CARDIAC CONTROL IN THE PACIFIC HAGFISH (EPTATRETUS STOUTII)

by

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ABSTRACT

The Pacific hagfish (*Eptatretus stoutii*), being an extant ancestral craniate, possesses the most ancestral craniate-type heart with valved chambers, a response to increased filling pressure with increased stroke volume (Frank-Starling mechanism), and myogenic contractions. Unlike all other known craniate hearts, this heart receives no direct neural stimulation. Despite this, heart rate can vary four-fold during a prolonged, 36-h anoxic challenge followed by a normoxic recovery period, with heart rate decreasing in anoxia, and increasing beyond routine rates during recovery, a remarkable feat for an aneural heart. This thesis is a study of how the hagfish can regulate heart rate without the assistance of neural stimulation.

A major role of hyperpolarization-activated cyclic nucleotide-activated (HCN) channels in heartbeat initiation was indicated by pharmacological application of zatebradine to spontaneously contracting, isolated hearts, which stopped atrial contraction and vastly reduced ventricular contraction. Tetrodotoxin inhibition of voltage-gated Na^+ channels induced an atrioventricular block suggesting these channels play a role in cardiac conduction.

Partial cloning of HCN channel mRNA extracted from hagfish hearts revealed six HCN isoforms, two hagfish representatives of vertebrate HCN2 (HCN2a and HCN2b), three of HCN3 (HCN3a, HCN3b and HCN3c) and one HCN4. Two paralogs of HCN3b were discovered, however, HCN3a dominated the expression of

HCN isoforms followed by HCN4. All HCN isoforms bar HCN3b were dominantly expressed in the atrium, likely to support greater atrial excitability ensuring synchronous contractions. Phylogenetic analysis suggested that HCN3 is the ancestral isoform supporting previous observations.

Studies with β-adrenoreceptor agonists and antagonists in isolated, spontaneously beating hearts showed that the routine normoxic heart rate may involve maximal catecholamine stimulation of heart rate through cAMP stimulation of HCN channels via transmembrane adenylyl cyclase (tmAC). Loss of this tonic β-adrenorecptor cardiac stimulation during anoxia reduces heart rate, but restoring β-adrenoreceptor stimulation during normoxic recovery does not produce the previously observed increase above routine heart rate *in vivo*. Instead, bicarbonate-stimulated, soluble adenylyl cyclase (sAC) mediated cAMP production was found to produce this tachycardia in addition to the reinstated tmAC produced cAMP. This is the first time sAC has been implicated in heart rate control.

PREFACE

This work represents original research conducted by the author (Wilson, C.M.) primarily at the University of British Columbia, Point Grey Campus (UBC), Bamfield Marine Sciences Centre, Bamfield (BMSC), and the University of British Columbia and Department of Fisheries and Oceans Canada, Centre for Aquaculture and Environmental Research, West Vancouver (WVL).

A version of Chapter 2 has been published [Wilson, C.M., Farrell, A.P., 2012. Pharmacological characterization of the heartbeat in an extant vertebrate ancestor, the Pacific hagfish, *Eptatretus stoutii*. Comp. Biochem. Physiol. 164A:258-263]. All experimental work was conducted at BMSC and WVL by C.M. Wilson, who also produced the first draft of the manuscript, designed apparatus, analyzed data and produced the figures. As supervisor, A.P. Farrell assisted C.M. Wilson in experimental concept formation, experimental design, result interpretation and manuscript edits.

A version Chapter 3 has been published as part of a collaboration with the University of Oslo, Norway (UO), with experiments being conducted at BMSC, WVL and UO [Wilson, C.M., Stecyk, J.A.W., Couturier, C.S., Nilsson, G.E., Farrell, A.P., 2013. Phylogeny and effects of anoxia on hyperpolarization-activated, cyclic nucleotide-gated channel gene expression in the heart of a primitive chordate, the Pacific hagfish (*Eptatretus stoutii*). J. Exp. Biol. 216:4462-4472]. As lead investigator, C.M.

Wilson contributed to experimental design, primer design, performed all experiments, analyzed data, and produced both the figures and the first draft of the manuscript. J.A.W. Stecyk contributed to experimental design. C.S. Couturier assisted with data analysis and, with J.A.W. Stecyk, assisted with primer design and taught C.M. Wilson real-time PCR and phylogenetic analyses under the supervision of G.E. Nilsson. A.P. Farrell conceived the investigation and contributed to experimental design and production of the first draft of the manuscript. All authors contributed to data interpretation and manuscript edits.

