Cation-pi interactions as a possible mechanism for controlling the closing of Hyperpolarization-activated cyclic nucleotide-modulated ion channels

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The hyperpolarization-activated, cyclic nucleotide-modulated (HCN) channel gene family is known to contribute significantly to cardiac pacemaking via pacemaker currents. To date, there are four mammalian HCN isoforms (HCN1–4) identified. The importance of HCN channel function to normal cardiac automaticity in mice was recently corroborated in humans diagnosed with idiopathic sinus node dysfunction. While the importance of HCN channels is widely recognized amongst cardiologists and scientists of the field, the specific mechanism of HCN channel opening or closing remain elusive. It is known that HCN channels are activated via hyperpolarization, and it is recently discovered that there is a voltage-independent step in the opening or closing of the channel (and at the same time a rate-limiting step). One of the leading theories for HCN channel closing is the cation-pi interaction between Phenylalanine and a positively charged component in the channel. In this proposal, we introduce some basic HCN structures and channel dynamics, then outline the basic principles of measuring current traces with patch clamping. Finally, we characterize the use of new constructs of mutant HCN channels expressed in *Xenopus laevis* (i.e., frog) oocytes. We also present a four-state cyclic allosteric scheme in the absence of cAMP for the mechanism in regulating HCN channel opening and closing. Our goal is to compare and contrast mutant HCN channel current traces with wild type HCN channels in order to gain a better understanding of HCN regulation of currents and ultimately suggest a fitting model for its mechanisms.

PACS numbers:

I. MOTIVATION

To date, heart disease is still the leading cause of death in the United States. Many such physiological disorders originate from dysfunctional rhythmicity of the heart. These include, but not limited to, different forms of arrhythmias such as supraventricular tachycardia, atrial fibrillation, and ventricular fibrillation. Understanding the direct and indirect causes of these disorders is one of the most actively researched topics in medicinal and scientific research. Eric Accili's lab at UBC is particularly interested in studying the underlying mechanism for the pacemaking sinoatrial node cells.

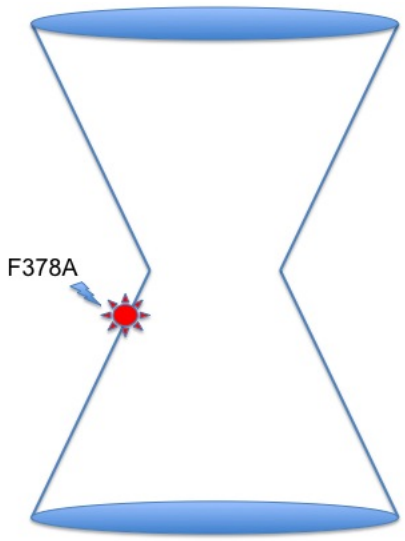

FIG. 1: Simplistic drawing of HCN channel and its pore region. The drawing shows the approximate location of the F378A mutation in the protein channel.

The motivation behind this project is to gain a better understanding of the specific mechanisms in the closing of the HCN channels. This is traditionally done using patch clamp [1], a electrophysiological technique where current from a tiny area of surface membrane of a living cell can be recorded. By setting a wide range of voltage in which HCN channels are normally opened or closed, current traces from different HCN channels can be recorded and compared against one another. In addition, one can gain insight into the model for the activation and deactivation of the channel.

What we hope to do with this project, is to use patch-clamping to measure current traces from different new mutant HCN channels. These mutant HCN channels are generated with the purpose of analyzing the possible cation-pi interaction exhibited in the phenyl group of the amino acid phenylalanine in the pore region (Figure 1. It has previously been shown that the mutation F378A slows down channel closing, and controls directly voltage-independent step of the cyclic model. However, the mutant channel showed very little difference in the activation of the channel (opening rate of the channel stays relatively constant). Since the mutation only slows down the closing of the channel, it is logical to conclude that a two-state system is too simple to model the channel mechanism. To test and confirm the effects of the phenyl group on phenylalanine, the newly generated mutants will include changes from phenylalanine to isoleucine, to leucine, to tryptophan, to tyrosine, and to a stop condon, for reasons detailed in the subsequent sections.

