoon view of a tetrameric HCN channel with four cyclic-nucleotide binding sites.
A

Conserved core

HCN2 132 .AS.L.L.L..F...F.F...T.A......222 HCN2
HCN4 210 QSGFMQRQFGMLQPGQVNFSLRMFGSQRVQERVKQFAGELHGYSDRFYKULTIMMLLVMNLIIIIPVGIITFKDENTPIVWENV 300 HCN4

..M....................EK.RT..V.............M.C........342 HCN2
VSDTFFLIDELVINFPGQVVEDNTWEILDEPQR13MKYLSNFYVDFISSIPDYIFLIVFETFIDVSEVKTAANL..V377 HCN4
S2
..S......................S.N.H.E.S.E.ES.T.I........462 HCN2
LIGMMLLCHWDGCLQLFVPMLQDFPFDCCWSTINGMVNSNWGKQYSYALPKAMSHMLCTIGGRQAPVQMSDVWLTMLKMTVATCYMFMFGHTALGSISSRROQYQEYKQVEQNYMF 540 HCN4
S5
..A.F.K..................D..N.G.V....A..K............582 HCN2
ELPPDTRQHDRYEHYQGKMFDEESILGELSELFREDILINFNCRLIVASMPLEAMADPMFVTSMLTFLREFFPOPDYI13REGT1GKMYF1QHGVSVLTKKGKETKLADGQYFG 660 HCN4
C-linker

..K...E.E.K...VQQ.ELC.682 HCN2
ICLLTRRGRRTASVRADTYCRLYSI.DVNFMVKVLEKYMERRHAFKTVADI.LD1R1GKMK1.T1JKVQHDNLSKGVNYQNEII1QQIIVRHDREMAHCAHVRQ 760 HCN4

CNBD

B

Shaker

KAT1

rKv1.2
t4HCN
mHCN3
mHCN2
mHCN1

mCNGA2
hCNGA2
mCNGA2

mCNGA1
hCNGA1
hCNGA1

HERG

0.1
**Figure 2:** Sequence identity and phylogeny of HCN channels. (A) ClustalW sequence alignment of core regions (S1-CNBD) of mouse HCN2 (SwissProt primary accession number (SPID) O88703) and mouse HCN4 (SPID O70507). Dots indicate conserved residues. Positively charged S4 residues are shown in red and residue K381 is boxed. Locations of helices S1-S6, C-linker and CNBD were assigned as reported in Baruscotti et al. (10) and Zagotta et al. (44). (B) Unrooted neighbour joining phylogenetic tree showing relationships between HCN, CNG, Kv, inward K+ rectifiers and EAG-related channels. HCN family members: mouse HCN1 (mHCN1), SPID O88704; mouse HCN2 (mHCN2), SPID O88703; mouse HCN3 (mHCN3), SPID O88705; mouse HCN4 (mHCN4), SPID O70507; sea urchin HCN (spHCN), SPID O76977. CNG family members: mouse CNGA1 (mCNGA1), SPID P29974; rat CNGA1 (rCNGA1), SPID Q62927; human CNGA1 (hCNGA1), SPID P29973; mouse CNGA2 (mCNGA2), SPID 62398; rat CNGA2 (rCNGA2), SPID Q00195; human CNGA2 (hCNGA2), SPID Q16280. EAG-like channels: human-ether-a-go-go (hERG), SPID Q12809. Plant K+ channels: arabidopsis KAT1 (KAT1), SPID Q39128. Kv channels: rat KCNA2 (rKv1.2), SPID P63142; drosophila KCNAS (Shaker) SPID P08510. Core (S1-CNBD) of HCN sequences and full sequences of all other constructs aligned with ClustalX (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/), and neighbour joining tree drawn with TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/rod.html).
Figure 3: **Inside-out excised patch-clamp technique.** mRNA is first transcribed in vitro from plasmid DNA and then injected into oocytes. High resistance seals are formed between the aperture of a narrow glass pipette and intact oocyte membranes. A membrane patch is then excised in the inside-out configuration, voltage-clamped and perfused with test solutions to elicit currents, which are digitally recorded.
Figure 4: HCN4Ax and HCN4AxK381E display normal voltage activation. (A) Diagram showing extreme COOH-truncations and location of K381E mutation. (B and C) Activation currents obtained from excised inside-out patches. Currents were elicited by 3 s hyperpolarizing pulses applied in -10mV increments between -70mV and -160mV from a holding voltage of -40mV.
Figure 5: K381E stabilizes an open state in the absence of cAMP. (A) Conductance-voltage (G-V) relationships for pooled HCN4Δx (circles, black line) and HCN4ΔxK381E (squares, red line) data. Peak conductance (G) values were calculated from peak tail currents upon return to a -40 mV holding voltage after a series of hyperpolarizing pulses, normalized to the maximal conductance (G/G_{max}) and plotted as a function of activating voltage pulse. Solid black and red lines represent best fits of each data set with a Boltzmann function: \( G/G_{max} = 1/(1+\exp(V_{1/2}-V)/\text{slope}) \), where \( V_{1/2} \) is the midpoint activation voltage, \( V \) is the voltage in mV. Data points are mean, error bars are ± S.D.
Mean V_{1/2} values from pooled data are -124±8 mV (n=3) for HCN4Δx and -123±9 mV for HCN4ΔxK381E. Boltzmann slope factors are 8.8±0.5 and 6.9±1, respectively. (B) Activation half-time (T_{1/2, act}) values for HCN4Δx (circles) and HCN4ΔxK381E (squares) from -130 mV to -160 mV. It should be noted that not all -130 and -140 mV traces reached steady state; calculated T_{1/2, act} values might therefore be an underestimate of the true activation time course. T_{1/2, act} values are ±S.D., n=3 for both constructs. (C) K381E speeds activation in HCN4 channels. Activation traces elicited with a -160 mV activation pulse and normalized to peak steady state current are qualitatively faster in HCN4ΔxK381E than in HCN4Δx. (D) K381E slows deactivation in HCN4. Tail currents were elicited by a return to a -40 mV holding voltage after a 3 s pulse to -160 mV, normalized to peak tail current, and fitted with a single exponential function (shown in red) after an initial delay (see methods) to obtain τ_{deact} values. τ_{deact} values for tail currents shown in (D) are 130 ms (HCN4Δx) and 743 ms (HCN4ΔxK381E).
zero current level

