ENERGETIC AND STRUCTURAL IMPACT OF CYCLIC NUCLEOTIDE BINDING TO HYPERPOLARIZATION-ACTIVATED CYCLIC NUCLEOTIDE-GATED CHANNELS

by

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Abstract

Hyperpolarization-activated Cyclic Nucleotide-gated, HCN, channels contribute to the membrane potential of excitable cells including pacemaker cells of the heart and neurons in the brain. By binding to the inner side of the HCN channel, cAMP facilitates channel opening, but the underlying mechanism has been mainly inferred from relating cAMP concentration to the degree of facilitation. Concentration-response relations reflect the tightly coupled process of cAMP binding and channel opening. The strength of binding and how it is linked to channel opening is not known. Furthermore, cAMP facilitation is not equal among the four mammalian HCN isoforms and the extent to which cAMP binding affinity contributes to these differences is not known.

My experiments support the conclusion that cAMP binds to one site of the isolated tetrameric C-terminus of HCN2 and HCN4 with high affinity and to three sites with low affinity revealing negative cooperativity. In contrast, only low affinity binding was observed in HCN1 with energetics of binding that were similar to those of the low affinity binding to HCN2. Cyclic AMP enhanced oligomerization of the HCN2 C-terminus in solution, but had a negligible effect on oligomerization of the HCN1 C-terminus. Oligomerization in solution is thought to reflect the formation of a gating ring in the intact channel that facilitates opening. Together, this suggests that HCN1 functions as though already disinhibited, explaining its easier opening in the absence of cAMP, its smaller facilitation of opening, and lack of negative cooperativity upon cAMP binding.

Lysine substitution at residue 488 of HCN2, initially identified in an individual with idiopathic generalized epilepsy, eliminated negative cooperativity and reduced oligomerization of the isolated C-terminus upon cAMP binding. This likely reflects a decrease in its ability to
form a gating ring in the intact channel and explains the reported inhibition of opening by this mutation.

The work presented in this thesis demonstrates the value of studying the C-terminus of the HCN channel in isolation to uncover the mechanism by which the HCN C-terminus and cAMP binding control channel opening that would otherwise be hidden by functional experiments.
Preface

This thesis is made up of work that has been published and prepared for publication in a peer-reviewed journal. Chapter 2 is a published article and presented in its original form, however the introduction has been modified to preserve readability. Chapter 3 contains work that is to be submitted within this year. It is presented in the most up-to-date form at the time of completion of this thesis.


S.S. Chow was responsible for designing and performing experiments, data analysis, producing figures, tables, and writing of the manuscript in partnership with E.A. Accili and F. Van Petegem. Additional experiments, figures, and tables were also performed by S.S. Chow as requested by journal reviewers. F. Van Petegem and E.A. Accili were involved in developing the project.

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S.S. Chow was responsible for developing, designing and performing experiments, data analysis, producing figures, tables, and writing the manuscript in partnership with E.A. Accili and F. Van
Petegem. L. Ng performed experiments and was involved in analyzing the data. F. Van Petegem provided guidance and E.A. Accili was involved in developing the project.
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## List of abbreviations

### Amino Acid One Letter Code

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<tr>
<th>Amino Acid</th>
<th>One Letter Code</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ala</td>
<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>Cys</td>
<td>C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Asp</td>
<td>D</td>
<td>Aspartate/Aspartic Acid</td>
</tr>
<tr>
<td>Glu</td>
<td>E</td>
<td>Glutamate/Glutamic Acid</td>
</tr>
<tr>
<td>Phe</td>
<td>F</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Gly</td>
<td>G</td>
<td>Glycine</td>
</tr>
<tr>
<td>His</td>
<td>H</td>
<td>Histidine</td>
</tr>
<tr>
<td>Ile</td>
<td>I</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>Lys</td>
<td>K</td>
<td>Lysine</td>
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<tr>
<td>Leu</td>
<td>L</td>
<td>Leucine</td>
</tr>
<tr>
<td>Met</td>
<td>M</td>
<td>Methionine</td>
</tr>
<tr>
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<td>Asparagine</td>
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<td>Threonine</td>
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<tr>
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<td>V</td>
<td>Valine</td>
</tr>
<tr>
<td>Trp</td>
<td>W</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Tyr</td>
<td>Y</td>
<td>Tyrosine</td>
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### Other abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>BCNG</td>
<td>Brain cyclic nucleotide-gated</td>
</tr>
<tr>
<td>βME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cCMP</td>
<td>Cyclic cytidine 3’,5’-monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>cNMP</td>
<td>Cyclic nucleotide monophosphate</td>
</tr>
<tr>
<td>CAP</td>
<td>Catabolite activator protein</td>
</tr>
<tr>
<td>CNBD</td>
<td>Cyclic nucleotide binding domain</td>
</tr>
<tr>
<td>CNG</td>
<td>Cyclic nucleotide-gated (channel)</td>
</tr>
<tr>
<td>C-terminus</td>
<td>Carboxy terminus</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>Effective concentration</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
</tr>
<tr>
<td>F5M</td>
<td>Fluorescein-5-malemide</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GIRK</td>
<td>G protein-coupled inwardly-rectifying potassium (channel)</td>
</tr>
<tr>
<td>HAC</td>
<td>Hyperpolarization-activated channel</td>
</tr>
<tr>
<td>HCN</td>
<td>Hyperpolarization-activated cyclic nucleotide-gated (channel)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HMT</td>
<td>His$_6$-Maltose binding protein-Tobacco etch virus (tag)</td>
</tr>
<tr>
<td>I$_f$</td>
<td>Funny current</td>
</tr>
<tr>
<td>I$_h$</td>
<td>Hyperpolarization-activated current</td>
</tr>
<tr>
<td>ISO</td>
<td>Isoproterenol</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>MBP</td>
<td>Maltose binding protein (tag)</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)-ethanesulfonic acid</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut off</td>
</tr>
<tr>
<td>N-terminus</td>
<td>Amino terminus</td>
</tr>
<tr>
<td>P$<em>{Na}$/P$</em>{K}$</td>
<td>Sodium and potassium permeability ratio</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>r.m.s.d.</td>
<td>Root mean squared deviation</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SAN</td>
<td>Sinoatrial node</td>
</tr>
<tr>
<td>S.D.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>S.E.</td>
<td>Standard error</td>
</tr>
<tr>
<td>SpIH</td>
<td>Streptococcus pneumonia hyperpolarization-activated (channel)</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
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## List of symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>$K_d$</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>$\Delta G$</td>
<td>Gibbs free energy</td>
</tr>
<tr>
<td>$\Delta H$</td>
<td>Change in enthalpy</td>
</tr>
<tr>
<td>$\Delta S$</td>
<td>Change in entropy</td>
</tr>
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Acknowledgements

I would like to begin by thanking my supervisor Dr. Eric Accili, to whom I am forever grateful. His guidance, mentorship, advice, patience and encouragement has helped me immensely throughout my doctoral studies. I would also like to thank Dr. Filip Van Petegem for his endless support and scientific advice, and acknowledge the members of my supervisory committee, Drs. Christopher Ahern, Lawrence McIntosh and Kenneth Baimbridge for their guidance.

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To Mom, Papa and Tim
Chapter 1: Introduction
Overview

In the adult mammalian heart, each heartbeat is triggered by pacemaker cells that reside in the sinoatrial node, SAN, which is located at the top of the right atrium of the heart. Pacemaker cells are specialized cardiomyocytes that spontaneously initiate and generate electrical impulses that are conducted throughout the heart to make it beat. This spontaneous electrical activity is characterized by action potentials that are separated by a slowly depolarizing phase known as the diastolic depolarization or pacemaker potential. A contributor to the generation and modulation of this diastolic depolarization is the hyperpolarization-activated or funny current \((I_h\) or \(I_f\)). This current is known as funny because it conducts both sodium and potassium, is activated by membrane potential hyperpolarization, and its activation is promoted and inhibited by \(\beta\)-adrenergic and muscarinic stimulation, respectively. Control of \(I_f\) by autonomic stimulation occurs by direct binding of the intracellular messenger cyclic adenosine monophosphate, cAMP, to the cytoplasmic C-terminus of \(I_f\) channels. A cyclic nucleotide binding domain, CNBD, was identified in the cloned subunits that conducts \(I_f\), called HCN1-4. The C-terminal region including the CNBD was shown to tonically inhibit opening, which was relieved by cAMP binding. Movements in the C-terminus induced upon cAMP binding have been studied, but the molecular events that link cAMP binding to channel opening remain obscure.

This introduction will briefly discuss the history of \(I_f\), its proposed role in excitable tissues such as the SAN, the role cAMP plays in facilitating \(I_f\) opening, and the current understanding of the molecular mechanisms underlying cAMP facilitation.
A brief history of the funny current

The funny current, $I_f$, was initially described in spontaneously active Purkinje fibers, however, at the time, it was thought to be a pure outwardly conducting potassium current called $I_{K2}$ to distinguish itself from $I_{K1}$, an inwardly rectifying potassium current and $I_K$, an outwardly conducting potassium current (Noble and Tsien, 1968). $I_{K2}$ was thought to contribute to the diastolic depolarization phase of SAN (Figure 1.1A) by activating during the action potential and decaying during the repolarization phase (Noble and Tsien, 1968; Hauswirth et al., 1968a). However depolarization triggered by a decaying potassium current was difficult to understand, as an inward current was required to depolarize the membrane within the pacemaker range of -60 to -80 mV.

During this time, Noma and Irisawa reported a slow transient inward current that activated at hyperpolarized potentials in SAN cells. This slow inward current was thought to be a sodium current that slowly depolarized the cell between action potentials (Noma and Irisawa, 1976). Brown et al. (1980) later confirmed that this inward sodium current depolarized the cell within the pacemaker range of -60 to -80 mV and that large hyperpolarizing pulses produced a large inward current that translated to a steeper upstroke of the action potential, thus foreshadowing its role in pacemaking (Brown and DiFrancesco, 1980). They named this slow inward sodium current $I_f$.

In 1981, DiFrancesco showed that $I_{K2}$ of Purkinje fibres and $I_f$ of SAN were the same current. He reported that Purkinje fibers, where $I_{K2}$ was initially measured, was mistaken for a potassium current because Purkinje fibers contained large inwardly rectifying currents, $I_{K1}$, thereby masking the inward current of $I_{K2}$. Because only small amounts of $I_{K1}$ are conducted in SAN cells, $I_{K2}$ could be measured more accurately. Barium was also used to block $I_{K1}$ in Purkinje
fibers to further confirm $I_{K2}$ as an inward current that activated at hyperpolarized potentials below -50 mV in the same way as $I_f$ (Difrancesco, 1981).

**Properties of $I_f$ and its proposed role in the heart and neurons**

In SAN cells, multiple studies have shown $I_f$ activates over a range of -50 to -100 mV (Nakayama et al., 1984; Difrancesco, 1984; Denyer and Brown, 1990; Van Ginneken and Giles, 1991), and deactivation occurs at depolarized potentials and completely closes at potentials more positive than -30 mV (Difrancesco et al., 1986). The fully activated current reverses near -20 mV, which reflects its mixed permeability to sodium and potassium ratio, $P_{Na}/P_K$, of 0.24-0.31 (Ishii et al., 1999; Ludwig et al., 1998; Moroni et al., 2000). During diastole, sodium moves inward to slowly depolarize the membrane potential, a process referred to as the diastolic depolarization or pacemaker potential (Figure 1.1A). Eventually, the membrane potential depolarizes to the point at which the L-type voltage-gated calcium channel opens to cause the rapid upstroke of the action potential.

There is some variability in the activation range of $I_f$. In some cases, the activation range of $I_f$ appears to be outside the pacemaker range of -60 to -80 mV and the amount of $I_f$ activated within the pacemaker range may be too small to contribute to pacemaking (Denyer and Brown, 1990). Some of the activation range variability may be due to so-called “run-down”. DiFrancesco et al. (1986) found that $I_f$ activation range progressively shifted to more negative potentials over a period of 20 minutes (Difrancesco et al., 1986). It has also been suggested that PIP$_2$ is necessary to keep the $I_f$ activation range at less negative potentials (Pian et al., 2006).

The neuronal equivalent of $I_f$, known as $I_h$, has also been described in a number of cells types such as dorsal root ganglion and thalamic neurons. In general, neuronal $I_h$ has been found
to maintain a slightly depolarized membrane potential, thus increasing resting membrane conductance (Bal and McCormick, 1997), and normalizing inputs from different dendritic locations as excitatory postsynaptic potentials varies inversely with the distance from the soma (Magee, 1999).

**Adrenaline increases the open probability of the \( I_f \) channel by stimulating the production of cAMP acting on the cytoplasmic side**

Upon perfusion of adrenaline, an increase in \( I_{K2} \) in Purkinje fibres within the pacemaker depolarization range was seen that steepened the diastolic depolarization slope and raised the frequency of action potentials (Hauswirth et al., 1968b) (Figure 1.1A). This effect was extended to \( I_f \) in SAN, where adrenaline was shown to increase the probability of channel opening, as well as a decrease in the latency period between openings upon hyperpolarization (Brown et al., 1979; Difrancesco, 1986).

To determine whether the effect of adrenaline on \( I_f \) was due to phosphorylation by Protein kinase A (PKA) or cAMP, each was individually applied to excised patch SAN cells. In the presence of 100 U/mL of the catalytic subunit of PKA alone, no current activation was seen. However in the presence of 100 µM of cAMP alone, \( I_f \) was stimulated, and the addition of PKA did not increase it further. Application of cAMP showed a maximum shift of approximately 11 mV and an effective concentration, \( EC_{50} \), of 0.211 µM (Difrancesco and Tortora, 1991).

While the application of adrenaline increases the open probability of \( I_f \) and positively shifts the activation potential by 11 mV (Figure 1.1B) (Difrancesco, 1986; Difrancesco et al., 1986; Hauswirth et al., 1968b), acetylcholine is seen to have the opposite effect: it depresses \( I_f \) and shifts the activation curve to more hyperpolarized potentials (Difrancesco and Tromba,
The effects of adrenaline and acetylcholine are mediated by cAMP attaching to a cytosolic region of the channel (Barbuti et al., 1999; Difrancesco and Tortora, 1991; Difrancesco and Mangoni, 1994). This was determined through experiments where the enzyme pronase was added to the SAN. Not only was the effect of cAMP eliminated, it also shifted the activation curve to voltages that were even more positive than those encompassing the $I_f$ activation curve in the presence of cAMP (Barbuti et al., 1999).

Cyclic AMP facilitation in neurons

The role of cAMP facilitation of $I_h$ in neurons is complex and has not been as extensively studied as in the pacemaker of the heart. Nevertheless, a role for $I_h$ and cAMP facilitation has been proposed in some neuronal processes.

Cyclic AMP facilitation of $I_h$ is thought to regulate the sleep-wake cycle through the rhythmic generation of action potentials in the thalamocortical neurons. During sleep, these oscillatory bursts are slow (<15 Hz), however during wakefulness, when cAMP levels are increased, bursts increase in frequency to ~40 Hz (Kanyshkova et al., 2009; Luthi, 1998). Calcium spikes are thought to activate adenylyl cyclase to increase intracellular cAMP levels, which positively shifts the activation curve and maintains resting membrane potential stability by keeping $I_h$ channels constitutively open (Kuisle et al., 2006).
Figure 1.1 Cyclic AMP modulates $I_f$ in response to the addition of adrenaline or acetylcholine

A) Acceleration and slowing of the SAN action potential induced by 10 nM isoproterenol (ISO) or 3 nM of acetylcholine (ACh). Figure adapted from Difrancesco (1993) and reprinted with permission from the Annual Review of Physiology. B) Hyperpolarizing voltage ramp protocol (top) from -35 mV to -145 mV applied to inside-out SAN patches to produce the normalized $I_f$ probability curve (below). The addition of 10 µM of cAMP facilitated channel opening, while removal shifted curve back to return. Figure adapted from Difrancesco and Mangoni (1994), reprinted with permission from Wiley.
Subunits underlying hyperpolarization-activated currents are cloned

Subunits that underlie $I_f$ were discovered independently by three separate groups of investigators, Santoro et al. (1997), Ludwig et al. (1998) and Gauss et al (1998). While searching for proteins that interacted with the SH3 domain of a neural specific form of Src using yeast two-hybrid screening, Santoro et al. (1997) identified and isolated a new protein from mouse brain named BCNG-1, for brain cyclic nucleotide-gated 1 (Santoro et al., 1997). With a combination of RT-PCR, library screening and a BLAST search within the mouse and human expressed sequence tag (EST) database, another three isoforms were discovered. They were cloned from the brain and heart and named BCNG-2, 3 and 4 (Santoro et al., 1997). The same clones were also found by Ludwig et al. (1998) via BLAST search among the EST database using the CNBD of cyclic nucleotide-gated, CNG, channels, but these clones were named HAC 1, 2, and 3, for hyperpolarization-activated channel (Ludwig et al., 1998). During this time, Gauss et al. (1998) also identified and cloned a channel with similar properties as $I_h$ from sea urchin testis called SpIh. This channel is involved in rhythmic firing of central neurons as well as controlling pacemaking in the heart (Gauss et al., 1998).