Chapter 4 was a collaboration between UBC, the Scripps Institution of Oceanography, University of California San Diego (UCSD) with experiments being conducted at UBC, BMSC, WVL and UCSD. As lead investigator, C.M. Wilson designed the experiment and apparatus, conducted all experiments bar immunofluorescence and cAMP production assay, analyzed the data and produced the figures and first draft of the manuscript. J. Roa taught C.M. Wilson molecular techniques and conducted bar immunofluorescence and cAMP production assay experiments. Figures 4.3 and 4.4 were produced using data obtained by J. Roa in these experiments. Molecular experiments were conducted under the supervision of M. Tresguerres who also assisted in experimental design and teaching. Plasma pH was measured by G.C. Cox. As supervisor, A.P. Farrell contributed to experimental concept formation, experimental design, result interpretation and manuscript edits.

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ANOVA	Analysis of variance
AR	Acetylation reagent
ATP	Adenosine triphosphate
BMSC	Bamfield Marine Sciences Centre
Bpm	Beats per minute
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
$CaCl_2$	Calcium chloride
cCMP	Cyclic cytosine monophosphate
cDNA	Complimentary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
CNBD	Cyclic nucleotide binding domain
CNG	Cyclic-nucleotide-gated gene
Cq	Crossing point/threshold cycle
DDT	Dithiothreitol
dfSAC	Dogfish soluble adenylyl cyclase
DMSO	Dimethyl sulfoxide
E_{mean}	Average priming efficiency
ERG	Ether-à-go-go-related gene
H^+	Hydrogen ion
HCN	Hyperpolarization-activated cyclic nucleotide-gated channel
HCO_3^-	Bicarbonate ion

HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IBMX	Isobutylmethylxanthine
I_{Ca}	Calcium current
I_f	Funny current
I_h	Hyperpolarization-activated current
I_{Na}	Sodium current
I_{NaTTX}	Tetrodotoxin-sensitive inactivation-resistant sodium current
I_{NCX}	Inward sodium/calcium exchanger current
K^+	Potassium ion
KCl	Potassium chloride
$MgSO_4$	Magnesium sulfate
mRNA	Messenger ribonucleic acid
N_2	Nitrogen gas
Na^+	Sodium ion
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaH_2PO_4	Mososodium phosphate
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
NCX	Sodium/calcium exchanger
Ni^{2+}	Nickel ion
PCR	Polymerase chain reaction
PKA	Protein kinase A

RT-PCR	Real-time reverse transcriptase-polymerase chain reaction
RyR	Ryanodine receptor
sAC	Soluble adenylyl cyclase
SD	Sample diluents
s.e.m	Standard error of the mean
SERCA	Sarco/endoplasmpic reticulum calcium ATPase
SL	Sarcolemma
SR	Sarcoplasmic reticulum
tmAC	Transmembrane adenylyl cyclase
TRIP	Traf interacting protein
TTX	Tetrodotoxin
UBC	University of British Columbia
UCSD	University of California, San Diego
UO	University of Oslo
VG	Voltage-gated
VIP	Vasoactive intestinal peptide
WVL	Department of Fisheries and Oceans, West Vancouver Laboratory

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Without doubt, this work would not have been possible without the support, assistance and love of many people.

I am particularly grateful to Dr. Tony Farrell, who gambled on a Mancunian undergraduate and started to teach me science and experimental design back in 2005. Your support has been both professional and personal and I look up to you as a mentor, role model, excellent scientist and trusted friend. Your ability to stick it through the rough times when experiments failed has given me a steadfast determination to get through it and, at the same time, move on when appropriate. This has lead to, what in my opinion is, a thesis containing fantastic results.

My supervisory committee, Dr. Bill Milsom, Dr. Bob Harris and Dr. Colin Brauner has been a force to reckon with, dragging me over the coals when needed, and congratulating me on success. Your insight has been invaluable and your suggestions lead me to tweak experiments and add content to my thesis to increase its value and impact.

In addition, the work of coauthors, mentioned in the preface above, is greatly appreciated, the experience and techniques gained has already proved to be fruitful.