50 pA

500 ms

G/Gmax

Vm (mV)

1.0

0.0

-160

-120

-80

100 nm

10 pM cAMP

52
Figure 6: CAMP effects on voltage activation and kinetics of HCN4. (A) G-V relationships from pooled data in the absence (open symbols) and presence (filled symbols) of saturating (10μM) cAMP for HCN4Δx (circles, black lines) and HCN4ΔxK381E (squares, red lines). G/G_{max} values were normalized, plotted and fitted as described in Fig. 5. V_{1/2} values in the absence of cAMP are given in Fig. 5; cAMP shifts V_{1/2} values ∼+13 mV in both constructs. (B) cAMP shifts V_{1/2} values in HCN4Δx. Activation current at a non-saturating voltage (-130 mV) is increased in the presence of saturating (10μM) cAMP. (C) cAMP increases maximal conductance in HCN4Δx. Amplitude of steady state activation current at a saturating voltage (-160 mV) is increased in the presence of cAMP.
Figure 7: CAMP enhances sustained activation in HCN4 channels. HCN4Δx tail currents in the absence and presence of saturating (10μM) cAMP. Tail currents were elicited by a 2 s activation pulse to -160 mV followed by a return to a -40 mV holding voltage and fitted with a single exponential function (shown in red) as described in Fig. 5. τ_{dcact} values for tail currents shown are 119 ms (no cAMP) and 224 ms (10 μM cAMP), respectively.
Figure 8: K381E enhances cAMP-dependent sustained activation. (A) HCN4ΔxK381E tail currents in the absence and presence of saturating cAMP. Tail currents were elicited by a 2 s activation pulse to -160 mV followed by a return to a -40 mV holding voltage. Tail currents were fitted with a single exponential function (shown in red) as described in Fig. 5. τ\textsubscript{act} values for tail currents shown are 743 ms (no cAMP) and 6236 ms (cAMP); the τ\textsubscript{act}/τ\textsubscript{deact} ratio is 8.4. Note time scale difference compared with Fig. 7. (B) Expanded time scale showing HCN4ΔxK381E (black) tail current decay relative to HCN4Δx (grey, as shown and described in Fig. 7).
Figure 9: K381E effects on cAMP-dependent sustained activation are pulse-length dependent. (A) K381E effect on sustained activation is abolished with sufficiently short activation pulses. HCN4ΔxK381E tail currents, in presence of saturating CAMP, resulting from activation pulses of varying lengths (3 s, 500 ms, 50 ms). Currents were normalized to mean peak tail current values. (B) K381E effect on sustained activation in the absence of CAMP is also pulse length dependent. Tail currents, in the absence of CAMP, resulting from activation pulses of varying lengths (3 s, 500 ms, 50 ms). Currents were normalized to mean peak tail current values.
Figure 10: K381E effects on cAMP-dependent sustained activation are voltage-dependent. (A) HCN4ΔxK381E +40 mV tail currents in the absence and presence of saturating cAMP. Tail currents were elicited by a return to a holding voltage of +40mV after a 2 s activation pulse to -160 mV. $\tau_{\text{dact}}$ values for tail currents shown are 190 ms (no cAMP) and 538 ms (cAMP); the $\tau_{\text{dact,cAMP}} / \tau_{\text{dact,basal}}$ ratio is 2.8. (B) HCN4Δx +40 mV tail currents shown for comparison. Tail currents were elicited as above. $\tau_{\text{dact}}$ values for tail currents shown are 40 ms (no cAMP) and 45 ms (cAMP); the $\tau_{\text{dact,cAMP}} / \tau_{\text{dact,basal}}$ ratio is 1.1.
No cAMP
10 pM CAMP