In 1998, Clapham coined the term Hyperpolarization-activated Cyclic Nucleotide-Gated, HCN, channel to described these ion channel subunits (Clapham, 1998). The four mammalian HCN isoforms are now known as HCN1, 2, 3 and 4. Since the original reports of HCN cloning, other HCNs have been cloned in rainbow trout, (Cho et al., 2003), the honey bee (Gisselmann et al., 2003), fruit fly (Krieger et al., 1999; Marx et al., 1999), lobster (Gisselmann et al., 2005) and sea squirt (Jackson et al., 2012).
The predicted HCN channel structure is similar to those of voltage-gated potassium channels

Although the crystal structure of the HCN channels has yet to be solved, they are predicted to have a structure similar to that of the voltage-gated potassium channel because of their similarities in sequence. Their primary structure is comprised of six transmembrane segments, a pore between the S5 and S6 segment and a coordination of voltage sensors between S1 and S4. The pore contains a GYG sequence similar to that of the voltage-gated potassium channel. HCN channels also have a cytosolic N and C-termini (Figure 1.2A).

Similar to voltage-gated potassium channels, HCN channels are predicted to form tetramers. However, unlike these channels, HCN channels do not have a primary sequence similar to that of the T1 domain that is responsible for tetramerization, but may have a similar domain in their N-terminus. Using yeast-two hybrid assays, Proenza et al. (2002) found the N-termini of HCN1 and HCN2 interacted amongst themselves, with each other, and its deletion removed both current and cell surface expression. This data concluded that the N-terminus is important for functional expression of the channel (Proenza, 2002).

The C-terminus contains the cyclic nucleotide binding domain, CNBD and C-linker that connects the S6 transmembrane segment to the CNBD. More on the role of these two segments will be discussed in more detail later in this introduction.
Figure 1.2 Predicted topology of HCN channels and sequence alignment

A) Cartoon representation of the proposed topology of one of the four subunits. Included are 6 putative transmembrane segments (S1-S6), including voltage sensor (S4) and pore between S5 and S6. C-terminal end comprises the C-linker and CNBD where cAMP is known to bind. B) Sequence alignment of the C-linker and CNBD of the four HCN isoforms. Grey horizontal bars and black arrows represent α-helices and β-strands respectively. Residues highlighted in black are identical, dark grey are conserved and white indicate at least a difference of one amino acid among the isoforms.
Proposed role for the HCN isoforms in the heart and brain

HCN channels are expressed predominately in the brain and heart, however the distribution of the isoforms varies. HCN4 makes up 80% of the HCN population in the SAN, thus presumably plays an important role in heart rate regulation. Deletion of the HCN4 gene was embryonically lethal at day 10 to 11.5, and showed a 40% decrease in beating frequency with no effect of cAMP (Stieber et al., 2003). To assess its role in adult pacemaking, HCN4 was temporally knocked down using the Cre/loxP-system. These mice had cardiac arrhythmias with recurrent sinus pauses. Aside from a few sinus pauses, the addition of isoproterenol showed no difference in heart rate acceleration between HCN4 deficient mice and wild type. Inducible cardiac-specific HCN4 knockout mice elicited a similar response. They showed progressive development of severe bradycardia and AV block attributed by a 70% reduction of I_f. This led to the death of mice at day 5. Similar to the HCN4 deficient mice, I_f showed normal sensitivity to cAMP (Baruscotti et al., 2011).

A more severe cardiac phenotype is seen with muscarinic stimulation. The addition of carbachol enhanced SAN dysfunction by a decrease in heart rate and an increase in sinus pauses (Herrmann et al., 2007). It was therefore proposed while HCN4 is not required for sympathetic stimulation, HCN4 may contribute to stabilizing the resting heart rate (Herrmann et al., 2007; Baruscotti et al., 2011). HCN4 may also be important in the development of adult pacemaking cells which require the ability to respond to autonomic stimulation, but is not important in the development of primitive pacemaking cells (Stieber et al., 2003; Wahl-Schott and Biel, 2008; Baruscotti et al., 2011).

Because HCN2 accounts for ~20% of the HCN population in the SAN, it seems to take on a smaller role in pacemaking in the heart. The average heart rate at rest and during activity in
HCN2 deficient mice was similar to that of wild type, however the intervals between successive heartbeats varied widely. A 28-35% reduction in \( I_f \) was also seen. Similar to that of HCN4, HCN2 deficient mice showed normal sensitivity to cAMP, also suggesting HCN2 is not essential for autonomic control of cardiac activity (Ludwig et al., 2003).

In neurons, HCN2 knockout mice have been shown to reduce locomotor activity, and hypersensitivity to pain. These mice experienced less horizontal movement as well as decreased rearing, i.e. standing on hind legs. These mice also had bursts of action potential firing in their thalamocortical relay neurons, which is caused by a destabilization of the resting membrane potential (Ludwig et al., 2003).

Mice with HCN2 knocked out of their nociceptors lose their hypersensitivity to pain. It is speculated this is due to the reduced amount of \( I_h \) seen and the loss of sensitivity to cAMP which is the mode of action of Prostaglandin E2, an inflammatory mediator (Emery et al., 2011). They found mice showed normal baseline sensitivity to pain from heat, cold and mechanical stimuli, but were protected from hypersensitivity to these pain inducing stimuli (Emery et al., 2011).

In contrast to HCN2 and HCN4, HCN1 expression is primarily focused in the cerebral cortex, hippocampus and cerebellum (Santoro et al., 1998; Moosmang et al., 1999) where it plays a role in motor learning, memory (Nolan et al., 2003; 2004) and epileptic seizures (Santoro et al., 2010). To test motor learning and memory, wild type and HCN1 knock out mice were trained to swim to a visible platform. Upon subsequent training sessions, wild type mice were able to locate the platform faster than HCN1 knock out mice. HCN1 knock out mice swam in circles and maintained physical contact to the pool walls. Similar results were seen when the platform was hidden, suggesting spatial memory of HCN1 knock out mice was unaffected, but their ability to coordinate movement involved in swimming was impaired (Nolan et al., 2003).
This was also seen when knock out mice were placed on a rotating rotarod. At speeds greater than 30 rpm, wild type mice were able to adapt, while knockout mice did not due to their inability to coordinate the fast movement required to stay on the rotarod (Nolan et al., 2003).

HCN1 knock out mice also experienced greater severity of seizures, as well as an increase in seizure related deaths, however this only occurred when seizures were induced electrically or chemically (Santoro et al., 2010).

Unlike the other HCN isoforms, the role of HCN3 has not been as extensively studied. So far, studies have shown HCN3 expression was not found in the SAN, but in the ventricular muscles where it may play a role in regulating the ventricular resting membrane potential (Moosmang et al., 1999; Santoro et al., 1998; Fenske et al., 2011). HCN3 deficient mice had an accelerated late repolarization phase of the ventricular action potential causing it to shorten. This result was attributed to the 30% decrease in total $I_h$ seen in HCN3 deficient mice. Resting membrane potential was also shifted to hyperpolarized potentials. From these results, it was concluded that HCN3 plays a role in regulating the ventricular resting membrane potential by controlling the potassium current influx upon the repolarization phase of the action potential (Fenske et al., 2011).

**Biophysical properties of HCN channels**

Each mammalian HCN isoform display the defining characteristics of $I_f$, which include conducting inward current at hyperpolarized potentials and mixed permeability to sodium and potassium ratio, $P_{Na}/P_K$, of ~0.24-0.31 (Ishii et al., 1999; Moroni et al., 2000; Ludwig et al., 1998). However, the potentials at which they are activated and their response to cAMP differ (Figure 1.3A). The half maximal voltage, $V_{1/2}$ of HCN1 is ~78 mV (Stieber et al., 2005a;
Stieber, 2003) whereas the $V_{1/2}$ values of HCN2 and HCN4 are at more hyperpolarized potentials, $\sim$-97 mV (Tran, 2002; Mistrik, 2005; Stieber, 2003; Stieber et al., 2005a) and $\sim$-99 mV (Stieber et al., 2005a; b) respectively. The $V_{1/2}$ for HCN3 falls between at $\sim$-91 mV (Mistrik, 2005; Stieber, 2003).

While all HCN isoforms have a CNBD with similar sequences (Figure 1.2B), they do not respond equally to cAMP. HCN2 and HCN4 channel opening are facilitated by cAMP by shifting $V_{1/2}$ values to more depolarized potentials by $\sim$17 mV (Mistrik, 2005; Stieber et al., 2005a; Stieber, 2003) (Stieber et al., 2005a; Stieber, 2003) (Figure 1.3A). Concentration-response curves reported half maximal effective concentration, $EC_{50}$ of $\sim$0.2 µM (Zagotta et al., 2003) for HCN2 and $\sim$1.53 µM for HCN4 (Milanesi et al., 2006). On the other hand, cAMP has very little or no effect on HCN1 and HCN3 (Figure 1.3B); $EC_{50}$ value of HCN1=0.06 µM (Wang et al., 2001).
Figure 1.3 Activation curves of inside-out patches in the presence and absence of cAMP

A) Activation curves for HCN2 and HCN4 in the absence (white circles) and presence (black circles) of saturating (10 µM) concentrations of cAMP. B) Activation curves of HCN1 and HCN3 (Mistrik, 2005) in the absence (white circles) and presence (black circles) of saturating (10 µM) concentrations of cAMP. HCN1 and HCN2 figure adapted from © Chen et al. 2001. Originally published in Journal of General Physiology. Vol 117:491-503. HCN4 figure adapted from Trends in Cardiovascular Medicine, Vol.12, Cardiac HCN channels: Structure, Function, and Modulation, Biel et al., 206-213 (2002), with permission from Elsevier.
Control of HCN opening by the C-linker and CNBD

As previously discussed, cAMP directly affects $I_f$ by increasing the open probability of the channel and positively shifting the activation curve (Difrancesco and Mangoni, 1994; Difrancesco and Tortora, 1991) (Figure 1.1B). By exposing the cytosolic side of the $I_f$ channel to pronase, Barbuti et al. (1999) had proposed that a cytosolic region of the channel tonically inhibited opening and that cAMP binding relieved inhibition. Subsequently, it was shown that the C-linker and CNBD exerted an inhibitory effect on the channel, which was indeed relieved upon cAMP binding to the C-terminus.

Through serial deletions of the C-terminal region of both the HCN1 and HCN2 isoforms, they found that removal of the C-helix portion of the CNBD, a region known to play an important role in channel opening in CNG channels, removed the effect of cAMP in both channel isoforms. However, removal of the CNBD, not only removed the effect of cAMP on the channel, it also shifted $V_{1/2}$ levels beyond that of the wild type channel with cAMP. Similar to the effect of cAMP, the removal of the CNBD of HCN1 produced a smaller overall shift compared to removing the CNBD of HCN2 (Wainger et al., 2001). Thus, the tonic inhibition by the HCN1 CNBD appeared to be less than that of the HCN2 CNBD.

Exchange of CNBDs between the HCN1 and HCN2 isoforms had little effect on basal gating and cAMP facilitation on channel opening (Wang et al., 2001). However, when both the C-linker and the CNBD were swapped between HCN1 and HCN2, HCN2 behaved like that of HCN1 and HCN2 behaved like that of HCN1. Thus, the C-linker region appears to play an important role in the differences in tonic inhibition by the CNBD between HCN1 and HCN2.

Less is known about the lack of response to cAMP in HCN3. Similar to HCN1, HCN3 activates at more depolarized potentials than HCN2 and HCN4 (Stieber et al., 2005a), however it
is unknown whether this depolarized shift in activation is due to a smaller tonic inhibition of opening by the C-linker and CNBD. It is known that the CNBD of HCN3 can bind to cAMP. When the CNBD of HCN3 and HCN4 were swapped, HCN3 gained HCN4-like properties including responding to cAMP by positively shifting $V_{1/2}$ by $\sim 17$ mV (Stieber et al., 2005a).

**The structure of the HCN channel C-terminus has been solved by x-ray crystallography**

Although a structure of the HCN channel has yet to be solved, a C-terminal fragment of the HCN1, HCN2, HCN4 and SpIH channel have been solved with x-ray crystallography (Figure 1.4) (Lolicato et al., 2011; Zagotta et al., 2003; Xu et al., 2010; Flynn et al., 2007). Each fragment starts immediately after the S6 transmembrane segment and includes the C-linker and CNBD.

In general, these structures are similar among the isoforms. The structure of the C-linker is comprised of six $\alpha$-helices labeled A’ to F’ which lean on neighbouring C-linker subunits in an “elbow-on-shoulder” configuration to form the tetramer (Figure 1.5) (Zagotta et al., 2003). It is followed by the CNBD which includes four $\alpha$-helices (A, P, B, C) with a $\beta$-roll between the A- and B-helices. The $\beta$-roll is made up of eight $\beta$-strands, which Zagotta et al. (2003) describes as a “jelly-roll-like” topology. Cyclic AMP binds in the anti-configuration inside the jelly-roll and interacts with the $\beta$-roll and C-helix (Zagotta et al., 2003).

Analytical ultracentrifugation showed the addition of cAMP promoted tetramerization between subunits, whereas without cAMP, the population was predominately monomers. Together with the knowledge that cAMP disinhibits the channel, it was hypothesized that the addition of cAMP promotes the formation of a gating ring that facilitates channel opening.
(Zagotta et al., 2003). Therefore one would expect the unliganded structure to be different. However, this was not the case.

**Figure 1.4** *Crystal structure the HCN2 C-linker and CNBD construct bound to cAMP*

Ribbon representation of a single monomer of HCN2J with cAMP (left). HCN2J tetramer as viewed parallel to the four-fold axis (right). Each subunit is shown in a different colour.

The crystal structure of HCN2J without cAMP was also solved by x-ray crystallography in the tetrameric form, but only subtle differences were noted between it and the same piece with cAMP bound. Two bromide ions were found occupying the cyclic nucleotide binding pocket, the F’-helix was less helical and the C-terminal portion of the CNBD, the C-helix, was not well resolved suggesting it was less structured (Taraska et al., 2009). In addition, no difference was seen between the crystal structure of HCN1 (Lolicato et al., 2011) or HCN4 (Lolicato et al., 2011; Xu et al., 2010). It has been speculated that the crystal contacts required to crystallize this fragment are not necessarily reflecting what is seen *in vivo* (Taraska et al., 2009) or this conservation in structure is due to an intrinsically stable fold which can tolerate changes such as...
the addition of cAMP, or changes in residues (Xu et al., 2010). However, further studies on the crystal structure suggest that this may not be the case. Craven et al. (2004) discovered an important intersubunit salt bridge between K472 located in B’-helix of the C-linker and E502 in the neighbouring D’-helix and D542 in the β-roll. When these salt bridges were broken by charge reversal, K472E, E502K and D542K, a depolarizing shift was seen in the activation curve with K472E, whereas no shift was seen with E502K or D542K. They speculated that the reason K472E opened more easily than E502K and D542K was because K472E channels cannot form salt bridges, whereas with E502K at least one salt bridge could be formed.

Attempts to crystallize HCN2J-K472E and E502K were only successful with E502K. Overall the crystal structure was similar to that of wild type. It was speculated that K472E was unable to be crystallized because these salt bridges are important in stabilizing the protein and when broken, the structure becomes flexible. Conclusions were drawn that perhaps the C-linker in the crystal structure of HCN2J bound to cAMP is in the resting state and requires the attachment of the transmembrane segments to complete the transition to the active state. It was proposed that the conformational changes that facilitate channel opening upon cAMP binding can be thought of as a series of three interacting modules: the pore, C-linker and CNBD (Figure 1.5). Each module is in equilibrium between two possible conformations: pore can be closed or open, C-linker can be resting or active and the CNBD can by unbound or bound. They are also coupled, that is, one module affects the other. For example, when the CNBD is in the bound state, this shifts the C-linker equilibrium to the active state, thus shifting the pore to the open state. But because the role of the C-linker is to inhibit the pore, removing it would promote the resting state of the C-linker, hence the lack of difference seen between the crystal structure with and without cAMP (Craven and Zagotta, 2004).
**Figure 1.5 Model of modular gating scheme for HCN channels**

Pore can be closed (C) or open (O), C-linker can be resting (R) or activated (A), and CNBD can be unbound (U) or bound (B) with cAMP. Equilibrium constants are shown for each module: L for pore, M for C-linker, and K for CNBD. The pore and C-linker influence each other by allosteric factor C, and the C-linker and CNBD influence each other by allosteric factor F. Figure adapted from ©Craven and Zagotta 2004. Originally published in Journal of General Physiology. Vol 124:663-677.