By having a better understanding of the voltage-independent step of the HCN channels, one can see how the channel is specifically activated or deactivated. The presence of a rate-limiting voltage-independent step in both channel opening and closing may serve an important physiological function by preventing channel kinetics from becoming excessively rapid at extremely negative or positive potentials. A limit to the rate of opening of HCN could ensure that there is a limit to the rate of spontaneous firing in many cell types, including cardiac ventricular myocytes [5]. On the other hand, removal of the limiting voltage-independent step allows total voltage dependency. Furthermore, a more detailed view into the structural mechanism of the opening and closing of HCN channels may allow us to design drugs that specifically target these channels to treat against certain physiological disorders in the future.

II. THEORY

Since this project is heavily biology-orientated equipped with physical and mathematical analysis, it is important to bring everyone to the same page. In this section, we will explain the basic functions of the heart and its pacemaking capabilities, as well as outlining the structural and functional properties of HCN channels. Furthermore, a brief introduction of the cation-pi interaction and the mutants chosen for this experiment will be included to enhance our understanding of the subject. And finally some key concepts of the popular electrophysiology technique patch clamping will be addressed.

A. The Human Heart and Hyperpolarization-activated, Cyclic Nucleotide-modulated (HCN) Channel

The primary function of the heart is to supply blood and nutrients to the body. The regular beating, or contraction, of the heart moves the blood throughout the body. Each heartbeat is controlled by electrical impulses traveling through the heart. If the heart's electrical system fails to function properly, the heart cannot beat regularly and this results in a rhythm disorder, or arrhythmia. The electrical system regulating heartbeat consists of two main areas of control and a series of conducting pathways: The sinoatrial, or SA, node is located in the right atrium (Figure 2). It provides the main control and is the source of each beat. The SA node also keeps up with the body's overall need for blood and increases the heart rate when necessary, such as during exercise, emotional excitement, or illness such as fever. The SA node is sometimes called the "natural pacemaker" of the heart.

Hyperpolarization-activated cyclic nucleotide-modulated (HCN) ion channels are crucial in generating electrical rhythmicity in several types of neurons [1, 4, 7, 9, 14, 15] and in sinus node cells of the heart

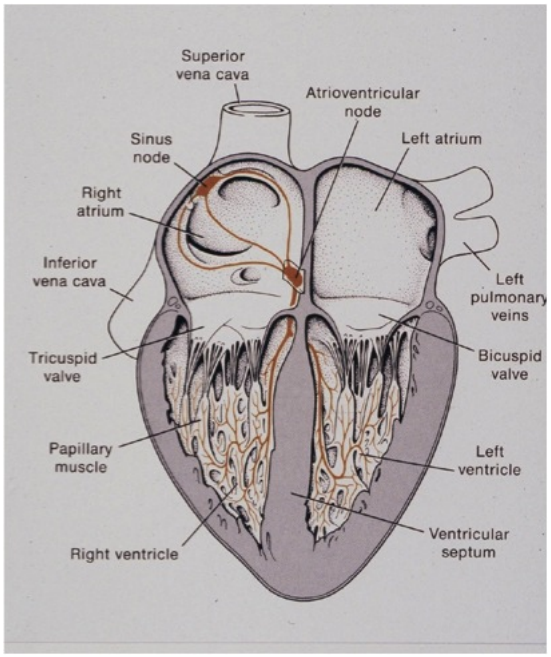

FIG. 2: Electrical system of the heart

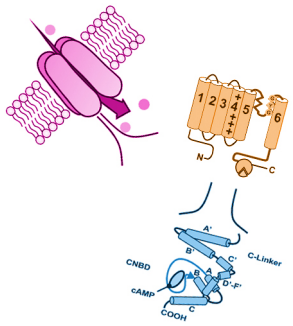

FIG. 3: Cartoon structure of HCN channel showing its structural components [2]