No cAMP
10 pM cAMP
Figure 11: HCN2-HCN4 chimeric channels exhibit normal voltage-dependence and $V_{1/2}$ shift due to cAMP. Constructs shown as inset. G-V relationships from pooled data for 242Δx (diamonds, black lines) and 242ΔxK381E (triangles, red lines) in the absence (open symbols) and presence (filled symbols) of cAMP. Data points were normalized and fitted as described in Figs. 5 and 6. Mean $V_{1/2}$ values and G-V slope factors from pooled data are 128±2 mV (n=6) and 5.8±1 (n=6) for 242ΔxK381E, and 122±8 mV (n=4) and 5.7±2 (n=6) for 242ΔxK381E. $V_{1/2}$ shift due to cAMP is ~+8 mV in 242Δx and ~+13 mV 242ΔxK381E. (B) cAMP shifts $V_{1/2}$ values in 242Δx. Current level elicited by a non-saturating voltage (-130 mV) is increased in the presence of saturating cAMP. (C) cAMP increases maximal voltage-saturated conductance in values in 242Δx. Amplitude of steady state activation current at a saturating voltage (-160 mV) is increased in the presence of cAMP.
Figure 12: Basal K381E effects are preserved in an HCN2-HCN4 chimera. (A) K381E speeds activation in an HCN2-HCN4 chimera. Activation traces elicited with a -160 mV activation pulse and normalized to peak steady state current are qualitatively faster in 242ΔxK381E than in 242Δx. (B) K381E slows deactivation in an HCN2-HCN4 chimera. Tail currents were elicited by a return to a -40 mV holding voltage after a 3 s pulse to -160mV, normalized to mean peak tail current values, and fitted with a single exponential function (fits shown in red) as described in Fig. 5. τ_{deact} values for tail currents shown are 113 ms (242Δx) and 1021 ms (242ΔxK381E).
Figure 13: S1-S6 contains necessary determinants for K381E effects on cAMP-dependent sustained activation. (A) 242ΔxK381E tail currents in the absence and presence of saturating cAMP. Tail currents were elicited by a 3 s activation pulse to -160 mV followed by a return to a -40 mV holding voltage. Tail currents were fitted with a single exponential function (fits shown in red) as described in Fig. 5. \( \tau_{\text{dact}} \) values for tail currents shown are 438 ms (no cAMP) and 4840 ms (cAMP); The \( \tau_{\text{dact}} \) ratio is 11.1. (B) Expanded time scale showing same current traces as (A).
Figure 14: 5-state model describing HCN gating. C and O denote status of activation gate (Closed or Open). R and A denote status of S4 voltage-sensor (Resting or Activated). Upon hyperpolarization, favoured activation pathway is CR-CA-OA1, and with sufficiently long hyperpolarizations, OA1-OA2 (this transition is enhanced by K381E). At very negative voltages, the opening rate is determined primarily by CA-OA1. Upon subsequent depolarization, the favoured deactivation pathway is OA2-OR-CR. OA2-OR is both voltage- and cAMP-dependent. At very positive voltages, the closing rate is determined primarily by OR-CR.
**Figure 15: Cartoon gating model describing cAMP- and K381E effects on sustained activation.** Voltage-sensor (VS, red) can exist in one of two configurations: “S4 in” or “S4 out”; Pore gate (blue) can exist in one of two configurations: closed (dark blue) or open (light blue); COOH region can exist in one of three configurations: resisting (“R-state”, resists gate opening, orange triangles), willing (“W-state”, favours gate opening, green circles), or persistent (“P-state”, strongly favours gate opening, purple circles). Starbursts indicate inhibitory interactions, black lines indicate conformation-specific interactions, lightning strikes indicate highly stabilizing synergistic interactions, and black arrows indicate favoured direction of equilibrium. (Top) Gating in the absence of cAMP. Resting channel (1) has VS out, pore gate closed and COOH-region in the R-state. Upon hyperpolarization and resultant movement of S4 in (2), gate opening proceeds through a voltage-independent step (2-3). Upon depolarization, channels close through a voltage-independent closing step (4-1) after movement of the VS position from in to out (3-4). (Bottom) Gating in the presence of cAMP. Resting channel (5) has VS out, pore gate closed and COOH region in W-state. Upon hyperpolarization and movement of S4 to in position (6), gate opening proceeds through a voltage-independent step (6-7). With sufficiently long hyperpolarizations, the P-state is able to form and interacts synergistically with a voltage-sensitive region (7-8); this interaction that may involve the S4-S5 and C-linkers. Upon depolarization, channels close through a voltage- and cAMP-dependent step (8-9) followed by a voltage-independent gate closing step (9-5). See Ch. 4 text for details.
Appendix 2: Tables