**Coupling of cAMP binding to channel opening is initiated by the C-helix of the CNBD**

The C-helix of the CNBD is of particular to note, as its deletion in HCN2 removed its effect on cAMP facilitation (Wainger et al., 2001). As previously mentioned, the C-terminus of the C-helix of the unbound HCN2J crystal structure was unresolved suggesting its need for cAMP for stability. Recent studies have focused their attention on the C-helix to understand its role in coupling to gating ring formation.

To map the conformation changes that occur upon cAMP binding, transition metal ion FRET was used to track HCN2J in solution (Taraska et al., 2009). Attaching the fluorophore, fluorescein-5-maleimide, F5M, to either the F'-helix of the C-linker or β-roll of the CNBD as the donor and nickel to various lengths of the C-helix as the acceptor, any movement can be tracked by the amount of fluorescence emitted (Taraska et al., 2009). The greatest amount of fluorescence was seen when the nickel was attached to the N-terminal portion of the C-helix than when attached to the centre or C-terminal end. From this it was proposed that upon cAMP
binding, the N-terminal portion of the C-helix moves towards the β-roll region of the CNBD, comes in close contact to the F’-helix of the C-linker, thus triggering changes in the C-linker to disinhibit the channel and facilitate opening (Taraska et al., 2009).

Zhou et al. (2007) characterized residues selected from molecular dynamic simulations to determine which residues contributed to efficacy and which contributed to binding (Zhou and Siegelbaum, 2007). It was hypothesized that residues involved in binding moved little, but those involved in cAMP efficacy moved a lot. Out of seven residues, only R632 located in the C-helix contributed to efficacy (Zhou and Siegelbaum, 2007). R632 forms a triad of stable contacts: a salt bridge with E582 and the ribose of cAMP. When mutated to an alanine, the channel becomes destabilized and cAMP efficacy is reduced (Zhou and Siegelbaum, 2007).

The above data showed that the C-helix plays an important role in translating cAMP binding to facilitate channel opening through its movement. However, these studies primarily focused on measuring the overall functional effect of cAMP on HCN, which does not provide the individual mechanistic processes that each module contributes to channel opening.

**Only two cAMP molecules are required to be bound for channel facilitation**

Although the crystal structure of HCN2J shows a 1:1 ratio of cAMP to CNBD, it is unclear as to how many cAMP molecules are required to bind to the channel to produce an effect, and what is the energetic contribution of each cAMP molecule to the gating ring when bound. Does each subunit act in an independent manner or is there cooperativity between subunits brought on by intersubunit contacts?

Ulens et al. (2003) created both tandem dimers and tetrameric dimers and controlled the number of cAMP bound per subunit by mutating an arginine to a glutamate, R591E. This
arginine has been shown to make contacts with the cyclic phosphate group of the cAMP molecule and when mutated to a glutamate, disrupts the electrostatic interaction and decreases cAMP binding affinity three-fold, however voltage gating is unaltered (Chen et al., 2001).

Upon linking the subunits as a tandem tetramer with either 1, 2, 3 or all 4 binding sites removed, cAMP sensitivity decreased, however there was no difference in sensitivity whether one or all four cAMP binding sites were removed. EC$_{50}$ values were ~0.14 µM (Ulens and Siegelbaum, 2003). Furthermore, Ulens et al. (2001) concluded that while only one cAMP is sufficient to produce a significant increase in $I_r$, all four are required to achieve maximal effect. Each successively bound cAMP independently contributed to channel opening energetics and in a non-linear fashion: i.e. one or three bound cAMP produced less than 25% and 75% the maximal voltage shift. When one cAMP bound to the CNBD it promoted the intersubunit interaction between the C-linkers to form the gating ring. This shifted the channel from a 2-fold symmetry of a dimer of dimers to a 4-fold symmetric tetramer, suggesting some form of cooperativity (Ulens and Siegelbaum, 2003).

Using a combination of patch-clamp fluorometry, confocal microscopy, and a fluorescent cAMP derivative, fcAMP, Kusch et al. (2010) compared normalized steady-state current with normalized fluorescence signal. They found upon application of 0.75 µM of cAMP at a maximum hyperpolarizing potential of -160 mV, where all channels were fully activated and maximum current is achieved, the normalized fluorescence signal suggested only 60% of the binding sites were occupied or only two cAMP molecules are required to be bound to produce the maximum amount of current (Kusch et al., 2010).

The above studies touched on the idea of cooperativity between subunits and that only two cAMP molecules are required for maximum effect, however the binding strength and the
energetic contributions of cAMP and its effect on intersubunit interaction is still unclear. This would help in determining the differences in cAMP response between the isoforms.

**Diseases associated with HCN channels**

To date there are four published disease-associated mutations in the C-terminus, one in HCN2 and three in HCN4. Studying disease-associated residues is important because they identify parts of the C-terminus that control gating. The HCN2 mutations, E488K, is a charge reversal mutation located in the C'-helix of the C-linker and is associated with idiopathic generalized epilepsy. Although the mutation shifts the activation curve to hyperpolarized potentials, cAMP facilitation is similar to that seen in wild type, $\Delta V_{1/2} = \approx 7$ mV. However the sensitivity, efficacy and strength of cAMP binding in this mutation are unknown and could explain the altered functional effect seen with this mutation.

D553N, S672R and L573X are all HCN4 disease mutations associated with sinus node dysfunction. Patients with D553N mutation located in the B'-helix of the C-linker experience recurrent syncope, QT prolongation and polymorphic ventricular tachycardia torsade de pointes. Trafficking of the mutant to the cell membrane was significantly lower than that of wild type. Upon co-expression with wild type, reduction in surface expression was also seen suggesting D553N effects normal channels in a dominant-negative manner. This was also reflected in the low amounts of current expressed in both mutant and co-expressed mutant with wild type (Ueda, 2004). Although S672R is located in the CNBD, cAMP facilitation on channel opening is not inhibited, however activation curves are shifted to negative hyperpolarized potentials mimicking that seen with low concentration of acetylcholine. Therefore, patients with S672R mutation experience chronic bradycardia (Milanesi et al., 2006). Lastly patients expressing L573X
experience sinus bradycardia along with intermittent atrial fibrillation. L573X is a 1-bp deletion (1631delC) when translated, leads to the truncation of the C-terminus, thus eliminating cAMP response in this mutant (Schulze-Bahr et al., 2003). Mice with the same mutation also had a reduction in heart rate at rest and during exercise. Their heart rate was unable to adapt to increased physical activity (Alig et al., 2009).

**Summary**

HCN channels play an important role in maintaining the rhythm of the heart by activating at hyperpolarized potentials to allow sodium ions to depolarize the cell during the diastolic depolarization phase to initiate the next action potential. Heart rate is also modulated by adrenaline and acetylcholine through binding directly to the channels from the cytoplasmic side. Four mammalian HCN isoforms have been identified, but they do not behave nor respond to cAMP equally. HCN1 has a very small response to cAMP compared to HCN2 and HCN4, whereas HCN3 has no response at all. Although all probably bind cAMP, the reasons for their differences in cAMP response is not clear. Because the EC50 values are a combination of binding and effect, the strength with which cAMP binds is also not known. Functional studies showed cAMP facilitates HCN2 channel opening and increases channel probability by disinhibiting the channel. HCN channels are probably tetrameric, with each of the four subunits containing a cAMP binding site. Binding of only one molecule is sufficient to produce some facilitation of opening and the maximum effect may require less than complete tetrameric occupancy. The crystal structure of the C-terminus of HCN1, HCN2 and HCN4 in the presence of cAMP helped visualize the cAMP binding site, its tetrameric form and pinpoint residues important for binding and selectivity. The combination of both functional and structural studies
led to the understanding that the CNBD and channel opening are coupled, and that binding may lead to conformational changes in the C-linker that promote a gating ring that facilitates opening. My thesis focuses on several unanswered questions of how strongly cAMP binds and how binding leads to variable facilitation of HCN opening among the four isoforms.

**Rationale and objectives of thesis hypotheses**

Most of the experiments examining the actions of cAMP on HCN channels have utilized electrophysiological approaches. These are useful and necessary as they directly assess the functional effects of the channels as a whole and translate the results to *in vivo*. The disadvantage of functional studies is its inability to separate cAMP binding from its effect, as EC$_{50}$ values are a combination of these events.

In order to directly measure binding affinity of cAMP, we isolated the C-linker and CNBD from the whole channel to more accurately study the cAMP binding event without interference from the rest of the channel. In chapter 2, I characterized the cAMP binding event in HCN1, HCN2 and HCN4 using primarily isothermal titration calorimetry, a technique that measures binding affinity and the energetics of the binding.

Because the C-terminus plays such an important role in channel function and cAMP facilitation, mutations within this region have led to channelopathies as mentioned in the introduction. Studying specific disease-associated mutations would help to uncover ways in which the function of mutant channels can be improved. In chapter 3, I focused on E488K in HCN2, a mutation associated with idiopathic general epilepsy. Because of its hyperpolarized shift in activation, we hypothesize cAMP binding, energetics and oligomerization states may be altered.
Techniques used in chapter 2 and chapter 3

Dynamic light scattering (DLS)

Dynamic Light Scattering (DLS) is a technique used to determine apparent molecular weight of a protein in solution by measuring the hydrodynamic radius of the protein. This radius is the radius of a hypothetical hard sphere with a hydration layer rotating in a solvent. This is measured using a laser that measures the time-dependent fluctuation in scattering intensity. Since all molecules in solution diffuse with random or Brownian motion, when the laser is scattered constructive or deconstructive inference occurs, which causes a change in light intensity. The faster the particles diffuse, the faster the intensity will change. This intensity of scattered light is directly related to the hydrodynamic radius of the protein. A standard plot of measured hydrodynamic radii of known molecular weights of proteins is determined and the hydrodynamic radii of unknown proteins in solution are compared to the graph to determine their apparent molecular weight (Wyatt Technology).

Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry (ITC) is a technique used to directly measure binding parameters, such as binding affinity ($K_d$), enthalpy ($\Delta H$) and stoichiometry ($n$) of ligand/protein interactions by monitoring the heat changes that occurs during an interaction. The ITC consists of two identical cells made of Hastelloy, a chemically inert super alloy efficient in conducting heat, enclosed in an adiabatic jacket and is maintained at constant temperature, such as 22°C. A syringe titrates small amounts of ligand in aliquots into the sample cell and the heat is either released or absorbed depending on the interaction. The power required to maintain a zero temperature difference between the sample and reference cell within the enclosed jacket is
measured and recorded. These inflection points decrease as binding site become saturated, thus less power is need to compensate for the temperature difference. The area under these inflection points are integrated and plotted on a graph, which is fit either to a one or two binding site equation (Appendix A.2) (Manual, 1991; Velázquez Campoy et al., 2004). From this fit, $K_d$, $\Delta H$ and n, is determined, and from these values, Gibbs free energy ($\Delta G$) and entropy ($\Delta S$) are calculated using the following equations:

$$\Delta G = -RT \ln K_a$$

$$\Delta G = \Delta H - T\Delta S$$

where $R$ is the gas constant and $T$ is temperature measured in Kelvin.

**Figure 1.6 Schematic diagram an ITC instrument**

The apparatus measures heat energy per unit time that must be added to the sample cell to maintain zero temperature difference between the two cells at the designated temperature for the experiment. Figure adapted from (Zhou et al., 2011) reprinted by permission from Macmillan Publishers Ltd: Nature Protocols.
Chapter 2: Energetics of cyclic AMP binding to HCN channel C-terminus reveal negative cooperativity\(^1\)

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Introduction

As discussed in chapter 1, binding of cAMP to the cytoplasmic side of the HCN channel facilitates channel opening (Difrancesco and Tortora, 1991); in the heart, this increases the rate and enhances conduction (Difrancesco, 1993). In the SAN, HCN channel opening is facilitated with a half-maximal cAMP concentration of 0.2 µM (Difrancesco and Tortora, 1991) and is marked by a depolarizing shift in the voltage range over which the channel opens (Difrancesco and Mangoni, 1994). There are four mammalian HCN isoform (HCN1-4) (Santoro et al., 1998; Ludwig et al., 1998; Gauss et al., 1998; Ishii et al., 1999) and each subunit contains a cyclic nucleotide binding domain (CNBD) in the cytosolic C-terminus and are predicted to have six transmembrane segments (S1-S6) with a cytosolic N- and C-termini. They combine as tetramers to form the ion-conducting channel containing four cAMP binding sites.

The structure of a fragment of the HCN2 C-terminus was solved by x-ray crystallography and forms a symmetrical tetramer (Zagotta et al., 2003). The structure hangs below the pore and is derived from sequences that begin just after the end of the S6 transmembrane segment, and includes the C-linker, a region that connects the CNBD to the cytosolic side of the channel pore. Cyclic AMP is thought to bind and induce a conformational change that propagates through the CNBD, as well as to the C-linker of an adjacent subunit (Zhou and Siegelbaum, 2007; Berrera et al., 2006). This allows interactions of select α-helices to form a “gating ring” that disinhibits channel opening (Zagotta et al., 2003; Zhou et al., 2004; Wainger et al., 2001).

Curiously, facilitation of opening by cAMP differs among the mammalian HCNs. HCN2 and HCN4 opening is strongly facilitated by cAMP, whereas that of HCN1 and HCN3 is only weakly affected or not affected at all, respectively (Wainger et al., 2001; Mistrik, 2005). This variation among the isoforms is thought to arise, at least in part, from subtle differences in
primary structure of the CNBD and the C-linker (Wainger et al., 2001; Chen et al., 2001; Stieber et al., 2005a). Notably, opening is facilitated even in chimeric HCN2 and HCN4 channels containing the CNBDs from HCN1 and HCN3 (Chen et al., 2001; Stieber et al., 2005a), suggesting that all HCNs bind cAMP. Nevertheless, the molecular basis for the differences in cAMP effect among the isoforms remains unknown. In this chapter, we set out to directly quantify and compare the energetics of cAMP binding among HCN1, HCN2 and HCN4 using the C-terminus in solution.

**Experimental approach**

*HCN mutagenesis and protein purification*

HCN constructs were cloned in a modified pET28 vector (Novagen), containing a His6-tag, maltose binding protein, and a cleavage site for the tobacco etch virus protease (pET28 HMT). The constructs utilized were mouse HCN1J (residues 390 – 580), ΔAB-HCN1J (432 - 580), mouse HCN2J (443 – 645), ΔAB-HCN2J (458 – 645), rabbit HCN4J (522 – 724) and ΔAB-HCN4J (563 - 724). The R591E mutation in HCN2J was obtained using QuikChange protocol (Stratagene). GCN4LI, a leucine zipper protein that forms an artificial parallel tetramer when amino acids in the “a” and “d” positions of the coiled coil are mutated to a leucine and an isoleucine respectively (Harbury et al., 1993), and HCN2J were amplified by PCR with a BamHI site and an additional five glycines at the 3’ and 5’ end respectively making a 10 glycine linker between the two pieces. After ligation, the fusion construct was amplified and cloned into the pET28 HMT vector.

All proteins were expressed in *Escherichia coli* Rosetta (DE3) pLacI grown in 2xYT media at 37°C, except for HCN1J and ΔAB-HCN1J that were grown in 25°C, and induced with
0.4 mM IPTG. Cells were lysed by sonication in 250 mM KCl + 10 mM HEPES (pH 7.4) (buffer A), except in the case of HCN1J and GCN4LI-HCN2J where 500mM NaCl plus 30 mM HEPES (pH 7.4) was used, supplemented with 100 mM PMSF, 25 mg/mL Lysozyme, 25 mg/mL DNase I, 10% glycerol, and 0.1 M EDTA. Cell debris was removed by centrifugation for 30 minutes at 35000xg, and the filtered supernatant was loaded onto a Talon (Clontech) column, washed with buffer A and eluted with buffer A + 500 mM imidazole. His-tagged TEV protease was used to cleave the tag for 2 hours at room temperature and dialyzed against buffer A + 10 mM β-mercaptoethanol (βME). To remove the tag and TEV protease, the protein solution was re-run on talon resin. For GCN4LI-HCN2J, the tags were not removed, and run on an amylose column instead. The flow-through was collected, dialyzed against 10 mM KCl, 20 mM MES (pH 6.0) + 10 mM βME (buffer C) and further purified on a Resource S column (GE Healthcare) in buffer C with a linear gradient to 40% buffer D (1 M KCl, 10 mM MES (pH 6.0) + 10 mM βME) over 40 column volumes. In the case of the GCN4LI-HCN2J, a Resource Q column (GE Healthcare) was used with 10 mM KCl + 10 mM Tris (pH 8.0) with a linear gradient to 40% of 1 M KCl + 10 mM Tris (pH 8.0) over 40 column volumes. Protein purity was confirmed by SDS-PAGE and MALDI-TOF Mass Spectroscopy. Homogeneity of the protein was confirmed on HiLoad Superdex 200 (GE Healthcare) (Figure A.1).