[3, 12]. HCN channels belong to the superfamily of cyclic nucleotide-gated channels [15]. Structurally, HCN channels are composed of four subunits, encoded by a family of related genes HCN1 HCN4 [10, 15], arranged around a central pore. Each subunit contains a cyclic nucleotide binding domain (CNBD) located at the intracellular C terminus [17], as well as a cytosolic N- and C- termini, 6 transmembrane segments (S1-6) with a putative voltage sensory (S4) and pore region between S5 and S6. HCN channels are primarily activated by hyperpolarizing membrane voltage. In addition, they can be further activated by the second messenger cAMP [6, 13] (Figure 3). Furthermore, measurements of HCN2 tail current kinetics revealed a voltage-independent closing step that becomes rate limiting at hyperpolarized voltages; and the rate of this closing step is decreased by cAMP. These results are consistent with a cyclic allosteric model in

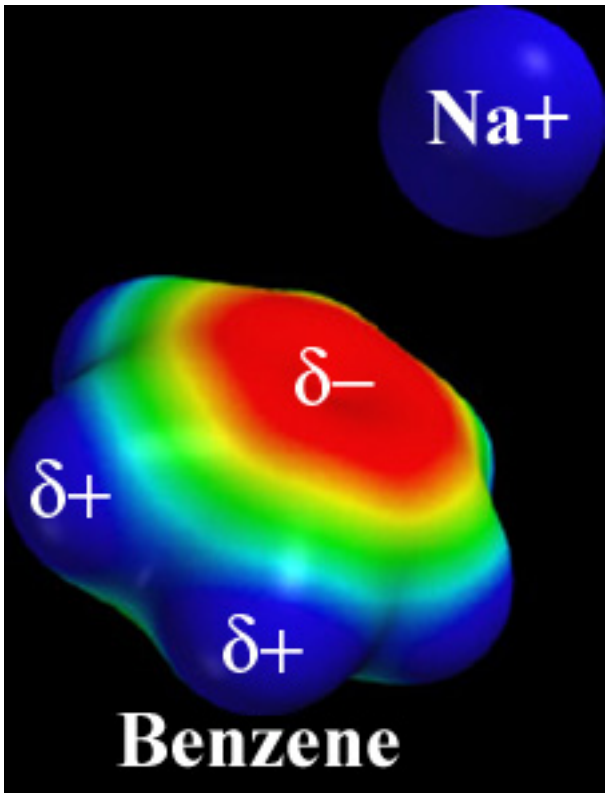

FIG. 4: Cation-pi orbital interaction. Surfaces colored by electrostatic potential. Image adapted from [8]

which voltage independent closed-open transition is subject to allosteric regulation by both voltage sensor movement and cAMP binding [5]. Moreover, in the hyperpolarizing phase of the action potential cAMP would progressively bind to the HCN channels, thereby increasing their conductance. This first slows hyperpolarization and later promotes pacemaker depolarization when the cAMP unbinds [11]. With relevance to the rate of opening and closing, recent data shows that when phenylalanine (F) at position 378 in HCN1, an amino acid in the pore region, was exchanged with alanine (A) (henceforth denoted as F378A), the closing rate of the channel when hyperpolarized is slowed considerably.

B. Cation-pi interaction and HCN Mutant Channels

Back in 1999, Gallivan and Dougherty reported results from a quantiative survey of cation-pi interactions in high-resolution structures in the Protein Data Bank [8]. Through their studies, they concluded that when a cationic side-chain is near an aromatic side-chan, the geometry is biased toward one that would experience a favorable cation-pi interaction (Figure 4). These interactions are involved in control of ion channels, G-protein-coupled receptors, transporters, and enzymatic catalysis.

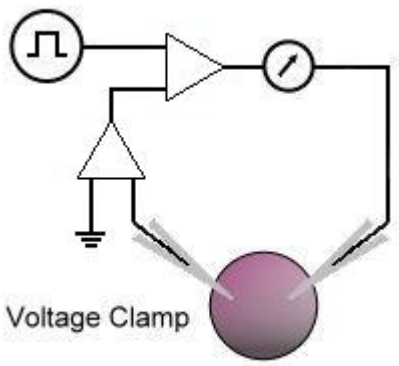

FIG. 5: Two-electrode voltage clamp. Two microelectrodes impale a Xenopus oocyte. One electrode monitors membrane potential (V_m) and the other passes enough current (I_m) through the membrane to clamp V_m to a predetermined command voltage ($V_{command}$).