I would also like to offer thanks to the Farrellites past and present for their help with experiments, keeping me laughing, smiling and tanked up on Tim Horton's. I would like to thank Linda Hanson, Erika Elisson, Christine Verhille, Gina Galli, Georgina Cox and Adam Goulding in particular. Additionally, this work would have been impossible if it were not for the help, time and expertise of Janice Oakes over at West Vancouver Laboratory. I would like to share my gratitude for the companionship and advice of Dr Greg Goss and his lab members, and Dr Chris Wood, at Bamfield who made that trip particularly enjoyable.

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DEDICATION

In loving memory of Clifford Percival, for teaching me the values of hard work, politeness and how to drive, while being the provider of whiskey, stories, laughs, love and friendship.

To my son, Luke, I hope that this work will one day inspire you to follow your dreams. Love Daddy.

CHAPTER 1: INTRODUCTION

1.1 Preface

The craniate heart is a muscular pump tasked with delivering blood around the body, allowing for the exchange of gases, nutrients, immune cells and ions throughout. All craniate hearts share four main characteristics: 1) They are made up of specialized muscle cells known as cardiac myocytes; 2) they contain multiple chambers, each guarded by valves; 3) they follow the Frank-Starling Law of the heart (increased cardiac filling leads to an intrinsic increase in stroke volume); and 4) they are myogenic, meaning that the contraction of the muscle is generated in the heart muscle itself.

The contraction of cardiac muscle is tightly regulated to an animal's blood flow needs (cardiac output; blood ejected from the heart per unit time). When an animal increases its activity, cardiac output can increase over 3-fold in athletic animals such as humans (Shin et al., 1995) and sockeye salmon (*Oncorhynchus nerka)* (Eliason et al., 2013). Cardiac output is regulated through changes in the rate at which the heart beats (the heart rate) and the volume of blood ejected from the heart with each heartbeat (the cardiac stroke volume). The aim of this thesis was to discover how the heartbeat is initiated and regulated in the Pacific hagfish, *Eptatretus stoutii*, an animal that belongs to a group of chordate animals that predate vertebrates, the

craniates.

My interest in the hagfish heart is that it shares all of the above characteristics of the vertebrate heart, but differs by having no cardiac innervation (it also lacks an intact pericardium). Indeed, the earliest known craniate heart with the above mentioned characteristics belongs to the hagfishes (Axelsson et al., 1990; Bloom et al., 1963; Farrell, 2007a; Ota and Kuratani, 2007). For comparison, the heart of the more ancestral urochordate, *Ciona sp.*, consists of a contractile tube devoid of valves and although it is myogenic, contractile impulses can start at either end of the tube to reverse the flow direction (Anderson, 1968). The valves of chambered hearts ensure a one-way flow. Hagfishes are also unique in that they possess another myogenic heart. In addition to the branchial heart, which is analogous to the systemic heart of all other vertebrates and the subject of this thesis, hagfishes posses a portal vein heart designed to overcome the vascular resistance encountered in the liver as venous blood returns to the branchial heart (Cole, 1926; Farrell, 2007a). Hagfish additionally possess neurogenic hearts, termed the caudal and cardinal hearts, that are powered by skeletal muscle contractions while the animal swims (Cole, 1926; Farrell, 2007a). These hearts aid in venous return in their low pressure circulatory system.

The focus of my thesis, the branchial heart, has three chambers, each guarded by valves. These are the sinus venosus, atrium and ventricle. The sinus venosus arises from the posterior cardinal vein and internal jugular vein and drains into the

atrium. Blood from the atrium empties into the ventricle, which drains into the ventral aorta (Fig 1.1; Forster et al., 1991; Farrell, 2007a). Hagfish lack an intrapericardial outflow tract (conus or bulbus arteriosus). Instead, a swelling of the ventral aorta seems to take its place and posseses a pair of semilunar valves (Farrell, 2007a).

Despite the evolutionary importance of the hagfishes, very little is known about the control of the aneural hagfish heart. Instead, the vast majority of our knowledge on craniate cardiac control comes from research conducted on mammals, with a smaller (yet significant) portion done on other taxa, including teleost and elasmobranch fishes. All of these species possess some form of cardiac innervation. As summarized below, cardiac control in hagfishes differs from both mammalian and piscine systems, even though a common feature to all is the initiation of the heartbeat by a pacemaker region, where the intrinsic rate of the heartbeat can be controlled.