Direct measurements of cyclic nucleotide binding by isothermal titration calorimetry (ITC)

Samples were concentrated and dialyzed against 150 mM KCl, 10 mM HEPES (pH 7.4) + 10 mM βME overnight at 4°C. Titrations were performed in an ITC200 (GE Healthcare) at 25°C or 10°C, using a concentration of cAMP that was 10X that for the HCN protein and 1 µl injections over 40 injections. Protein concentrations were determined by absorption at 280 nm
under denaturing conditions and calculated extinction coefficients. The binding isotherms were analyzed using single or two independent site binding models implemented in MicroCal Origin 7.0.

Binding isotherms are described by the following equation $c = K_a[M]^n$, where $K_a$ is the association constant, $[M]$ is the total protein concentration in the cell, and $N$ is the stoichiometry of interaction. The value for “c”, a unitless parameter, was between 1 and 1000 for all of the data used for this study, which ensures an accurate fitting.

Dynamic light scattering (DLS)

Samples were centrifuged for 10 minutes to remove dust particles. DLS measurements were performed on a DynaPro Plate Reader (Wyatt Technology). Acquisition was performed at 25ºC with Dynamics 7.0 with 5 scans of 5 seconds each. Analysis was performed using Dynamic 7.0 and Origin 7.0. Values for molecular weight and polydispersity were obtained by DLS. Molecular weights of oligomeric forms of protein were easily separated from aggregated protein, which was identified by its relatively immense value.

Results

*Cyclic AMP binds to the cyclic nucleotide binding domain of the HCN2 C-terminus with apparent high and low affinity*

We examined the binding of cAMP to the isolated C-terminus of the HCN2 channel, a region corresponding to the first six $\alpha$-helices of the C-linker, and the three $\alpha$-helices and eight $\beta$-barrel strands that constitute the CNBD. Examination of this region alone, which we refer to as HCN2J from the initial study of this region (Zagotta et al., 2003), allows us to isolate the
actual binding event, which is not possible in full-length channels. Although inherently limited because of the absence of the transmembrane and N-terminal regions of the channel, use of the C-terminal fragment also avoids reciprocal interactions between the C-terminus and transmembrane portions of the full-length channel that could influence binding. Ample quantities of HCN2J were obtained and binding of cAMP to HCN2J was assessed by ITC.

Adding cAMP to purified HCN2J at 100 or 200 µM produced two clear transitions, which could readily be fit with a two independent binding site model (Figure 2.1A) and yielded binding affinities of approximately 1 and 0.1 µM (Figure 2.1B). This model gave a better fit than the sequential model for four binding sites, also implemented in MicroCal Origin 7.0, in which the initial binding of the ligand to one site affects the affinity of the others. These two models, as applied in Origin, are described in detail in appendix A, along with examples of how each model fit the data (Figure A.2) and a state diagram illustrating a possible molecular interpretation for the negative cooperativity (Figure A.3).

The energetics of binding was different for each binding event (Figure 2.1C); the apparent low affinity binding event was driven by favourable enthalpy and unfavourable entropy, whereas both were favourable for the apparent high affinity binding event. Thus, the biphasic pattern is due to release of heat specific to each of two binding events, both of which are exothermic, but proceed and saturate over different and well-separated ranges of cAMP concentration. The lower affinity binding event occurs with a more negative enthalpy, resulting in a falling phase. The rising phase that follows occurs as both sets of binding sites saturate and the amount of heat released decreases.

The stoichiometry of binding, which is the ratio of cAMP to individual HCN2J subunits, was different between binding events, with values of ~0.25 and ~0.75 (Table A.1) for the high
and low affinity events, respectively. As these values sum up to an overall stoichiometry of ~1, this suggests that both binding events occur to the same site with altered geometries.

For the low affinity binding event, the values for $c$ are ~114, and the values for $\Delta H$ are >15 kcal/mol, within the optimal experimental guidelines suggested by Wiseman et al. (1989) (Wiseman et al., 1989). However, for the high affinity binding event, the values for $c$ are high (>400), and the values for $\Delta H$ are <6 kcal/mol. To examine the profile of the heat changes due to only the high affinity binding event, the $\mu$cal/mol versus molar ratio curves were simulated with the experimental data obtained for only this event. These simulated curves show that the decrease in heat over time was comparatively steep. Thus although the data are within the acceptable range of $c$-values and the fit to the two independent binding site model is good, it is probable that the value obtained from the fitting for the high affinity binding is not as accurate as that for the low affinity binding event (Leavitt and Freire, 2001).

Lowering temperature (to 10°C from 25°C) still resulted in two transitions and affinities much like those at the higher temperature, although the total amount of heat released was considerably smaller and the double transition is less pronounced (Figure A.4, Table A.1). To determine whether both events reflected binding to the CNBD, we introduced the R591E mutation, which is located in the highly conserved phosphate binding cassette and coordinates the cAMP phosphate group (Zagotta et al., 2003; Chen et al., 2001; Tibbs et al., 1998; Ulens and Siegelbaum, 2003). This mutation eliminated both binding events, consistent with two transitions resulting from binding of cAMP to the known CNBD (Figure 2.1D).
Cyclic AMP binds to HCN2J with an apparent high and low affinity

**Figure 2.1** Cyclic AMP binds to HCN2J with an apparent high and low affinity

A) Plots of heat produced and measured by ITC upon progressive injections of cAMP into 200 µM HCN2J. In the upper plot, the broadening of peaks causes an initial increase in the overall heat per injection. The solid line through the values of the lower plot represents a two independent site binding model, which yielded values for affinity and energetics (ΔG, ΔH and ΔS) (Table A.1). B) Bar graph comparing high and low cAMP binding affinity as determined from the fit in ‘A’. Error bars indicate S.D. C) Bar graph comparing energetics between the low and high affinity binding events, determined from the fit in ‘A’. Error bars indicate S.D. D) Plots of heat produced and measured by ITC upon progressive injections of cAMP into 200 µM HCN2J-R591E.
High affinity binding of cAMP occurs in concert with tetrameric forms of HCN2J

One of the two binding events could have been due to promotion of self-association of HCN2J by cAMP binding to form dimers and tetramers, which has been shown previously by analytical ultracentrifugation (Zagotta et al., 2003; Zhou et al., 2004). To minimize the extent of HCN2J self-association, ITC measurements were repeated using lower concentrations of protein. Because self-association requires the C-linker, ITC measurements were also carried out using HCN2J lacking the first two α-helices of this region (A’, B’) (ΔAB-HCN2J). Indeed, cAMP addition to lower concentrations of HCN2J and to ΔAB-HCN2J resulted in only the lower affinity binding event (Figure 2A,B), both with a stoichiometry of one (Table A.1), suggesting that self-association was necessary for the appearance of a high affinity binding event. Although ΔH values were <6 kcal/mol for the single binding event of 10 and 25 µM HCN2J, the c-values obtained were ~8 and ~19, respectively, which are within optimal range.

To approximate the extent of self-association and assess its contribution to the high affinity binding event, DLS of HCN2J at a range of concentrations, and ΔAB-HCN2J (200 µM), was performed with or without a saturating concentration of cAMP (Figure 2.2C). The apparent molecular weight obtained for ΔAB-HCN2J was ~26 kDa, close to the calculated weight of the monomer (22.4 kDa); this weight was unaffected by cAMP, consistent with the lack of cAMP-induced self-association. At the lowest concentrations of protein used (10 µM), the apparent molecular weight of HCN2J was also ~26 kDa, suggesting the protein was monomeric.

However, the apparent weight of HCN2J increased with increasing concentrations of protein, consistent with the appearance of larger forms. This interpretation is supported by values for polydispersity, the standard deviation of the distribution of molecular weights obtained by DLS. Values for polydispersity of greater than 30% are generally considered to indicate
populations of particles that are of significantly different sizes, or to be polydisperse, with a molecular weight corresponding to the peak of the distribution (Habel et al., 2001; Zulauf and D'Arcy, 1992). At lowest concentration of HCN2J and for ΔAB-HCN2J, polydispersity was <15%, which, along with the apparent molecular weight, is consistent with very small contributions from oligomeric forms of the protein. For intermediate protein concentrations, polydispersity values were ~27.5%, suggesting small but more significant contributions from multiple oligomeric forms. This is reflected in the gradual increase in apparent molecular weights ranging from ~42 to 59 kDa for 25, 50 and 100 µM HCN2J. At the highest concentration of HCN2J (200 µM), the apparent weight is about 83 kDa, close to the 89.6 kDa as predicted for the tetramer; this, along with a polydispersity value of ~24%, supports the predominance of the tetrameric form at the highest concentration. The higher HCN2J concentrations are likely a better reflection of its effective concentration when attached to the intact tetrameric channel at the plasma membrane.

Notably, the apparent weight was increased with cAMP at all concentrations of HCN2J except for the highest concentration; its effect was largest at the lowest concentration of HCN2J and progressively decreased as the protein concentration increased. In cAMP, values for polydispersity remained below 30% at all concentrations of HCN2J. At 200 µM, the absence of an effect of cAMP on the molecular weight of HCN2J is consistent with the predominance of tetramers. Thus, high affinity binding is apparent mainly when HCN2J is already a tetramer and is not due to self-association in itself. Together with the stoichiometry of cAMP binding to HCN2J at higher protein concentrations, these data suggest an inherent negative cooperativity, whereby cAMP binds initially to one subunit of the tetramer with high affinity and subsequently to the remaining three subunits with lower affinity. The stoichiometries of the two binding events
are calculated in relation to the concentration of individual HCN subunits, thus their summed value of one is consistent with negative cooperativity, in which one cAMP binds with high affinity and the remaining three cAMP molecules bind with low affinity.
Figure 2.2 Low affinity binding of cAMP remains at low HCN2J concentrations and when self-association is eliminated

A) Plots of heat produced and measured by ITC upon progressive injections of cAMP into indicated concentrations of HCN2J and 200 µM ΔAB-HCN2J, as indicated. The solid line through the values of the lower plots represents a single binding model for all of the above except for 100 µM HCN2J, which was fit with a two-site binding model. The fits yielded values for affinity and energetics (ΔG, ΔH and ΔS) (Table A.1). Note that along with the experiment using 10 µM HCN2J on the far left, data is plotted from an experiment in which cAMP was titrated into buffer lacking HCN protein (grey trace in upper plot and grey points in lower plot); no discernible heat is released when cAMP is added to buffer alone. B) Bar graph comparing affinities among the single transitions determined from the fits in ‘A’ and the lower affinity determined from the two site binding model fit for 100 µM HCN2J in ‘A’. Error bars indicate S.D. C) Plot of apparent molecular weight versus concentration of HCN2J with or without a saturating concentration of cAMP (2 mM), as indicated, determined by DLS. At 200 µM, open (-cAMP) and solid (+cAMP) circles overlap. The molecular weight of 200 µM ΔAB-HCN2J, with or without cAMP, is also indicated in the plot. Error bars indicate S.D.
Low affinity binding of cAMP remains when the C-terminus is constrained as a tetramer

To further examine binding of cAMP to the HCN2J tetramer, we fused it to the C-terminus of GCN4L1, a protein known to form a tetrameric coiled-coil (Harbury et al., 1993), and confirmed it was a tetramer by gel filtration chromatography (Figure 2.3D). As pre-tetramerization reduces the amount of tag-free protein after cleavage with TEV protease, and hence the yield, we kept the HMT tag attached for both constrained and unconstrained forms of the HCN2J tetramer.

Unlike the unconstrained HCN2J tetramer, titration of cAMP with the constrained HCN2J tetramer revealed only a single binding transition (Figure 2.3A). The possibility thus exists that the HCN2J fragments were unable to form a tetramer. However, the binding is characterized by a low binding affinity and energetic signature that were identical to those of the low affinity binding event in the unconstrained HCN2J tetramer (Figure 2.3B,C). The binding affinity of both the constrained and unconstrained tetramer were similar to the single binding event determined for the monomeric ΔAB-HCN2J, but the energetics were different; the absolute values of entropy and enthalpy were much smaller after deletion (Figure 2.3C). Thus, the CNBDs are able to make tetrameric contacts, and the changes induced by high affinity binding of cAMP in the unconstrained HCN2J tetramer have probably already taken place in the constrained tetramer.
Figure 2.3 Only the low affinity binding transition is observed in a constrained HCN2J tetramer

A) Plots of heat produced and measured by ITC upon progressive injections of cAMP into 200 µM of HMT-HCN2J or HMT-GCN4LI-HCN2J, as indicated. The solid line through the values of the lower plots represents a two-site binding model and a single binding site model for HMT-HCN2J or HMT-GCN4LI-HCN2J, respectively. This fit yielded values for affinity and energetics (ΔG, ΔH and ΔS). For the high affinity binding event in HMT-HCN2J, the c-values were ~ 200. B) Bar graph comparing affinities between the single binding transition of HMT-GCN4LI-HCN2J and the lower affinity transition determined from the two site binding model fit for HMT-HCN2J, in ‘A’. Error bars indicate S.D. C) Bar graph comparing the energetics determined from the single binding transition of HMT-GCN4LI-HCN2J and of ΔAB-HCN2J, and from the lower affinity transition of HMT-HCN2J. Error bars indicated S.D. D) Size exclusion column profile of HMT-GCN4LI-HCN2J (black) and HMT-HCN2J (grey). mAU, milliabsorbance units.
Cyclic AMP elicits only a single low affinity binding transition in the HCN1 isoform

HCN2 and HCN4 channel opening is strongly facilitated by cAMP whereas that of HCN1 and HCN3 is only weakly affected or not affected at all, respectively (Wainger et al., 2001; Mistrik, 2005). We therefore wondered whether the conformational changes in HCN2J, as seen in the double transitions of cAMP binding, might reflect the stronger effect of cAMP. Therefore, cAMP binding to purified versions HCN1 and HCN4 C-termini was next examined. For HCN4J and ΔAB-HCN4J, the biphasic heat signature, apparent binding affinities of cAMP, and associated energetics were similar to those for HCN2J (Figure A.3).

In contrast, for HCN1J, only a single transition was observed upon cAMP binding in both low and high concentrations (Figure 2.4A), with values of binding affinity and energetics (Figure 2.4B,C) that were similar to those for the single binding event in the constrained HCN2J and for the low affinity binding event of the unconstrained HCN2J tetramer. Notably, the energetics of binding for HCN1J was different from those of ΔAB-HCN1J. Although both exhibit a single binding transition of low affinity and similar free energy of binding, the absolute values for both enthalpy and entropy were larger, paralleling the differences in energetics noted between tetrameric HCN2J and ΔAB-HCN2J.

Dynamic light scattering across a range of HCN1J concentrations (Figure 2.4D), under the same conditions as those utilized for ITC, showed that, as for HCN2J, the apparent molecular weight increased with increasing concentrations, consistent with the appearance of larger forms. However, in the absence of cAMP, the apparent weights for HCN1J were larger than those for HCN2J at intermediate and high concentrations of protein, suggesting that self-association and tetramer formation occurred to a greater extent under these conditions. Furthermore, the
molecular weight of HCN1J at all concentrations tested, was unaffected by saturating levels of cAMP, suggesting the cAMP is unable to induce significant conformational changes.

The value of stoichiometry for the single binding event of HCN1J is ~0.37 and ~0.68, at 200 µM and 25 µM, respectively; that for ΔAB-HCN1J is ~0.88 (Table A.1). While a stoichiometry near one would be expected for a single binding event, the lower values probably reflect an underestimation of concentration that results from non-specific aggregation that renders the cAMP binding site inaccessible. Nevertheless, the molecular weights for HCN1J constructs are reliable as they were obtained from DLS experiments in which polydispersity values for the non-aggregated portions were ≤ 31%. Together, the data suggest that the changes induced by high affinity binding of cAMP in the unconstrained HCN2J tetramer have already taken place in the HCN1 C-terminus.
Figure 2.4 Cyclic AMP binds to HCN1J similar to the constrained HCN2J tetramer

A) Plots of heat produced and measured by ITC upon progressive injections of cAMP into indicated concentrations of HCN1J and 200 µM ΔAB-HCN1J. The solid line through the values of the lower plots represents a single binding model for all of the plots above, which yielded values for affinity and energetics (ΔG, ΔH and ΔS). B) Bar graph comparing affinities among single transitions determined from the fits in ‘A’. Error bars indicate S.D. C) Bar graph comparing energetics among the low affinity binding events for indicated concentrations of HCN1 and ΔAB-HCN1J, determined from the fit in ‘A’. Error bars indicate S.D. D) Plot of apparent molecular weight, determined by DLS, versus concentration of HCN1J, with or without a saturating concentration of cAMP (2 mM), as indicated. Values obtained under the identical conditions for HCN2J, from Figure 2.2C, are re-plotted in open (- cAMP) and solid (+ cAMP) squares. Error bars indicated S.D.
Discussion

In this chapter, I show that cAMP binding to HCN channels can be assessed in detail using the isolated C-terminus and interpreted in the absence of downstream changes in the transmembrane segments associated with channel gating. Cyclic AMP bound to one subunit of tetrameric HCN2 with high affinity, and subsequently to the remaining three subunits with lower affinity. The greater similarity of energetics of the single binding event of the constrained HCN2J tetramer to those of the low affinity binding event of the unconstrained HCN2J tetramer, than to those of the HCN2J monomer (ΔAB-HCN2J), suggests that the changes induced by high affinity binding of cAMP in the unconstrained HCN2J tetramer have already taken place in the constrained tetramer.