We hypothesize that cation-pi interaction between the face of an electron-rich system like benzene and a charged amino acid in the pore region of HCN may be crucial in the voltage-independent closing step.

To confirm, or reject, the contribution of cation-pi interactions in channel closing, we will proceed to generate several channel mutants and collect current traces from them. The mutant selection was chosen with regards to their structural properties. The chosen amino acid in the pore region at position 378 is originally phenylalanine (F), a molecule that contains a benzene group. We decide to substitute it with alanine (A), isoleucine (I), leucine (L), tryptophan (W), tyrosine (T), or stop condon (STP) due to their respective amino acid structure similarities or differences. We predict that the mutation F378A will show the same data as previously observed; while F378I and F378L will also slow down the closing mechanism due to the absence of phenyl groups on either amino acid On the other hand, the mutation F378W and F378T should have minute or no effect on the closing of the HCN channel due to their possession of a phenyl group, as well as the fact that they are both large non-polar amino acids, similar to phenylalanine. This will also test whether the orientation of the pi-electron cloud has any contribution to the closing of the channel because the phenyl groups are orientated differently in these amino acids. Finally, mutation F-to-STP may very well result in a non-functional channel.

C. Patch-Clamp Technique

The patch-clamp technique is a variation of the voltage-clamp technique which measures current across cell membranes. This approach for recording whole-cell currents was introduced by Erwin Neher and Bert Sakmann, who received the Nobel Prize in Physiology or

Medicine in 1991. In this voltage-clamping technique, specialized electronics are used to inject current into the cell to set the membrane voltage to a value that is different from the resting potential. The device then measures the total current required to clamp V_m (membrane voltage) to this value. A typical method of voltage clamping involves impaling a cell with two sharp electrodes, one for monitoring V_m and one for injecting the current (Figure 5). When the voltage-sensing electrode detects a difference from the intended voltage, called the command voltage, a feedback amplifier rapidly injects opposing current to maintain a constant V_m. The magnitude of the injected current needed to keep V_m constant is equal, but opposite in sign, to the membrane current. Thus, this provides an accurate measurement of the total membrane current (I_m).

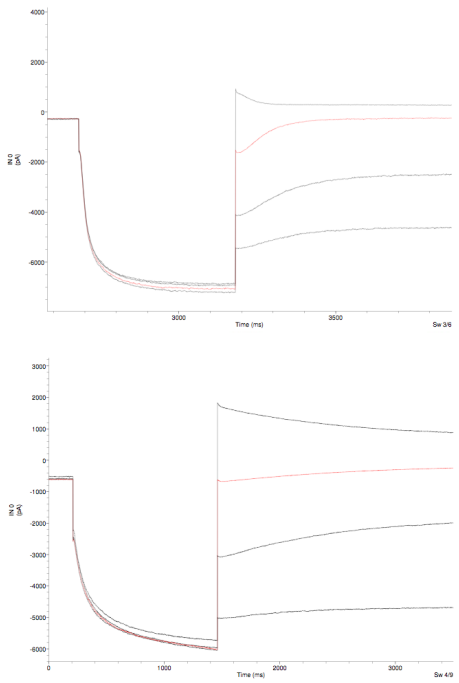

FIG. 6: The two graphs are raw data from whole-cell patch clamp electrophysiology. (Top is the wild type channel; bottom is the mutant F378A channel.) Each injected cell is stimulated by different voltages to induce channel opening. Five replica were done for both mutant and wildtype. In both cases, the initial increase in the current magnitude is caused by a hyperpolarizing voltage resulting in HCN channel opening. The current returns to normal at different rates when normal membrane voltage is applied due to different rates of channel closing. As seen above, the mutation in phenylalanine causes a slower deactivation where the channel closes more slowly.