Therefore, this thesis will document my studies that detail how the heartbeat is initiated and regulated. Initiation of the heartbeat was explored with pharmacological, phylogenetic and gene-regulation studies, while the regulation of the heartbeat was investigated with pharmacological, gene regulation and metabolic studies. Together, the data obtained provide several novel as well as illuminating experimental insights into aneural cardiac control. The discoveries within may have broader implications for the vertebrate taxa as a whole.

My thesis examines three aspects of cardiac control: 1) The initiation of the heartbeat; 2) Synchronization of cardiac contraction; and 3) Control of heart rate. Each topic is introduced below. The limited literature on cardiac control in hagfish generated questions that are organized into testable hypothesis which then form the basis for my thesis.

1.2 The initiation of the craniate heartbeat: Funny currents vs. Calcium clocks.

A myogenic heartbeat is usually initiated in specialized pacemaker cells located in the primary pacemaker at the sinus venosus/atrium border. In some species, such as birds and mammals, the sinus venosus is no longer required to promote atrial filling. In these animals, the sinus venosus fuses with the atrium becoming atrialized and is referred to as the sinus venarum. In these cases the pacemaker area is localized in an area called the sinoatrial, or sinus, node (Jensen et al., 2014). Pacemaker activity is also found in the cardiac conduction system of birds and mammals and, under certain conditions, can be elicited from normally non-pacing cardiomyocytes (Yu et al., 1993a; Yu et al., 1995; Yasui et al., 2001; Marionneau et al., 2005). In these tissues, pacemaker activity is usually lower than, and therefore overridden by, the primary pacemaker.

All cardiac excitability, including pacemaker activity, is generated by transmembrane ion movements through ion-specific channels in the cell membrane (a process

collectively termed electrophysiology). These movements create ionic currents that then stimulate cardiac muscle contraction via the interaction of the proteins troponin and myosin (excitation-contraction coupling, reviewed by Bers, 2002). Ionic current flows are driven by electrochemical gradients. For example, the concentration of Na^+ is much higher outside the cell compared to the inside, and the inside of the cell is also negatively charged. Therefore, there is a large electrochemical gradient driving Na^+ into the cell. Thus, when ion channels specific to Na^+ open, positively charged Na ions enter the cell causing the cell membrane to become less negatively charged. As the membrane potential approaches 0 volts, it is said to depolarize, while repolarization refers to the restoration of the resting membrane potential (Fig. 1.2). During an action potential, depolarization of the muscle cell membrane (sarcolemma; SL) is caused by Na^+ and Ca^{2+} entering the cell, and repolarization occurs when K^+ moves out of the cell.

Such ion movements across the SL depend on the open state of ion-selective channels. These channels are voltage-gated (VG), which means that the open probability of the channel (the likelihood that a channel will spontaneously open, or activate) varies with the cell membrane potential. When a channel opens, it allows a current to flow across the SL and the cell membrane potential changes. Each ion channel also requires the cell membrane to be at a particular potential, termed the threshold potential, before activation occurs. In the case of a typical cardiac muscle cell (cardiomyocyte) at rest, the membrane potential remains relatively stable compared to the primary pacemaker cells. Upon stimulation, a rapid depolarization

due to Na^+ influx occurs. This opens Ca^{2+} channels allowing Ca^{2+} to enter the cell during the plateau phase, a period when changes in membrane potential are relatively small compared to the rest of the action potential. This Ca^{2+} comes from the extracellular fluid and triggers further Ca^{2+} release from the sarcoplasmic reticulum (SR). It is this increase in cytosolic Ca^{2+} that activates contraction. Following the plateau phase, repolarization occurs due to the opening of K^+ channels returning the membrane potential to the resting state, awaiting the next stimulation.

Pacemaker cells have different electrophysiological events compared to working cardiomyocytes (Brown and DiFrancesco, 1980; Doerr et al., 1989; Baker et al., 1997; DiFrancesco, 2010). In pacemaker cells, the normally relatively resting membrane potential of cardiomyocytes is replaced with a gently depolarizing membrane potential (Fig. 1.2). This gentle depolarization is termed the pacemaker potential, and it is this depolarization that produces the autorhythmicity of the stimulation of the pacemaker cell to produce action potentials, which then drives the myogenic craniate heartbeat as a whole (Fig. 1.2). The pacemaker action potential has a Ca^{2+}-mediated upstroke compared to the more rapid Na^+ mediated upstroke of cardiac myocytes. For when the membrane potential reaches the threshold potential for the Ca^{2+} channel, it triggers the next upstroke, a process detailed below.