Cyclic AMP binds to HCN1, HCN2, and HCN4 with similar low affinity, but we found no evidence for a high affinity binding transition in HCN1. Thus, as for the constrained HCN2J tetramer, our data suggest that changes induced by the high affinity binding of cAMP have already occurred in the HCN1 C-terminus. This “pre-activation” of HCN1 explains both the small effect of cAMP on facilitation of its opening and the opening of the unliganded channel, which occurs as though it has already been disinhibited by the C-terminus (Wainger et al., 2001; Wang et al., 2001).

Two recent studies found that binding of cAMP to the HCN C-terminus produced only a single transition when examined by ITC, with values very close to the low affinity binding values obtained in our study. In one study (Xu et al., 2010), the amount of HCN4 protein utilized was 75 µM, suggesting the proportion of tetramers was low; this would explain the absence of a high affinity binding transition. In another study (Wu et al., 2011), the amount of HCN2 protein used was not reported. Temperature was also not reported in those studies. We found that
lowering temperature from 25°C to 10°C reduced the amount of heat released considerably, although two transitions were still resolved provided that high concentrations were used. Nevertheless, reduced heat at lower temperatures could make it more difficult to resolve the transitions, particularly at lower concentrations at which tetramer formation is already low.

The biphasic pattern of heat released when cAMP was added to HCN2J was fit very well using a two independent site model, with three parameters per binding each of two sites, (K_a, ΔH and N), and not very well using a sequential model, with two free parameters (K_a and ΔH) for each of four binding sites. The comparatively better fit for the two independent binding site model was probably due to the availability of the parameter N for each site of the two sites, which was free to adjust independently during the fit. The stoichiometry obtained from fitting the data to the two independent binding site model was close to 1:3, high affinity relative to low affinity.

A possible interpretation for the negative cooperativity inferred by the two independent binding site models is offered in Figure A.3. Binding of cAMP to the unoccupied HCN2J tetramer changes the conformation of one subunit, resulting in subunit interfaces with increased stability. This hampers the conformational changes of the other subunits upon binding cAMP, resulting in an observed negative cooperativity (a sequential model (Koshland, 1996)). An alternative mechanism would involve one high affinity site and three low affinity sites that “pre-exist” within a tetramer. However, such asymmetry is not supported by the structures of the holo and apo C-terminus obtained by x-ray diffraction, which are 4-fold symmetrical tetramers (Zagotta et al., 2003; Taraska et al., 2009).

Our data support functional assays (Wu et al., 2011; Kusch et al., 2010) and modelling (Difrancesco, 1999; Chen et al., 2007) suggesting that HCN2 can switch between states of low
(~1-2 μM) and high (sub-micromolar) cAMP affinity, but these studies suggest that this switch occurs when the channel goes from closed to open. We show that a transition from high to low affinity can also be induced solely by cAMP binding to the C-terminus without influence from the transmembrane segments. A similar switch has been described in the catabolic activating peptide (CAP) dimer, a structurally related cAMP-binding protein in which ITC measurements have shown that binding of the second cAMP molecule occurs with lower affinity but more favourable enthalpy than binding of the first molecule (Popovych et al., 2006); thus, for both CAP and the HCN2 C-terminus, the thermodynamic basis for negative cooperativity is completely entropic. It remains to be seen how this entropic C-terminal switch, and resulting changes in conformation and/or dynamics interact with the transmembrane elements to facilitate HCN opening.
Chapter 3: Inhibition of C-terminus oligomerization and negative cooperative binding of cAMP by an epilepsy-associated mutation in the HCN2 channel

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Introduction

As mentioned in chapter 1, HCN2 is found in the central nervous system and controls neuronal excitability and rhythmicity (Wahl-Schott and Biel, 2008; Robinson and Siegelbaum, 2003). Consistent with its role in key areas of the brain, HCN2 dysfunction has been proposed to contribute to pathological conditions such as epilepsy (Bender and Baram, 2008; Dubé et al., 2009; Ludwig et al., 2003; Tang et al., 2008; Reid et al., 2009). Most recently, the first epilepsy-associated mutation in human HCN2 was reported and found in the C-linker region of the C-terminus that physically connects the more distal CNBD with the inner gate of the pore (DiFrancesco et al., 2011). Hyperpolarization-induced opening of this mutant HCN2 channel is strongly inhibited when compared to opening of the wild type HCN2 channel, but the molecular mechanism underlying this functional defect is not known.

The HCN2 C-termini interact to form a symmetrical tetramer that is thought to hang below the channel pore (Zagotta et al., 2003). Binding of cAMP to the CNBD of the C-terminus has been suggested to promote interactions among C-termini to ultimately form a gating ring that disinhibits channel opening (Zagotta et al., 2003; Ulens and Siegelbaum, 2003; Zhou and Siegelbaum, 2007; Wainger et al., 2001; Taraska et al., 2009). Evidence for the formation of a gating ring comes from studies of the purified HCN2 C-terminus using analytical ultracentrifugation showing that the proportion of tetramers increases in the presence of cAMP (Zagotta et al., 2003). The correspondence between facilitation of opening and the degree of in vitro tetramer formation has been noted and compared between HCN1 and HCN2 channels, i.e. in the absence of cAMP, the C-termini of HCN2 form tetramers less efficiently and opening of this isoform is comparatively more inhibited (Wainger et al., 2001; Lolicato et al., 2011).
Several pieces of evidence point to the C-linker as important for gating ring formation. In the resolved tetrameric structure of the HCN2 C-terminus, the C-linker is responsible for much of the contact that is made among the subunits (Zagotta et al., 2003). The C-linker contributes significantly to the differences in the degree of inhibition of channel opening between HCN1 and HCN2 (Wang et al., 2001). In chapter 2, we found that oligomerization, along with negatively cooperative binding of cAMP, was eliminated when the first two helices of the C-linker were removed from either the HCN1 or HCN2 C-terminus. Breaking either of two putative salt bridges identified in the crystal structure of the HCN2 C-terminus between the C-linker and the C-linker and CNBD of the adjacent subunit surprisingly facilitated channel opening. This suggests that the C-linker can exist in both a resting or active conformation (Craven et al., 2008; Craven and Zagotta, 2004). Finally, experiments using transition ion metal FRET have shown that the HCN2 C-linker moves relative to the CNBD when cAMP binds (Taraska et al., 2009).

According to the proposed model of HCN2 opening (refer to figure 1.5), the epilepsy mutation may disrupt intersubunit interactions favouring the resting conformation of the C-linker and reducing the stability of a gating ring, and thus inhibit opening. Here, we solved the structure of the mutant HCN2 C-terminus and assessed its ability to oligomerize. The resolved crystal structure shows modified interactions in and around the mutation, consistent with an impact on tetramer formation. We also found that the mutant C-terminus displayed a reduced ability to oligomerize in solution, suggesting that the inhibition of channel opening is due to a gating ring that is less stably formed. Finally, negatively cooperative binding of cAMP is also eliminated by the epilepsy mutation, consistent with our previous suggestion that intersubunit interactions mediated by the HCN2 C-linker control both the pattern of binding and channel opening.
**Experimental approach**

**HCN mutagenesis and protein purification**

Using Quikchange protocol (Stratagene) mutation E488K and E488A were inserted into the mouse HCN2J (residues 443-645) construct cloned into the modified pET28 vector (Novagen) containing an His$_6$, maltose-binding protein tag and a cleavage site for the tobacco etch virus protease denoted as an HMT tag. Residue E488 is the mouse equivalent to residue E515 in the human isoform. They were then expressed in *Escherichia coli* Rosetta (DE3) pLacI, grown and purified as mentioned in chapter 2. Homogeneity of the protein was confirmed on HiLoad Superdex 200 (GE Healthcare).

**Crystallization, data collection and structure determination**

Before crystallization protein was exchanged with 50 mM KCl, 10 mM HEPES (pH 7.4) + 10 mM dithiothreitol (DTT) and concentrated to 10 mg/mL using a 10,000 MWCO concentrator (Amicon, Millipore). Crystals were obtained using the hanging-drop method at 4°C. 1 µL of protein solution was mixed with 1 µL of reservoir solution of 200 mM NaCl, 0.1 mM citric acid and 15% PEG 400. Crystals were cryoprotected in reservoir solution supplemented with either 20% glycerol or 20% glucose and flash frozen in liquid nitrogen. Diffraction data sets were collected at the Advance Photon Source (APS) beamline 23-ID-D-GM/CA. Data was processed with XDS, then refined using REFMAC5 with TLS restraints and successive rounds of manual modeling in COOT. Data collection and refinement statistics are available in (Table 3.1). Analysis of interactions between intersubunit interfaces was done by PDBePISA (http://www.ebi.ac.uk/msd-srv/prot_int/).
Direct measurements of cyclic nucleotide binding by isothermal titration calorimetry (ITC)

Samples were concentrated and dialyzed against 150 mM KCl, 10 mM HEPES (pH 7.4) + 10 mM βME overnight at 4°C. Titrations were performed in an ITC200 (GE Healthcare) at 25°C, using a concentration of cAMP that was 10X that for the HCN protein and 1 µl injections. Protein concentrations were determined by absorption at 280 nm under denaturing conditions and calculated extinction coefficients. The binding isotherms were analyzed using single site binding models implemented in MicroCal Origin 7.0.

Dynamic light scattering (DLS)

Samples were centrifuged for 10 minutes to remove dust particles. DLS measurements were performed on a DynaPro Plate Reader (Wyatt Technology). Acquisition was performed at 25°C with Dynamics 7.0 with 5 scans of 5 seconds each. Analysis was performed using Dynamic 7.0 and Origin 7.0. Values for molecular weight and polydispersity were obtained by DLS. Molecular weights of oligomeric forms of protein were easily separated from aggregated protein, which was identified by its relatively immense value.

Results

Structure of the epilepsy mutant

To visualize any changes that might be induced by substitution of glutamate, E488, in the C’-helix of the C-linker with lysine, K, we first solved the crystal structure of E488K-HCN2J (Figure 3.1A,C). Upon initial crystallization and structure determination of E488K-HCN2J with cAMP, we observed two electron densities around residue 488 which were not observed in the wild type structure (Zagotta et al., 2003). These two electron densities were found to best-fit two
glycerol molecules as 20% glycerol was used as a cryoprotectant (Figure A.6A,B). To confirm that these electron densities were glycerol, we re-crystallized E488K-HCN2J using 20% glucose as a cryoprotectant and no electron densities were found around residue 488. The absence of electron densities when using glucose confirmed that they were glycerol molecules. Since there was no difference between the crystal structure with glycerol or glucose (r.m.s.d. 0.3 Å) the crystal structure with glycerol was chosen to be further refined because of its higher resolution of 2.35 Å as compared to 2.70 Å with glucose.

The refined structure of E488K-HCN2J showed that it formed a tetramer similar to that of wild type HCN2J when fully-liganded by cAMP (r.m.s.d. 0.6 Å) (Figure 3.1A,B), however lysine substitution modified some interactions between adjacent C-linkers. Three new hydrogen bonds were observed between K452 in the A’-helix with S490 in the adjacent C’-helix, as well as between Q458 in the A’-helix and S444 and S445 in the adjacent A’-helix (Figure 3.1D). Hydrogen bonds between adjacent A’ helices were not seen in the wild type structure. A new salt bridge was also formed between K452 and E494 in adjacent C’-helices.

Because the solved structure of the wild type is thought to represent the resting conformation of the C-linker (Craven et al., 2008; Craven and Zagotta, 2004), modified interactions noted among mutant subunits suggest that the strength with which they interact in the resting state is modified.
Figure 3.1 *Crystal structure of E488K-HCN2J in the presence of cAMP.*

A) Ribbon representation of a single subunit of E488K-HCN2J. B) Overlay of HCN2J (light grey) and E488K-HCN2J (red) C) Ribbon representation of the tetrameric assembly of E488K-HCN2J perpendicular (left) and parallel (right) to the four fold axis. D) Close-up view of HCN2J (left) and E488K-HCN2J (right) showing the region around residue 488. Hydrogen bonds are shown in black and salt bridges are shown in green. Helices A’ and B’ are shown in red and helices C’ and D’ of neighbouring subunit in blue.
<table>
<thead>
<tr>
<th><strong>Data Collection</strong></th>
<th>E488K-HCN2J</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Space Group</strong></td>
<td>P42,2</td>
</tr>
<tr>
<td><strong>Cell parameters (Å,°)</strong></td>
<td>a = b = 97.087, c = 46.243, α = β = γ = 90</td>
</tr>
<tr>
<td><strong>Resolution (Å)</strong></td>
<td>50-2.35 (2.41-2.35)</td>
</tr>
<tr>
<td><strong>R_sym (%)</strong></td>
<td>8.7 (94.9)</td>
</tr>
<tr>
<td><strong>I/σ (I)</strong></td>
<td>14.11 (2.13)</td>
</tr>
<tr>
<td><strong>Completeness (%)</strong></td>
<td>99.7 (100.0)</td>
</tr>
<tr>
<td><strong>No. of unique reflections</strong></td>
<td>9667 (700)</td>
</tr>
</tbody>
</table>

**Refinement**

| Resolution (Å) | 2.35 |
| No. reflections | 10327 |
| R_work / R_free | 0.1907 (0.2447) |

| **No. atoms** | |
| Protein       | 1633 |
| Ligand/ion    | 22   |
| Water         | 71   |

| **B - factors** | |
| Protein        | 37.476 |
| Ligand/ion     | 53.125 |
| Water          | 57.185 |

| **R.M.S. Deviations** | |
| Bond lengths (Å)     | 0.0081 |
| Bond angles (°)      | 1.1963 |

| **% Residues in core/allowed regions of Ramachandran plot** | 99.5/0.50 |

Table 3.1 Data collection and refinement statistics for E488K-HCN2J
The E488K-HCN2 epilepsy mutant C-terminus self-associates less efficiently than wild type HCN2 C-terminus

Modified interactions between subunits identified in the crystal structure of the resting mutant C-terminus suggest that oligomerization is modified. Because the propensity to oligomerize correlates with facilitation of opening, we suspected that oligomerization of the mutant channel would occur at higher concentrations compared to those required to promote oligomerization of the wild type C-terminus.

We examined the oligomeric status of the E488K-HCN2 as a function of protein concentration using DLS. The apparent weight of both the wild type and mutant HCN2 C-terminus increased with increasing concentrations, consistent with the appearance of larger oligomeric forms (Figure 3.2A). However, while the wild type protein is found mainly as a tetramer at 200 µM (based on the apparent weight), the mutant approached a molecular weight for a tetramer at higher concentration of protein, close to 400 µM.

To determine if charge reversal is responsible for reduced ability to self-associate, we mutated E488 to alanine, A. Like E448K-HCN2J, E488A-HCN2J must be present in comparatively higher concentrations than wild type HCN2J in order to form oligomers in solution (Figure 3.2B). The similarity in concentration-dependence of oligomerization between of E488K and E488A indicates that the loss of E, rather than the introduction of K, leads to impaired oligomerization.

Unlike that of wild type HCN2 C-terminus, the apparent molecular weight of E488K-HCN2J and E488A-HCN2J at lower protein concentrations did not increase in the presence of cAMP, suggesting that oligomerization of the mutant was not affected by this cyclic nucleotide.
Figure 3.2 Self-association is eliminated by substitution of E488 with K or A

A) Plots of apparent molecular weight versus concentration of HCN2J (squares) and E488K-HCN2J (circles) with (black) or without cAMP (white). Error bars indicate S.E. B) Plots of apparent molecular weight versus concentration of HCN2J (squares) and E488A-HCN2J (triangles) with (black) or without cAMP (white). Error bars indicate S.E.

Cyclic AMP binding does not occur with negative cooperativity in E488K-HCN2

The inability of cAMP to promote oligomerization of E488K-HCN2J led us to examine the ability of this cyclic nucleotide to bind to the tetrameric forms of this mutant. The addition of cAMP to 400 µM of E488K-HCN2J elicited a pattern of heat production that was fit with a single binding site model, as opposed to a more complex pattern observed with wild type HCN2 C-terminus that was fit with a two site model (refer to Figure 2.1A). Fitting the heat pattern of the E488K-HCN2 with a one binding site model yielding a binding affinity of ~ 2.5 µM (Figure 3.3A,B); a high binding affinity site was no longer apparent.

Curiously, the energetics of binding for the single cAMP binding event in E488K-HCN2J was not similar to those of the low affinity binding event for HCN2J, but was reminiscent of cAMP binding to the monomeric form of HCN2J that lacks the first two α-helices of the C-linker (Table 3.2). This suggests that the binding sites of the individual C-termini within the tetramer
behave more like their monomeric counterparts (refer to Figure 2.3C), as though intersubunit cooperativity is removed (Figure 3.3C). Similar to E488K-HCN2J, negative cooperativity upon cAMP binding was not observed. Heat patterns were fit to a single binding site model with no high affinity binding apparent. Energetics also reflected that of the monomeric counterpart of HCN2J (Figure 3.3B,C).