Table 1: Summary of voltage-activation of mouse HCN channels. Values are for channels heterologously expressed in *X. laevis* oocytes and measured using the excised inside-out patch clamp technique.

<table>
<thead>
<tr>
<th></th>
<th>No cAMP</th>
<th>Saturating cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V$_{1/2}$ (mV)</td>
<td>G-V slope</td>
</tr>
<tr>
<td>mHCN1</td>
<td>-110.6±1.3</td>
<td>5.3±0.3</td>
</tr>
<tr>
<td></td>
<td>-117.1±0.9</td>
<td>6.2±0.2</td>
</tr>
<tr>
<td>mHCN2</td>
<td>-125±2.0</td>
<td>3.76±0.3</td>
</tr>
<tr>
<td></td>
<td>-129.6±1.9</td>
<td>4.5±0.3</td>
</tr>
<tr>
<td></td>
<td>-137.2±1.0</td>
<td>4.7±0.3</td>
</tr>
<tr>
<td>mHCN4Δx</td>
<td>-124±8 (n=3)</td>
<td>8.8±0.5 (n=3)</td>
</tr>
<tr>
<td>mHCN4ΔxK381E</td>
<td>-123±9 (n=5)</td>
<td>6.9±1.4 (n=5)</td>
</tr>
<tr>
<td>242Δx</td>
<td>-128±2 (n=6)</td>
<td>5.8±1.0 (n=6)</td>
</tr>
<tr>
<td>242ΔxK381E</td>
<td>-122±8 (n=4)</td>
<td>5.7±2.0 (n=4)</td>
</tr>
</tbody>
</table>

2 Wainger et al., 2001  
3 Wang et al., 2001  
4 Craven and Zagotta, 2004
Table 2: Deactivation time constant values for HCN4 and 242 constructs. Time constants were obtained fully deactivated tail currents resulting from a return to a holding voltage of -40 mV or +40 mV after a 2 s (for HCN4 constructs) or 3 s (for 242 constructs) activation pulse to -160 mV. Tail currents were fitted with a single exponential function after an initial delay as described.

<table>
<thead>
<tr>
<th></th>
<th>r\text{deact at }-40\text{mV}</th>
<th>r\text{deact at }+40\text{mV}</th>
<th>r\text{deact ratio}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No cAMP</td>
<td>cAMP</td>
<td>r\text{deact ratio}</td>
</tr>
<tr>
<td>mHCN4Δx</td>
<td>180±140 (n=4)</td>
<td>260±130 (n=4)</td>
<td>1.6±0.4 (n=4)</td>
</tr>
<tr>
<td>mHCN4ΔxK381E</td>
<td>830±190 (n=4)</td>
<td>7400±2300 (n=4)</td>
<td>8.9±0.6 (n=4)</td>
</tr>
<tr>
<td>242Δx</td>
<td>95±30 (n=4)</td>
<td>270±65 (n=4)</td>
<td>3.1±1.2 (n=4)</td>
</tr>
<tr>
<td>242ΔxK381E</td>
<td>1200±500 (n=4)</td>
<td>8500±2500 (n=4)</td>
<td>7.4±1.7 (n=4)</td>
</tr>
</tbody>
</table>
Table 3: Maximal conductance values in the absence of cAMP. $G_{\text{max, basal}}/G_{\text{max, cA}}$ values were calculated from peak tail conductances upon a return to a holding voltage of -40mV after a 2 or 3 s maximally activating voltage pulse to -160mV.

<table>
<thead>
<tr>
<th></th>
<th>$G_{\text{max, basal}}/G_{\text{max, cA}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>mHCN2</td>
<td>0.76±0.05 $^5$</td>
</tr>
<tr>
<td>mHCN4Δx</td>
<td>0.87±0.09 (n=3)</td>
</tr>
<tr>
<td>mHCN4ΔxK381E</td>
<td>0.88±0.08 (n=4)</td>
</tr>
<tr>
<td>242Δx</td>
<td>0.75±0.05 (n=4)</td>
</tr>
<tr>
<td>242ΔxK381E</td>
<td>0.85±0.06 (n=4)</td>
</tr>
</tbody>
</table>

$^5$ Shin et al., 2004.
REFERENCES