Currently, there are three competing models that predict how the pacemaker potential is bought about, the membrane clock, the calcium clock and an integrated

model containing components of both clocks, each of which are introduced along with details of pacemaker electrophysiology. They differ in the precise cellular processes that are involved in generating the autorhythmicity of the pacemaker potential and the types, and locations, of the channels involved.

1.2.1 The membrane clock

The membrane clock posits that all cardiac pacing activity is restricted to electrical events associated with the SL. The core of this hypothesis is that the pacemaker potential is triggered by the opening of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels following hyperpolarization of the cell membrane. HCN channels allow Na^+ and K^+ to slowly enter the cytoplasm, which produces a current known as the funny current, I_f, or less frequently, the hyperpolarization-activated current, I_h (Brown et al., 1979a; Brown and DiFrancesco, 1980; Di Francesco and Ojeda, 1980; Yanagihara and Irisawa, 1980; Doerr et al., 1989; Baker et al., 1997; DiFrancesco, 2010). This slow influx of positive ions that gradually depolarizes the membrane, creating the pacemaker potential, until it reaches the opening threshold for T-type Ca^{2+} channels (Fig. 1.2). These channels then open quickly and briefly, allowing Ca^{2+} to rush into the cells and causing rapid depolarization of the membrane and the upstroke of the pacemaker action potential. As depolarization progresses, the threshold potential for L-type Ca^{2+} channels is reached, allowing even more calcium to enter the cell (Hagiwara et al., 1988; Doerr et al., 1989; Zhou and Lipsius, 1994). This electrical activity then quickly spreads to cardiomyocytes in other regions of the heart (by means of electrically coupled cells or specialized

conduction fibres) and produces contraction of the heart chambers. At the same time, slowly opening and closing VG K^+ channels bring about repolarization of the pacemaker cell, termed the delayed rectifier K^+ current. This repolarization results in hyperpolarization, which closes the delayed rectifier K^+ channels and reactivates both the HCN channels and the action potential cycle of the pacemaker cell (Fig. 1.2; Lei et al., 2000). With this membrane clock hypothesis, only ion channels found in the SL are involved in generating the pacemaker current.

1.2.2 The calcium clock

A second model to explain spontaneous pacing of sinoatrial cells was prompted by the discovery that ryanodine application to feline pacemaker cells reduced the rate of spontaneous depolarization during the pacemaker potential (Rubenstein and Lipsius, 1989). Since ryanodine inhibits Ca^{2+} release from ryanodine receptors (RyR) in the SR, it was suggested that periodic local Ca^{2+} release from the SR in the late diastolic phase was responsible for the pacemaker potential. This suggestion was further supported by evidence from confocal and fluorescence microscopy studies showing spontaneous Ca^{2+} realeases (sparks) from the SR (Huser et al., 2000; Maltsev and Lakatta, 2007). These Ca^{2+} sparks from the SR were independent of SL ion channels (Vinogradova et al., 2004).

A second player in the calcium clock model involves a SL protein, the sodium/calcium exchanger (NCX). NCX can generate current in both directions by exchanging 1 Ca^{2+} for 3 Na^+, operating in either a forward mode (1 Ca^{2+} out of the

cell in exchange for 3 Na^+ in) or a reverse mode, depending on the relative electrochemical gradients of Ca^{2+} and Na^+ across SL (Shigekawa and Iwamoto, 2001). Together with localized Ca^{2+} release from the SR into the cytoplasm, NCX could generate a pacemaker potential as follows. In response to the spontaneous increase in intracellular Ca^{2+} concentration from SR Ca^{2+} release, NCX operates in the forward mode to expel the excess Ca^{2+} from the cytoplasm. This will result in the cell gradually depolarizing because 3 Na^+ ions enter the cell per Ca^{2+} expelled. Therefore, inhibiting Ca^{2+} release into the cytoplasm with ryanodine prevents this exchange, which would then produce the observed reduction in pacemaker potential and the pacing of the cell. Co-localization of RyR in the SR and NCX channels in the SL allows NCX to be very responsive to changes in intracellular Ca^{2+} concentration due to SR Ca^{2+} release via RyR (Lyashkov et al., 2007). This process will proceed until the threshold potential for the T-type Ca-channel is reached to trigger a pacemaker action potential as described above.