Together, these data suggest that the mutation inhibits interactions that promote alterations in binding site associated with negative cooperative binding, as well as formation of a gating ring.
Figure 3.3 Negative cooperativity is eliminated by substitution of E488 with K or A

A) Plots of heat produced upon progressive injections of cAMP into E488K-HCN2J (400 µM) (left) and E488A-HCN2J (400 µM) (right). The solid line through the values of the lower plots represents a single binding model. The fits yielded values for affinity and energetics (ΔG, ΔH, and -TΔS) (Table 3.2). B) Bar graph comparing affinities among the single transitions determined from the fits in B. Error bars indicate S.D. C) Bar graph comparing the energetics of E488K-HCN2J and E488A-HCN2J determined from fitting the data in ‘A’ with a single binding equation. Error bars indicate S.D.
Table 3.2 Thermodynamic parameters for cAMP interactions with E488K-HCN2J and E488A-HCNJ

<table>
<thead>
<tr>
<th></th>
<th>$K_d$ (µM)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>-T$\Delta S$ (kcal/mol)</th>
<th>$\Delta G$ (kcal/mol)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>E488K-HCN2J</td>
<td>2.56 ± 0.08</td>
<td>-7.60 ± 0.08</td>
<td>-0.03 ± 0.09</td>
<td>-7.63 ± 0.02</td>
<td>0.88 ± 0.09</td>
</tr>
<tr>
<td>E488A-HCN2J</td>
<td>10.29 ± 2.46</td>
<td>-6.45 ± 0.49</td>
<td>-0.41 ± 0.59</td>
<td>-6.86 ± 0.13</td>
<td>1.00 ± 0.13</td>
</tr>
</tbody>
</table>

Discussion

By inserting the epilepsy mutation into the isolated C-terminus of the HCN2 channel, we were able to assess in detail the effect of E488K on structure, oligomerization and cAMP binding. The resolved crystal structure did not dramatically differ from wild type, both of which demonstrate a C-linker region that is in its resting state. However, the ability of E488K-HCN2J to oligomerize is hindered as compared to wild type HCN2 C-terminus. These findings suggest that the mutant channel C-linker is less able to promote a C-terminal gating ring to facilitate opening of the HCN2 channel by hyperpolarization. This interpretation fits with the finding that the opening of the intact mutant HCN2 channel occurs at more negative voltages (DiFrancesco et al., 2011).

We also show that cAMP binds to the mutant C-terminus without negative cooperativity unlike when it binds to the wild type C-terminus. The energetics of cAMP binding to the mutant are reminiscent of cAMP binding to ΔAB-HCN2J, a form of the HCN2 C-terminus that lacks the A’ and B’ α-helices of the C-linker, remains monomeric even at high protein concentrations and shows no negative cooperativity upon cAMP binding. Together, these data suggest that intersubunit interactions mediated by the C-linker are important for both oligomerization and negative cooperative binding of cAMP, and are mainly eliminated by mutation of E488 to K or to A.
Although it is bound, cAMP did not promote oligomerization of E488K-HCN2J, unlike the wild type C-terminus, consistent with the importance of the C-linker in self-association. However, cAMP was shown to facilitate opening of both the full length E488K and wild type HCN2 channel, producing shifts in $V_{1/2}$ of $\sim 7$ mV in both cases (DiFrancesco et al., 2011). How can the inability of cAMP to promote oligomerization of the mutant C-terminus, and presumably gating ring formation, be reconciled with retention of its ability to fully facilitate opening of the intact channel? Binding of cAMP to the mutant channel may only produce modest oligomerization that is not discernible in our assay, but is still sufficient to facilitate opening of the intact channel. Also, gating ring formation may be promoted by cAMP more effectively when the C-terminus is attached to the pore of the channel; because the C-terminus tonically inhibits pore opening, pore opening might be expected to promote a gating ring (Craven and Zagotta, 2004). Alternatively, cAMP may produce some of its effects in a manner that is independent of a gating ring.

The absence of negative cooperativity in E488K-HCN2J suggests that the potency of cAMP on this mutant channel may be reduced, but this has not yet been examined. Our data also identify other sites in the C-linker that may contribute to intersubunit interactions, controlling both gating and potency of cAMP, which remain to be explored.
Chapter 4: Discussion
Cyclic AMP binds to the HCN2 channel with negative cooperativity

Previous methods of determining cAMP binding and cooperativity among subunits has been limited to functional studies such as concentration-response studies where increasing amounts of cAMP are applied until a maximal shift in the range over which the channel opens is reached. These amounts and their corresponding shifts in channel opening, $V_{1/2}$ values, are plotted then fitted to a Hill equation where an apparent binding affinity of cAMP, Hill coefficient (h) and half-effective concentration ($EC_{50}$) are determined. However, facilitation of HCN opening is a complex process where both binding and channel opening are tightly coupled and can contribute to $EC_{50}$ values. Binding affinity of cAMP itself has not been determined for HCN channels. Furthermore, cAMP facilitation is not equal between the four HCN isoforms and it is not clear to what extent cAMP binding affinity contributes to these differences. Are differences in binding affinity among cyclic nucleotides responsible for differences in potency? Do differences in binding affinity contribute to the differences in facilitation of opening among the HCN isoforms? In this thesis, the C-terminal fragment of mammalian HCN channel isoforms was isolated and cyclic nucleotide binding was directly assessed using isothermal titration calorimetry, ITC.

I found that at 200 µM of HCN2J, when the majority of the protein is in the tetrameric form, two binding events occurred: one reflecting a high binding affinity and the other a lower binding affinity. Stoichiometry values calculated from the ITC indicated cAMP bound to one subunit at high affinity, while the three other subunits bound with low affinity. From these results, I concluded cAMP binds to the CNBD of HCN2 in a negatively cooperative manner, the first time cooperativity in binding has been proposed for HCN channels.
My findings were surprising because previous studies on cooperativity of cAMP binding to HCN channels have been mixed. The first study of cyclic nucleotide binding to SAN HCN reported a concentration-response curve with a Hill coefficient of just under one (h=0.85), suggesting subtle negative cooperative binding. Concentration-response data for HCN2, expressed heterologously, fitted with the Hill equation yielded h-values greater than one (Wang et al., 2001; Chen et al., 2001) indicating positive cooperativity, while others reported a value less than one (Ulens and Siegelbaum, 2003; Kusch et al., 2010). Subsequently, Ulens et al. (2003) used tetrameric constructs in which the four cAMP binding sites were progressively removed by substitution of a key residue R591 in the phosphate binding pocket with E, R591E, to determine whether cAMP binding to each subunit was energetically equal. They found one cAMP molecule bound to HCN2 was sufficient to produce an increase in channel opening, but the facilitation was less than 25%, while binding of three cAMP molecules facilitated opening by less than 75% (Ulens and Siegelbaum, 2003). Cooperativity binding was not apparent from these studies. Due to the tightly coupled nature of cAMP binding and channel opening, Hill coefficients derived from fitting concentration-response curves are a less reliable measurement of cooperativity. It is only accurate in a simple sequential, that is, each binding site is the same or independent binding, which is not the case with HCN channels (Kusch et al., 2011; Weiss, 1997).

More recently Kusch et al. (2011) demonstrated a more complex pattern of cooperativity, using a combination of patch-clamp fluorimetry (with a fluorescent analogue of cAMP) and whole-cell electrophysiology to concurrently measure binding rates and rates of channel opening and closing, respectively. A global fit of a ten state allosteric model was used to define a pattern of cAMP binding to each subunit as negative-positive-negative; that is, the binding of the second
cAMP is facilitated by the binding of the first cAMP, however, the third cAMP binds unfavourably and the fourth binds again favourably. Overall, the values of affinity obtained by Kusch et al. (2011) are similar to those I found. Furthermore, the affinities they determined for binding to the first two sites are greater than the last two, with the largest change being a drop in affinity of the third binding site. Thus, I believe that my data and their data are not inconsistent, especially because their results come from a C-terminus that is attached to the pore; this makes their findings more realistic in that the pore may influence the conformation of the C-terminus and binding. However, their approach of combining binding rate data and opening and closing rate data with global fitting on a 10-state model is much more complex, making the resulting binding affinities more uncertain than my approach, which focuses only on binding.

**Negative cooperativity allows HCN2 channels to respond effectively to low intracellular concentrations of cAMP**

Negative cooperativity may be viewed as a way to maintain high sensitivity to a given ligand at low concentrations (Koshland, 2002). When viewed in this way, negative cooperativity allows HCN channels to have a greater impact at these lower concentrations. There is good evidence to suggest that basal cAMP levels in cardiomyocytes are high compared to the $K_d$ of the high affinity binding site and to the EC$_{50}$ determined from functional studies. Using FRET-based cAMP sensors, intracellular concentrations of 1-1.5 µM have been reported for mouse cardiomyocytes (Börner et al., 2011). In the SAN, the basal levels of cAMP are probably in the range of 0.2-0.5 µM based on a comparison of concentration-response curves of cAMP in isolated pacemaker cells of the SAN and of the ability of acetylcholine and isoproterenol to negatively and positively shift the HCN activation curve (Accili et al., 1997; Robinson et al.,
Together with my data showing that the values of $K_d$ for the high and low affinity sites of the HCN2 and HCN4 isoforms is in the range of 0.1 and 1 µM, respectively, it seems likely that the HCN channel of the SAN is at least partly bound to cAMP under basal conditions and that the greatest sensitivity of the channel to cAMP occurs at concentrations that are below its basal concentration.

There is evidence that the SAN HCN channel contributes mainly to basal heart rates. Mice overexpressing an HCN4 protein lacking the CNBD (Harzheim et al., 2008) demonstrate sinus bradycardia at rest, however sympathetic stimulation, which increases cAMP levels from basal levels, still increases heart rate, consistent with a contribution of HCN4 only to basal heart rate. There is also evidence to suggest that SAN HCN channels contribute to changes in heart rate when intracellular concentrations of cAMP are below basal levels. Difrancesco et al. (1989) found that at low nanomolar concentrations of ACh, 0.01 µM, $I_f$ is inhibited in isolated pacemaker cells and slows down the spontaneous rate (Difrancesco et al., 1989; Accili et al., 1997).

**Cooperativity in other cyclic nucleotide-gated channels**

Similar to HCN channels, cGMP binding in cyclic nucleotide-gated (CNG) channels is also coupled to channel opening. They have been shown to have both positive and negative cooperative binding. Cyclic GMP binds initially with a low affinity, 1 µM, which increases the open probability of the channel by a small amount. It is the binding of the second cGMP at 3.40 mM that causes the complete opening of the channel. The subsequent two cGMP bind with lower affinity, 1 µM, however are only required to stabilize the channel in the open conformation and to drive the binding equilibrium away from the second binding step (Biskup et al., 2007).
However, not all CNG channels exhibit cooperativity. Cyclic AMP binding to a bacterial form of a CNG channel called MlotiK1 from *Mesorhizobium loti* was non-cooperative. ITC $K_d$ values for cAMP binding to both the purified isolated, monomeric CNBD and the whole channel were similar ($107 \pm 11$ nM) (Cukkemane et al., 2007), suggesting binding was not affected by the addition of the rest of the channel to the CNBD. The crystal structure of the whole MlotiK1 channel also showed that the CNBD are spatially isolated from one another. This is thought to be due to the short C-linker that follows the S6 transmembrane segment (Chiu et al., 2007) and do not form intersubunit contacts or a gating ring as previously shown with HCN2 (Zagotta et al., 2003). These data indicate that the binding event is not influenced by intersubunit contacts and each binding site acts independently (Altieri et al., 2008; Biskup et al., 2007).

**The molecular basis of the comparatively smaller effect of cAMP on the HCN1 channel**

The opening of HCN1 and HCN3 isoforms are facilitated to a lesser extent or not at all, respectively, by cAMP, while the opening of HCN2 and HCN4 are facilitated more strongly by cAMP. Using dynamic light scattering, we found that the HCN1 C-terminus was better able to oligomerize in solution compared to the HCN2 C-terminus and that, unlike for the HCN2 C-terminus, cAMP had no effect on the ability of the HCN1 C-terminus to self-associate. Because the tendency to oligomerize correlates with facilitation of opening by the C-terminus, I suggested that the HCN1 channel functions as though already disinhibited, with a gating ring that is more stably formed in the absence of cAMP. A more stable gating ring in HCN1 explains its easier opening, the smaller effect of cAMP on opening and the absence of negative cooperativity upon cAMP binding.
Lolicato et al. (2011) also reported increased oligomerization of the HCN1 C-terminus using size exclusion chromatography and analytical ultracentrifugation. Without cAMP, HCN1 displayed a difference in oligomerization behaviour with a mixture of both tetramers and monomers, compared to HCN2 and HCN4, which only had monomers (Lolicato et al., 2011).

**Differences in oligomerization and binding between HCN1 and HCN2 channels reside in the C-linker**

Although there are some differences in primary structure between HCN1 and HCN2, there is reason to believe that the regions responsible for the differences in cooperativity and oligomerization reside in the C-linker. When cAMP was titrated into HCN2J fused with GCN4LI, a tetrameric domain, at the N-terminus of C-linker, the result was the same as HCN1, that is, binding reflected the low affinity binding event of cAMP binding to the HCN2J. Conversely, when the first two α-helices of the C-linker were removed from HCN2J, there was no cooperativity of cAMP binding and the subunits did not oligomerize.

Previous studies have pointed to the C-linker as the locus responsible for differences in gating. When the C-linker of the HCN2 channel was substituted for that of the HCN1 channel, voltage dependence positively shifted to that of the HCN1 channel. When the reverse was carried out, substituting the C-linker of the HCN1 channel for that of the HCN2 channel, the voltage dependence shifted in the negative direction. I interpreted these data to mean that, in the absence of cAMP, the C-terminus of the HCN1 channel is better able to oligomerize in solution, form a gating ring in the intact channel, and facilitate opening because of interactions mediated mainly by its C-linker.
When cAMP is applied, cAMP facilitation of channel opening reflected those of the exchanged HCN C-linker. For example, the shift in activation voltages of the HCN1 channel with the HCN2 C-linker was greater than wild type HCN1 (ΔV_{1/2} values 8.9 ± 0.4 mV and 4.4 ± 0.7 mV respectively) (Wang et al., 2001). Swapping the CNBD between HCN1 and HCN2 also produced a similar effect. However, exchanging both the C-linker and CNBD produced the maximum effect on cAMP facilitation on channel opening. Although this suggests that the C-linker is not enough to completely disinhibit the HCN channel, it plays an important role in voltage dependence of the HCN1 and HCN2 channel and is required to couple cAMP binding to channel opening (Wang et al., 2001).

**The C-linker controls oligomerization and cooperativity**

Based on the data presented in both chapter 2 and 3, HCN1 and HCN2 point to the C-linker as being an important determinant of both oligomerization of the C-terminus and negatively cooperative binding of cAMP. In chapter 3, I was able to identify a site in the C-linker that contributes to these processes by examining a mutation in this region of HCN2 that was identified in generalized idiopathic epilepsy (DiFrancesco et al., 2011). A glutamate is substituted for lysine (at position 488 in mouse HCN2, E488K), which negatively shifted the activation curve (DiFrancesco et al., 2011). I found that cAMP binds to E488K with a lower affinity and without negative cooperativity. Although at 400 µM the majority of the population of E488K is a tetramer, energetics reveal the characteristics of cAMP binding to the E488K HCN2 C-terminus is similar to that of the monomeric form of HCN2J, ΔAB-HCN2J. The similarity of energetics of cAMP binding between the mutant HCN2 C-terminus and the monomeric HCN2 C-terminus suggests a lack of intersubunit interaction in the former. Because
a high affinity binding site is absent in the mutant HCN2 C-terminus, I predict a lower potency of cAMP on the opening of the mutant channel, but this remains to be seen.

My results using DLS suggest that oligomerization of E488K-HCN2J was also inhibited. Reduced oligomerization suggests that the formation of a gating ring is reduced in the intact channel, which would explain inhibition channel opening by the epilepsy mutation (DiFrancesco et al., 2011).

DiFrancesco et al. (2011) found that the application of 10 µM cAMP positively shifted the activation curve to the same extent as in wild type HCN2. Curiously, my data showed oligomerization of the C-terminus in solution was not induced by addition of high amounts of cAMP. It may be that the total amount of C-terminus in the resting state is much larger for the mutant, thus binding of cAMP to the mutant channel may only produce modest oligomerization that is not discernible in my assay, but is still sufficient to facilitate opening of the intact channel (Figure 1.5). Alternatively, gating ring formation may be promoted by cAMP more effectively when the C-terminus is attached to the pore of the channel as the C-terminus tonically inhibits pore opening and may be required to promote a gating ring (Craven et al., 2008).