In the case of HCN channels, when membrane potential is hyperpolarized, the ion channel opens to allow diffusion of ions and thus current flows through. This can be detected using voltage clamp. (The detected current is called macroscopic current.) However, in our experiment we are interested in a single HCN channel activity, instead of the whole cell membrane which encompasses hundreds of such channels. This is where patch-clamp technique comes in. In this method, a glass micropipette electrode with a smooth, fine-polished tip that is approximately 1 micrometer in diameter is pressed onto the surface of the cell. Applying suction to the inside of the pipette forms a high-resistance seal between the circular rim of the pipette tip (a patch pipette) and the cell membrane (a patch). This allows us to potentially isolate one channel for recording. Figure 6 from shows an example of the current traces that can be obtained from patch-clamp (data from the Accili Lab). As mentioned, the mutant F378A channel shows a much slower closing rate than that of the wild type channel.

III. PROPOSED EXPERIMENTS

We adopted several approaches in generating the proposed mutants, including quick-change mutagenesis, two-step overlapping site-directed mutagenesis, as well as phusion PCR technique. In the end, we voted in favor of the two-step overlapping site-directed mutagenesis combined with phusion PCR technique, which showed the most promise in the few weeks of trial and error. Briefly, primers were designed to incorporate the mutation within them and ordered from Integrated DNA Techonlogies, Inc. Subsequent PCRs (using phusion technique) are performed to achieve the desired mutant DNA sequence. These DNAs are then digested and incorporated into maxi cells via transformation. Ultimately, they will be set to express in Xenopus oocytes. From there, we will perform patch-clamp experiments and record current traces from these mutants. Finally, we will try to fit these data to the four-state cyclic model proposed earlier.

IV. MATERIALS AND SCHEDULE

Though the F378A mutation has already been made and is readily available at the Accili lab, the proposed mutations have yet to be produced. The mutagenesis of the HCN channels have been reportedly difficult to achieve, and much of the time spent was focused on troubleshooting the failure to generate adequate mutant DNA sequences for sufficient expression of the mutant protein. Fortunately, there has been significant progress in generating these mutants and our goal is to have mutant stocks ready to use for testing by the end of November. All PCR reagents and patch-clamping equipments are provided by the Accili Lab at UBC.

V. ACKNOWLEDGEMENTS

Many thanks go to Dr. XX and YYYs (graduate student at UBC) for all the help they have

provided in mentoring me in the experimental approaches as well as the underlying concepts in this field of research. Numerous individual meetings have helped my academic progress tremendously and I appreciate and enjoy every single minute of it. Also thanks to Dr. Kiefl for his help and kindness in regards to the proposal and the course as a whole.

VI. PLANNED SCHEDULE

Following is the planned schedule, the months are in bold, items for each months are listed underneath.

September

dicussion of possible projects and direction of research design and order specific primers

October

Site-directed Mutagenesis via PCR
literature research and learning fundamental concepts of electrophysiology
Trouble-shooting in PCR protocols and trying various different methods

November

Continuation of mutagenesis / troubleshoot
Write up research proposal
Prepare a lab presentation for lab meeting and PHYS449 course

December

Finish generating the mutants
Oral presentation for Acilli Lab
Patch-clamp analysis

January

Fit already obtained data and possible new data generated
Process and correlate all data

February

Test cyclic model with data obtained
Write final Thesis paper
Start Presentation

March

Finish final Thesis paper
Finish final presentation

VII. APPENDIX - GLOSSARY OF TERMS

- Cardiac myocytes - The type of cell found in cardiac muscles.
- Depolarization - A change in a cell's membrane potential, making it more positive, or less negative. In neurons and some other cells, a large enough depolarization may result in an action potential.
- F378A - Mutation from Phenylalanine (F) to Alanine (A) at position 378 in the amino acid sequence of the protein.
- Hyperpolarization - Opposite of depolarization, and inhibits the rise of an action potential; a change in a cell's membrane potential that makes it more negative.
- Phusion PCR - Phusion High-Fidelity PCR Master Mix is a convenient 2X mix containing Phusion High-Fidelity DNA Polymerase, nucleotides and optimized reaction buffer including MgCl2. Only template and primers need to be added by the user.
- Transformation - In molecular biology transformation is the genetic alteration of a cell resulting from the uptake, incorporation and expression of exogenous genetic material (DNA) that is taken up through the cell wall(s).[1] Transformation occurs most commonly in bacteria and in some species occurs naturally.
- Wild type - refers to the phenotype of the typical form of a species as it occurs in nature. Typically the control in biological research.

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