1.2.3 Integrated model of cardiac pacemaking

A third model for cardiac pacemaking proposes that the processes involved with the membrane clock and the calcium clock interact with each other to generate a pacemaker potential (Monfredi et al., 2013). For example, if Na^+ and K^+ from HCN depolarize the cell, then Ca^{2+} release from the SR, triggering Na^+ influx from forward-mode NCX, simply adds to this depolarization. If an integrated model is the reality, the relative importance of particular ion channels or groups of channels will be debated likely for years to come. Indeed, to add more complexity, recent

evidence suggests that Ca^{2+} cycling through sinoatrial cell mitochondria may also play a role in pacing (Yaniv et al., 2012).

1.3 Cardiac pacemaking in fish

Rainbow trout (*Onchorhynchus mykiss*) pacemaker potentials and action potentials from pacemaker cells, as revealed by sharp-microelectrode studies (Havarninen and Vornanen, 2007), look very similar to those found in mammals. A role for HCN channels from the membrane clock model in setting heart rate has been supported in rainbow trout by the finding that application of HCN antagonists reduces resting heart rate by 11%, and maximal heart rate by 33% (Keen and Gamperl, 2012). Additionally, HCN channels have been sequenced in trout saccule hair cells and a defective HCN channel appears to be the cause of the *slow mo* mutation in embryonic zebrafish (*Danio rerio*), which is associated with a reduced heart rate (Cho et al., 2003; Backer et al., 2004; Jackson et al., 2007). HCN sequences can be found in the genomes of fugu (*Takifugu rubripes*), green puffer fish (*Tetraodon fluvatilis*), and zebrafish.

No literature exists on the role of the calcium clock on cardiac pacing in fishes. However, inferences can be made from Havarninen and Vornanen (2007) where ryanodine and thapsigargin (an antagonist of the SR-Ca^{2+} re-uptake ATPase, SERCA) were added to rainbow trout hearts. Heart rate was not reduced until the hearts were exposed to temperatures nearing the upper thermal limit (18°C) of the

species and beating at double the rate compared to 11°C, and three-times higher than that at 4°C. These data suggest that the calcium clock may become important at extremely high temperatures or heart rates. However, because pacemaker potentials have never been recorded under these conditions, the mechanism behind this reduction in heart rate at higher temperatures is unknown. In addition, studies on atrial and ventricular myocardium show that the relative role of SR Ca^{2+}-release compared to L-type Ca^{2+} influx in cardiac muscle contraction is species dependent, with more active fishes, such as tuna (*Thunnus sp.*), having a larger reliance on SR-sourced Ca^{2+} compared to more sluggish fishes, such as carp (*Cyprinidae sp.*) (Vornanen, 1989, Shiels et al., 1998, Shiels et al., 1999, Shiels and Farrell 2000). This could result in the calcium clock being important in setting intrinsic rate in these active teleost fishes, but of lesser or negligible importance in more sedentary species. Therefore, studies on Ca^{2+} cycling and the calcium clock among fish species would be of interest for further study beyond the scope of my thesis.

1.4 Initiation of the hagfish heartbeat

Previous work on pacemaker physiology of hagfishes is restricted to two electrophysiological studies that used intracellular recordings of pacemaker potentials from throughout the working myocardium in spontaneously contracting hearts (Jensen, 1965, Arlock, 1975). The well-defined pacemaker potential and action potential of hagfish pacemaker cells look very similar in shape to those in vertebrates. Additionally, as in other craniate hearts, further indication of pacemaker

ability throughout the heart is that when a hagfish heart is cut up, each isolated chunk will continue to beat and contract independently. However, Jensen (1965) showed that the diastolic membrane potential of hagfish cardiac cells is much less negative than that of vertebrates, being -41 mV and -48 mV in the atrium and ventricle, respectively, versus -65 and -75 in teleost fishes (Vornanen et al., 2002b).