**The crystal structure of the E488K-HCN2 C-terminus reveals altered interactions**

The crystal structure of both the wild type and mutant HCN2 C-termini with cAMP, reveals the “elbow-on-shoulder” configuration, where the A’ and B’ α-helices (elbow) rests on the C’ and D’ α-helices of the neighbouring subunit (shoulder), as shown previously for the wild type (Zagotta et al., 2003). The introduced lysine residue modified some interactions between adjacent C-linkers.
Three new hydrogen bonds were observed between K452 in the A’-helix with S490 in the adjacent C’-helix as well as between Q458 in the A’-helix and S444 and S445 in the adjacent A’-helix (Figure 3.1D). Hydrogen bonds between adjacent A’ helices was not seen in the wild type structure. A new salt bridge was also formed between K452 and E494 in the adjacent C’-helix.

Because the solved structure of the wild type is thought to represent the resting conformation of the C-linker (Craven et al., 2008; Craven and Zagotta, 2004), modified interactions noted among mutant subunits support my finding that the strength with which these subunits interact is modified, as measured by a reduction in the ability of the mutant C-termini to oligomerize.

**Limitations to my experiments**

Because cAMP binding and channel opening are tightly coupled, the C-linker and CNBD were isolated to directly characterize the cAMP binding site without the interference from the transmembrane segments. However, by isolating the binding site we eliminate the contribution of the transmembrane domains on binding and can only infer what is happening at the transmembrane segment level of the protein upon cAMP binding. Using a combination of confocal patch-clamp microscopy and mathematical modeling, Kusch et al. (2011) were able to measure cAMP binding and its effect on channel opening and determine cAMP binds with a negative-positive-negative pattern. Overall, the effects observed by Kusch et al support those that are reported in my thesis, but suggest that the transmembrane segments have an influence on binding of cAMP.

Running ITC experiments also requires the protein to be soluble and to be purified at high concentrations. Sometimes this is not possible, especially when it comes to membrane
channel proteins like HCN channels that tend to be insoluble and require detergents to increase solubility. Fortunately, the C-linker and CNBD is a soluble portion of HCN channels and I was able to purify this portion at high concentrations. However, it is possible to measure cAMP binding on an entire channel. Cukkemane et al. (2007) successfully purified the entire MlotiK channel, a bacterial version of CNG channels, and measured cAMP binding. They found that cAMP binding to the whole channel and to the isolated CNBD portion had similar binding affinity (Cukkemane et al., 2007).

**Future studies**

*Is there a role for cAMP binding to the HCN3 channel isoform?*

Although HCN3 channel opening is not facilitated by cAMP, there is evidence that this isoform is able to bind cAMP. Replacing the HCN4 CNBD with that of HCN3 resulted in a cAMP gating effect that was HCN4-like (Stieber et al., 2005a). I would expect the oligomerization behaviour of HCN3 and binding of cAMP to be similar to that of HCN1, that is, the HCN3 C-terminus would have a greater tendency to oligomerize in solution, that cAMP would not promote oligomerization and that cAMP would bind as though negatively cooperative changes were already induced.

This makes one wonder if cAMP binding to homomeric HCN3 channels is important for reasons other than for modifying channel opening. For example, HCN3 could act as a local buffer regulating cAMP concentration in the cell. Because cAMP is such a widely used second messenger that directly or indirectly impacts all areas of the cell, cAMP must be tightly regulated. The cell does this by organizing and compartmentalizing adenylyl cyclase and phosphodiesterases into cAMP microdomains, such as lipid rafts, to protect the rest of the cell.
from cAMP hyperactivity (Lefkimmiatis et al., 2009; Willoughby and Cooper, 2007). Interestingly, pacemaker channels have also been reported to localize in lipid rafts (Barbuti et al., 2004). Lefkimmiatis et al. (2009) isolated the RIβ subunit of PKA, which binds to cAMP with high affinity, and tagged it with mCherry a red fluorescent protein for tracking purposes. To determine its “bufferability” cell lysates containing PKA-RIβ were immunoprecipitated using Sp-2-AEA-CAMPS (Sp-CAMPS) agarose beads and expression was verified using western blot. Using FRET-based imaging, this PKA-RIβ segment blocked agonist-induced cAMP increases in the cell (Lefkimmiatis et al., 2009). A similar approach could also be used to determine if HCN3 could also act as a cAMP buffer.

Is there a role for cGMP binding to HCN channels?

The main focus of this thesis was to characterize cAMP binding to HCN2. However, another endogenously found cyclic nucleotide of interest is cGMP, which has also been shown to fully facilitate HCN channel opening, but is at least ten times less potent than cAMP (Difrancesco and Tortora, 1991; Zagotta et al., 2003). Because ligand binding and channel opening are so tightly coupled, it would be interesting to measure and characterize cGMP binding to determine if this loss in potency is due to binding or an event post-binding. Is negative cooperativity also seen with cGMP and at the same degree? Can both cAMP and cGMP compete for a CNBD? So far studies have show cGMP binds in a the syn-configuration, as opposed to the anti-configuration with cAMP (Zagotta et al., 2003), and that cGMP binds to water stronger than cAMP thus making it difficult for it to bind to the CNBD because of the amount of energy required to strip the waters from the ligand (Zhou and Siegelbaum, 2008).
Preliminary experiments show cGMP bound to HCN2J with negative cooperativity, and with a stoichiometry of 1:3 high to low binding affinity ratio (Figure B.1). The high binding affinity of cGMP is ~ 0.8 μM falls within the range of the high binding affinity of cAMP. Thus, cAMP and cGMP could compete for the initial CNBD binding site. Activation of HCN channels by cGMP was recently linked to modulating N-methyl-D-aspartate, NMDA receptors which modulate synaptic plasticity (Neitz et al., 2013).

Based on this finding, I hypothesized the degree of negative cooperativity might depend upon the identity of the first ligand bound. To test this, I added low concentrations of cAMP or cGMP to saturate the first high binding affinity site. I then titrated in either cAMP or cGMP to see if binding affinity would be altered depending on which cNMP bound to the initial site. Preliminary data showed the initial cNMP bound to the high affinity site affected binding affinity of the subsequent cNMPs. Saturating the first binding site with cAMP increased the binding affinity of cGMP to the other three binding sites. The reverse is also seen: saturating initially with cGMP decreased the binding affinity of cAMP to the other three sites (Figure B.2, Table B.1). A concern of this experiment is the confidence that only the first, high binding affinity site is saturated, and without spill over of cNMP into the other sites that could affect binding.

*Separating residues involved in binding and facilitation of opening using ITC*

Because cAMP binding and facilitation of channel opening are tightly coupled and concentration-response curves obtained by patch clamp electrophysiology reflect both processes, it is difficult to identify residues that contribute specifically to binding or facilitation. Specific roles for residues of interest have been examined almost exclusively by patch-clamp electrophysiology to generate concentration-response curves for mutant channel activity (Zhou
and Siegelbaum, 2007). By using ITC, in combination with functional information, it becomes possible to identify residues that contribute to either binding or facilitation. Identifying these residues is important because those that facilitate channel opening may undergo dynamic interactions with cAMP, whereas those that contribute to binding alone are static (Zhou and Siegelbaum, 2007). Categorizing these residues will help to define the characteristics required for a high binding affinity ligand and channel movement.

I chose residues in the HCN2 C-terminus that either make contact with the cyclic nucleotide or affect the $EC_{50}$ measured in patch clamp studies when they were individually substituted by alanine (Zhou and Siegelbaum, 2007). Using this approach, residues that make contact with cAMP could be categorized into three categories: 1) residues that affect binding, 2) residues that affect channel opening, or 3) residues that affect both processes.

D631A, D634A, L633A, and R591A were cloned into the HCN2 C-terminus, purified and the energetics of cAMP binding were determined by ITC (Figure B.3, Table B.2). Each of these mutations, except for D634A, has been shown to lower the potency of cAMP on channel opening (Zhou and Siegelbaum, 2007). D631A and D634A showed energetics and cAMP binding affinity was similar to that of wild type. The similarity of D634A binding to wild type is not surprising given the lack of any effect of this mutation on potency. However, the similarity of cAMP binding energetics of D631A to those of wild type is interesting because this mutation lowers potency of cAMP action on channel opening by approximately ten times. Together, the data suggest that D631 is a residue that undergoes a dynamic interaction with cAMP to affect a transition that follows binding.
**Future long-term studies**

*Phosphatidylinositol-4,5-bisphosphate binding to HCN channels*

Phosphatidylinositol-4,5-bisphosphate (PIP$_2$) has emerged as a regulator of HCN opening. Depletion of endogenous PIP$_2$ from the membrane causes a negative shift in voltage dependence upon patch excision (Flynn and Zagotta, 2011; Pian et al., 2006; Zolles et al., 2006). Application of exogenous PIP$_2$ or the soluble form, diC$_8$ PI(4,5)P$_2$, to HCN channels in inside-out patches (Pian et al., 2006) or by intrapipette dialysis (Zolles et al., 2006) facilitates opening. The effect of soluble diC$_8$ PI(4,5)P$_2$ in excised patches, at 25 µM, was significantly less when in the presence of a saturating concentration of cAMP (10 µM). It was a proposed that the two molecules bind to distinct sites, but recruit a common mechanism to promote HCN opening (Pian et al., 2006).

An alternative explanation for the reduced effect of PIP$_2$ in the presence of saturating cAMP is that they negatively interact. In the SpIH, PIP$_2$ enhances function by acting on the transmembrane regions of the channel, but it also inhibits facilitation of opening by cGMP, a partial agonist of SpIH, by binding to the C-linker close to its connection to the inner pore and the plasma membrane (Flynn and Zagotta, 2011). A similar binding site has been observed for PIP$_2$ in recent crystal structures of Kir 2.2 (Hansen et al., 2011) and GIRK channels (Whorton and MacKinnon, 2011), which require such an interaction for channel activation. Binding of PIP$_2$ to the C-terminus of the mammalian HCNs and inhibiting channel opening could explain the reduced effect of the combined PIP$_2$ and cAMP observed.
Crystallizing the complete HCN channel and cAMP bound HCN2J intermediates

So far only a fragment of the C-terminus of the HCN channel has been crystallized. The next step would be to crystallize the complete HCN channel to visualize the interactions the C-terminus has in relation to the rest of the channel. As mentioned throughout this thesis, the C-linker is speculated to be in the resting conformation and attachment to the transmembrane segments could be required to see the active conformation.

Chapter 2 shows cAMP bound to HCN2J initially with high binding affinity followed by low binding affinity, however the crystal structure of cAMP bound to HCN2J showed symmetrical binding. According to Popovych et al. (2006), to truly understand the allosteric process behind the unliganded and liganded states of a protein lies in the intermediate states. Proteins that have negatively cooperative binding are ideal as their intermediate states are well populated and can be more easily isolated (Popovych et al., 2006). The intermediate states of catabolite activator protein, CAP, a dimer that binds to cAMP with negative cooperativity, revealed most of the conformational changes occurred in the binding of the second cAMP (Popovych et al., 2006). Because cAMP also binds to HCN channels with negative cooperativity, crystallizing intermediates could be possible to provide further insight into the conformational changes that occur between unbound and bound cAMP states. I predict that binding of the first cAMP molecule will induce the biggest change followed by smaller changes to the subsequent three sites, as only two cAMP molecules are required to be bound to produce maximal effect (Kusch et al., 2010). This scenario was also predicted with CNG channels, as the last two ligands bound were only necessary for stability (Biskup et al., 2007).
Crystallizing and characterizing binding of the human ether-a-go-go related gene (hERG) and ether-a-go-go potassium (ELK) channels

Human ether-a-go-go related gene, hERG channels are voltage-dependent potassium channels that also play a role in cardiac excitability by repolarizing the membrane potential, \( I_{Kr} \). They are associated with long QT syndrome, particularly LQT2 (Curran et al., 1995). Curiously, hERG channels also have a CNBD, but are not affected by cAMP (Cui et al., 2000). Ether-a-go-go potassium, ELK, channels are in same family on hERG and its C-linker and CNBD has been crystallized. While the overall structure of ELK and HCN2J are similar, surrounding the CNBD are negatively charged residues that make cNMP binding difficult. Instead, a short \( \beta \)-strand occupies the binding pocket. Mutations in this strand shifted activation to depolarized potentials, suggesting it could be an intrinsic ligand for ELK (Brelidze et al., 2009). It would be interesting to compare the strength and energetics of binding of this short \( \beta \)-strand to ELK with that of cAMP in HCN2J to determine whether this ligand acts in a similar fashion as cAMP and whether binding is also negatively cooperative.

The hERG channel has also not been crystallized and it would also be interesting to see if it also has its own intrinsic ligand, similar to that of ELK, and its binding characteristics.
Using ITC to identify antagonists of HCN activity

Identifying antagonists for HCN channels is of potential benefit to reduce heart rate and treat certain types of cardiac disease. Currently, Ivabradine, an I_f channel pore blocker, is used to decrease heart rate to treat patients with angina pectoralis, sinus tachycardia and heart failure (Borer and Le Heuzey, 2008). By inhibiting HCN, Ivabradine reduces the slope of the diastolic depolarization phase and heart rate. The advantage of Ivabradine over other heart rate reducing agents like β-blockers, which reduce contractility and blunt exercise-appropriate increases in cardiac activity (Borer, 2004; DiFrancesco and Camm, 2004). However, Ivabradine has a number of side effects including luminous phenomena, where patients experience enhanced brightness in their visual field, and excessive bradycardia (Borer and Le Heuzey, 2008; Bucchi et al., 2002). Effects on vision are probably mediated by actions on the HCN1 isoform, which predominates in the retina of the eye (Demontis et al., 2008). By targeting the CNBD of HCN2 and HCN4 isoforms, a more subtle antagonist might be identified (because only the facilitation of opening is acted upon as opposed to completely blocking the channel pore) and retinal function could be spared because the HCN1 isoform is not greatly affected by cAMP.

Using the ITC and techniques developed in this thesis to study cAMP binding in isolation, binding affinities and energetics of cAMP analogues could be measured, as well as tested functionally using electrophysiological techniques. The goal would be to find an antagonist that bound with higher affinity than cAMP, maintained heart rate, but does not disrupt contractility of the heart. Because the pore is not blocked, the channel can still function by voltage. I would also hope this antagonist would outcompete cAMP to decrease cAMP facilitation and decrease heart rate. Cyclic AMP analogues can also be intelligently designed
using the knowledge that residues within the C-terminus can be separated into those that are involved in binding and those that move after binding, as previously mentioned.

_The effect of heteromerization of HCN channels on cAMP binding_

HCN channels have been proposed to heteromerize _in vivo_ because the properties in $I_f$ in SAN cells do not conform to one specific isoform (Altomare et al., 2003), nor does $I_h$ (Ulens and Tytgat, 2001). Because HCN isoforms are frequently expressed within the same location there is a high chance that they heteromerize. HCN2 and HCN4 have been shown to co-immunoprecipitate in the rat thalamus (Whitaker et al., 2007) and current recorded from HCN1 and HCN2 concatamers are similar to that seen in CA1 pyramidal neurons (Ulens and Tytgat, 2001; Chen et al., 2001). When co-expressed, HCN1 and HCN2 have a voltage-dependence that is intermediate, i.e. a combination of the two homomeric channel currents and a shift in cAMP response ranged between 7.2-14 mV (Chen et al., 2001; Ulens and Tytgat, 2001). From these results along with the results in this thesis, that HCN1 is less inhibited than HCN2, inhibition of the heteromeric channel is compromised and reflects that of a combination of the two isoforms. Cyclic AMP sensitivity is also measured as a combination of the two. But do these heteromeric channels have negative cooperativity when bound to cAMP?

Because of the large shift in activation upon addition of cAMP in the heteromeric HCN1-HCN2 channel, I would expect these channels to have an initial high then low binding affinity event, similar to homomeric HCN2 and HCN4 channels. One could test this hypothesis by creating HCN1J and HCN2J tandem tetramers, purify these constructs and measure cAMP binding using the ITC.
Summary

The experiments in this thesis highlight and address some of the major existing questions surrounding cAMP binding to HCN channels. The effect cAMP has on each channel differs; opening of HCN2 and HCN4 is facilitated by cAMP, however HCN1 and HCN3 are affected little or none, respectively. Because cAMP binding and channel opening are a tightly coupled process, it is difficult to isolate and directly measure cAMP binding which could help understand the differences of cAMP response between the isoforms. I was able to isolate and directly measure cAMP and characterize the binding site with energetics using ITC. We found HCN2 and HCN4 channel opening, which are facilitated by cAMP, bound to cAMP initially with high followed by low affinity to the other three subunits. HCN2 and HCN4 also displayed negative cooperativity in binding. In contrast, only low binding affinity was seen with HCN1 and no cooperativity. While the oligomerization of the C-terminus is enhanced in HCN2 and HCN4 it is inhibited in HCN1. When the C-terminus was fused to a tetrameric construct N-terminal to the C-linker region, a similar ITC profile as HCN1 and inhibition of oligomerization was seen. We concluded HCN1 was in a “pre-tetrameric” state and less inhibited than HCN2 and HCN4.

I also demonstrated the importance of the C-linker in oligomerization and cooperativity using an epilepsy-associated mutation, E488K, located in the C’-helix of the C-linker. This mutation inhibited oligomerization and negative cooperativity was not seen. Energetics were similar to that seen with the monomeric construct of HCN2 where the first two α-helices of the C-linker were removed suggesting that each subunit in E488K behaved independently from each other. However these differences are attributed to the removal of K instead of its addition as experiments with E488A pointed out.
My work has demonstrated that the tightly coupled processes of cAMP binding and channel opening, when isolated and measured can provide a more comprehensive look at the mechanism behind binding and its role in subunit interactions leading to channel opening. Therefore, the techniques used in this thesis could also benefit channels such as hERG, a voltage-gated potassium channel that was not facilitated by cNMP but has a CNBD in its C-terminus, and its role in channel opening.
References


Noma, A., and H. Irisawa. 1976. Membrane currents in the rabbit sinoatrial node cell as studied by the double microelectrode method. *Pflugers Arch*. 


Appendix A: Supplementary figures
Figure A.1 *Purified version of HCN C-terminus were monodispersed*

Size exclusion column profiles of the protein constructs utilized in our study.
Figure A.2 Fitting of two independent site and four sequential site models to the heat release data obtained from adding cAMP to 200 µM HCN2J

Plots of heat produced measured by ITC upon progressive injections of cAMP into 200µM HCN2J. The solid yellow line through values represents either a two-site binding model (above) or a four-site sequential model (below) fitted to the same data, using software from Origin. An estimate the goodness of fit is given by the $\chi^2$ value, which is defined as

$$\chi^2 = \frac{1}{n_{\text{eff}} - p} \sum [y_i - f(x_i;p_1,p_2,\ldots)]^2$$

where:

- $n_{\text{eff}}$ = the total number of experimental points used for the fitting
- $p_i$ = fitting parameters
\[ p = \text{total number of adjustable parameters} \]
\[ y_i = \text{experimental data points} \]
\[ f(x;p1,p2,p3 \ldots) = \text{fitting function} \]

The difference \( d = n^{\text{eff}} - p \) is the degrees of freedom. For each fit, \( \chi^2 \), is shown, along with the \( K_a \) (affinity constant), \( \Delta H \) and \( \Delta S \) for each binding event. For the two independent site model, \( N \) (stoichiometry) is also shown for each binding event. The fit for the two independent site model has a lower value for \( \chi^2 \), indicative of a better fit; this can also be appreciated visually by the greater deviation of the yellow line from the data in the lower plot. Although the standard errors of the affinity constants are high for both models, they are quite low for \( \Delta H \) and \( N \) for each binding event described by the two independent site model as compared to the sequential model.
Figure A.3 Possible state diagram to explain the binding of cAMP to HCN2J and negative cooperativity

In this sequential model, cyclic AMP binds to the unoccupied HCN2J tetramer and changes the conformation of one subunit, resulting in subunit interfaces with increased stability. This hampers the conformational changes of the other subunits upon binding cAMP, resulting in an observed negative cooperativity.
Figure A.4 Cyclic AMP binding to HCN2J at 10°C

Plots of heat produced and measured by ITC upon progressive injections of cAMP into 200μM HCN2J. The solid line through values of the lower plots represents a two-site binding model, which yielded values for affinity and energetics (ΔG, ΔH and ΔS) (Table A.1).
**Figure A.5** Cyclic AMP binds to HCN4 in a HCN2-like manner

A) Plots of heat produced and measured by ITC upon progressive injections of cAMP into indicated concentrations of HCN4J and 200µM ΔAB-HCN4J. The solid line through values of the lower plots represents a two-site binding model for 200µM HCN4J, and a single binding site model for 25µM HCN4J and 200µM ΔAB-HCN4J; fits yielded values for affinity and energetics (ΔG, ΔH and ΔS). B) Bar graph comparing affinities for the transitions determined from the fits in ‘A’. Error bars indicate S.D. C) Bar graph comparing energetics among 200µM ΔAB-HCN4J, 25µM HCN4J and 200µM HCN4J. Error bars indicate S.D.
Table A.1 *Full set of thermodynamic parameters for cAMP interactions with HCNs determined from isothermal titration calorimetry*. 

<table>
<thead>
<tr>
<th></th>
<th>Low Affinity Binding</th>
<th></th>
<th></th>
<th>High Affinity Binding</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_d (µM)</td>
<td>ΔH (kcal/mol)</td>
<td>-TΔS (kcal/mol)</td>
<td>ΔG (kcal/mol)</td>
<td>N</td>
</tr>
<tr>
<td>HCN2J</td>
<td>200µM</td>
<td>1.42 ± 0.30</td>
<td>-15.50 ± 1.61</td>
<td>7.47 ± 1.51</td>
<td>-8.00 ± 0.11</td>
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<tr>
<td></td>
<td>100µM</td>
<td>1.64 ± 0.20</td>
<td>-15.13 ± 1.17</td>
<td>7.30 ± 1.2</td>
<td>-7.90 ± 0.1</td>
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<td>25µM</td>
<td>1.49 ± 0.17</td>
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<td>-2.01 ± 0.23</td>
<td>-7.95 ± 0.07</td>
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<td>10µM</td>
<td>1.39 ± 0.09</td>
<td>-5.31 ± 0.76</td>
<td>-2.68 ± 0.73</td>
<td>-7.98 ± 0.04</td>
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<td>ΔAB-HCN2J</td>
<td>200µM</td>
<td>1.86 ± 0.23</td>
<td>-10.75 ± 2.62</td>
<td>2.64 ± 0.62</td>
<td>-7.83 ± 2.38</td>
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<tr>
<td>HCN1J</td>
<td>200µM</td>
<td>1.42 ± 0.47</td>
<td>-15.09 ± 0.50</td>
<td>7.09 ± 0.68</td>
<td>-7.98 ± 0.20</td>
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<tr>
<td></td>
<td>25µM</td>
<td>1.51 ± 0.80</td>
<td>-16.81 ± 6.90</td>
<td>8.75 ± 6.51</td>
<td>-8.05 ± 0.38</td>
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<tr>
<td>ΔAB-HCN1J</td>
<td>200µM</td>
<td>1.98 ± 0.50</td>
<td>-7.27 ± 0.50</td>
<td>-0.55 ± 0.32</td>
<td>-7.83 ± 0.06</td>
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<tr>
<td>HCN4J</td>
<td>200µM</td>
<td>1.11 ± 0.62</td>
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<td>4.20 ± 0.96</td>
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<td></td>
<td>25µM</td>
<td>0.75 ± 0.08</td>
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<tr>
<td>ΔAB-HCN4J</td>
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<td>1.44 ± 0.29</td>
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<td>-0.02 ± 0.004</td>
<td>-7.98 ± 0.13</td>
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<td>HMT-GCNC4-HCN2J</td>
<td>200µM</td>
<td>1.34 ± 0.03</td>
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<td>HMT-HCN2J</td>
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<tr>
<td>HCN2J 10°C</td>
<td>200µM</td>
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<td>-2.78 ± 0.39</td>
<td>-4.65 ± 0.31</td>
<td>-7.43 ± 0.08</td>
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To fit our data of cAMP binding to high concentrations of HCN2J, we invoked a two independent-site model and a four-site sequential model. The two independent binding site model assumes that saturation of each site depends only on the individual values of the two binding constants $K_i$, which are defined as

$$K_1 = \frac{\Theta_1}{(1 - \Theta_1)[X]} \quad K_2 = \frac{\Theta_2}{(1 - \Theta_2)[X]}$$

$$X_i = [X] + M_i(n_i \Theta_1 + n_2 \Theta_2)$$

where $n$ is the number of sites, $X_i$ and $[X]$ are the bulk and free concentrations of cAMP and $M_i$ is the bulk concentration of HCN2J and $\Theta$ is the fraction of sites occupied by cAMP.

Solving ‘1’ for $\Theta_1$ and $\Theta_2$, and substituting into ‘2’ gives

$$X_i = [X] + \frac{n_1 M_i [X] K_1}{1 + [X] K_1} + \frac{n_2 M_i [X] K_2}{1 + [X] K_2}$$

Clearing fractions and collecting terms yields a cubic equation,

$$[X]^3 + p[X]^2 + q[X] + r = 0$$

where

$$p = \frac{1}{K_1} + \frac{1}{K_2} + (n_1 + n_2) M_i - X_i$$

$$q = \left( \frac{n_1}{K_1} + \frac{n_2}{K_2} \right) M_i - \left( \frac{1}{K_1} + \frac{1}{K_2} \right) X_i + \frac{1}{K_1 K_2}$$

$$r = \frac{-X}{K_1 K_2}$$

Equations 4 and 5 are solved for $X_i$, and $\Theta_1$ and $\Theta_2$ are obtained from equation 1. The heat content $Q$ of the solution contained in Vo (cell volume) (determined as compared to zero for the unliganded protein) for any injection $i$ is equal to
\[ Q = M V_0 (n_1 \Delta H_1 + n_2 \Delta H_2) \quad 6 \]

A correction must be made for volume, which increases with each injection. Thus, the heat effect for the \(i\)th injection is

\[ \Delta Q = Q(i) + \frac{dV_i}{V_o} \left( \frac{Q(i) + Q(i-1)}{2} \right) - Q(i-1) \quad 7 \]

which is then minimized using the Levenberg–Marquardt algorithm to obtain the best values for the fitting parameters.

For the sequential binding model, the binding constants are defined relative to the progress of saturation.

\[ K_1 = \frac{[MX]}{[M][X]} \quad K_2 = \frac{[MX_2]}{[MX][X]} \quad K_3 = \frac{[MX_3]}{[MX_2][X]} \quad 8 \]

For identical sites, sequential binding creates statistical degeneracy because the first ligand has more sites to choose from as compared to the subsequent ligands. To distinguish between the phenomenological and intrinsic binding constants, in which the degeneracy has been removed, the following relationship is used;

\[ K_i = \frac{n - i - 1}{i} K_i \quad 9 \]

Because the concentrations of all liganded species \([ML_i]\) may be expressed in terms of concentration of non-ligated species \([M]\), the fraction of total macromolecule having \(i\) bound ligands, \(F_i\), is

\[ F_o = \frac{1}{P} \quad F_o = \frac{K_i[X]}{P} \quad F_2 = \frac{K_1K_2[X]^2}{P} \quad F_n = \frac{K_1K_2 \ldots K_n[X]^n}{P} \quad 10 \]

where

\[ P = 1 + K_1[X] + K_1K_2[X]^2 + \ldots + K_1K_2 \ldots K_n[X]^n \]

\[ 11 \]
\[ X_i = [X] + M \sum_{i=1}^{n} i F_i \]

Once \( n \) and values of fitting parameters \( K_1 \) through \( K_n \) are assigned, then equations 10, 11 are solved for \([X]\), after which all \( F_i \) are solved from equation 10, and the heat content is determined from

\[ Q = M_i V_o (F_1 \Delta H_1 + F_2 [\Delta H_1 + \Delta H_2] + ... + F_n [\Delta H_1 + \Delta H_2 + \Delta H_3 + ... \Delta H_n] \]  

Equation 7 is used to correct for the increase in volume and used in the Marquardt algorithm to obtain the best values for the fitting parameters.
Figure A.6 No significant difference is observed between E488K-HCN2J cryoprotected with glycerol or glucose.

A) Overlay of E488K-HCN2J cryoprotected with 20% glycerol (red) and 20% glucose (pale yellow). B) Close-up view of area (black box in A) surrounding glycerol.
Appendix B: Unpublished preliminary data for future studies
Figure B.1 *Cyclic GMP binds to HCN2J with high and low affinity*

A) Plots of heat produced and measured by ITC upon progressive injections of cAMP into 200 µM HCN2J. The solid line through the values of the lower plot represents a two independent binding model. B) Bar graph comparing high and low binding affinity of cAMP and cGMP as determined by the fit in A. Error bars indicated S.D. C) Bar graphs comparing energetics of high binding event (left) and low binding event (right). Error bars indicate S.D.
Figure B.1 Low affinity state depends on initial cyclic nucleotide bound
A) Plots of heat produced and measured by ITC upon progressive injections of cAMP or cGMP into HCN2J preincubated with either cAMP or cGMP as indicated. The solid line through the values of the lower plots represents a single binding model. B) Preincubated HCN2J with 2 mM cGMP then titrated in cAMP. C) Bar graph comparing affinities among the signal transitions determined from fits in A. Error bars indicated S.D. D) Bar graph comparing energetics between the binding events, determined from fit in A. Error bars indicate S.D.
Figure B.2 Characterization of residues involved in cAMP binding or movement

Plots of heat produced and measured by ITC upon progressive injections of cAMP in 200 μM of mutants as indicated. The solid line through the values of the lower plots represents a single binding site model.
Table B.2. Full set of thermodynamic parameters for cAMP and cGMP interactions with HCN2J determined from isothermal titration calorimetry

<table>
<thead>
<tr>
<th></th>
<th>K&lt;sub&gt;d&lt;/sub&gt; (μM)</th>
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<th>-TΔS (kcal/mol)</th>
<th>ΔG (kcal/mol)</th>
<th>N</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP</td>
<td>1.42 ± 0.30</td>
<td>-15.50 ± 1.61</td>
<td>7.47 ± 1.51</td>
<td>-8.00 ± 0.11</td>
<td>0.80 ± 0.12</td>
<td>3</td>
</tr>
<tr>
<td>cGMP</td>
<td>9.20 ± 0.61</td>
<td>-7.02 ± 0.70</td>
<td>0.15 ± 0.68</td>
<td>-6.87 ± 0.36</td>
<td>0.75 ± 0.04</td>
<td>3</td>
</tr>
<tr>
<td>cGMP + cGMP</td>
<td>12.13 ± 0.81</td>
<td>-8.58 ± 0.42</td>
<td>1.87 ± 0.46</td>
<td>-6.71 ± 0.04</td>
<td>0.40 ± 0.04</td>
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</tr>
<tr>
<td>cGMP + cAMP</td>
<td>16.07 ± 2.98</td>
<td>-18.33 ± 0.62</td>
<td>11.74 ± 0.64</td>
<td>-6.57 ± 0.12</td>
<td>0.40 ± 0.05</td>
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</tr>
<tr>
<td>cAMP + cAMP</td>
<td>2.09 ± 0.33</td>
<td>-17.50 ± 0.59</td>
<td>9.71 ± 0.52</td>
<td>-7.76 ± 0.10</td>
<td>0.32 ± 0.07</td>
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</tr>
<tr>
<td>cAMP + cGMP</td>
<td>6.59 ± 0.35</td>
<td>-10.52 ± 1.21</td>
<td>3.45 ± 1.24</td>
<td>-7.07 ± 0.03</td>
<td>0.24 ± 0.03</td>
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</tr>
</tbody>
</table>
### Table B.3

Full set of thermodynamic parameters for cAMP interactions with HCN2J mutants determined from isothermal titration calorimetry.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Kd (μM)</th>
<th>ΔH (kcal/mol)</th>
<th>-TΔS (kcal/mol)</th>
<th>ΔG (kcal/mol)</th>
<th>N</th>
<th>n</th>
</tr>
</thead>
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<tr>
<td>Low Affinity</td>
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<tr>
<td>D631A</td>
<td>1.42 ± 0.21</td>
<td>-16.67 ± 0.76</td>
<td>8.68 ± 1.18</td>
<td>-8.00 ± 0.09</td>
<td>0.85 ± 0.02</td>
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<tr>
<td>D634A</td>
<td>3.29</td>
<td>-21.22</td>
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<td>L633A</td>
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<td>-3.26</td>
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<td>R591A</td>
<td>44.58 ± 5.69</td>
<td>-11.00 ± 1.06</td>
<td>5.06 ± 1.04</td>
<td>-5.94 ± 0.07</td>
<td>0.79 ± 0.01</td>
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<tr>
<td>High Affinity</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.11 ± 0.02</td>
<td>-3.83 ± 0.46</td>
<td>8.68 ± 0.68</td>
<td>-9.50 ± 0.19</td>
<td>0.85 ± 0.02</td>
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<tr>
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<td>0.23</td>
<td>-7.93</td>
<td>-1.14</td>
<td>-9.07</td>
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</tr>
<tr>
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<td>0.12</td>
<td>-2.28</td>
<td>-7.19</td>
<td>-9.46</td>
<td>0.47</td>
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</tr>
<tr>
<td></td>
<td>1.51 ± 0.85</td>
<td>-4.72 ± 0.91</td>
<td>-3.31 ± 0.71</td>
<td>-8.02 ± 0.23</td>
<td>0.46 ± 0.07</td>
<td>3</td>
</tr>
</tbody>
</table>