Thus, like in fishes, the roles of the membrane and calcium clock have not been studied in hagfish. However, hagfish have the lowest power generating heart of all craniates (Satchell, 1986) and evidence suggests that there is low RyR density in hagfish hearts (Thomas et al., 1996), which implies a minimal role of SR Ca^{2+} release in hagfishes and therefore suggests a major role for the membrane clock. Therefore, my thesis specifically aims to characterize cardiac pacing by the membrane clock in hagfish hearts, deferring detailed investigation of the calcium clock model to future research.

1.4.1 Hyperpolarization-activated cyclic nucleotide-gated channels

To better introduce my working hypothesis that intrinsic heart rate in the Pacific hagfish is set by the membrane clock, regulation of I_f by HCN channels is introduced further.

HCN channels are part of the VG K^+ channel super family and are structurally and functionally closely related to the ether-a-go-go and cyclic nucleotide-gated K^+ channels. These channels consist of four protein subunits each of which consists of

six transmembrane segments, the fourth of which is a voltage sensor (Craven and Zagotta, 2006; Jackson et al., 2007). The ion-conducting pore lies between segments 5 and 6 and a cytoplasmic cyclic nucleotide-binding domain (CNBD) is joined to the rest of the protein by the C-linker, a portion of the protein involved in tetramerization (Fig. 1.3). cAMP binding causes conformational changes in the CNBD subunit and the adjacent C-linker, allowing the formation of a gating ring between closely positioned subunits (Chow et al., 2011). Binding of cAMP to the other three subunits of the ring becomes more difficult following binding of the first cAMP (Chow et al., 2011). Vertebrates have four isoforms of the HCN protein subunit, each encoded by a separate gene and numbered HCN1 to HCN4. To make up a complete channel, these isoforms can come together as homotetramers or heterotetramers. For example, HCN1 is known to form channels with HCN2 and HCN4, and HCN2 with HCN4, however, HCN2 has not been found as a heterotetramer with HCN3 (Much et al., 2003; Whitaker et al., 2007).

High sequence identities (80-90%) between the HCN isoforms suggest that they arose from gene duplications of an ancestral HCN3-like gene (Jackson et al., 2007). In *Ciona*, a tunicate, there have been lineage specific duplications of an ancestral HCN3-like gene, resulting in *Ciona* having three isoforms denoted as HCNa, HCNb and HCNc. Some teleost fishes, such as the green puffer, have multiple copies (paralogs) of some isoforms, likely a result of lineage specific gene or whole genome duplications common to teleost fishes (Jackson et al., 2007).

Nothing is known about HCN structure, expression or regulation in cardiac tissues of hagfishes, despite their key evolutionary position in the craniate lineage. My phylogenetic analysis of HCN structure and expression aims to highlight the evolution of HCN-channel isoforms in this ancestral craniate heart.

1.5 Regulating heart rate with HCN

The slope of the pacemaker current sets the frequency at which action potentials are generated in pacemaker cells. Consequently, anything that alters I_f can potentially alter heart rate. There are several known mechanisms that alter gating of HCN, and consequently I_f.

1.5.1 HCN gating by cAMP

The CNBD inhibition of I_f is inversely proportional to the concentration of cAMP in the cytoplasm. Thus, binding of cAMP increases the channel open probability, increasing I_f, and, in pacemaker cells, an increase in cAMP increases the slope of the pacemaker potential (DiFrancesco and Tortora, 1991; Wainger et al., 2001). cGMP and cCMP also gate HCN channels, however, much higher concentrations are needed compared to cAMP (DiFrancesco and Tortora, 1991). cAMP also activates protein kinase A, allowing for phosphorylation of the HCN channel to further increase its open probability (Chang et al., 1991; Yu et al., 1993b).

Clearly, changing the intracellular cAMP concentration and cAMP gating in pacemaker cells is an effective means to change heart rate. cAMP is produced by the metabolism of ATP catalyzed by adenylyl cyclase, with pyrophosphate as a byproduct (Kaumann et al.,1982; Waelbroeck et al., 1983; Ikezono et al., 1987; Guo et al., 2004). Consequently modulation of adenylyl cyclase activity can alter heart rate. Both the autonomic nervous system and hormones such as catecholamines can alter cytoplasmic cAMP through SL receptors as described below.

1.5.2 Modulation of adenylyl cyclase activity by the autonomic nervous system and catecholamines

Three primary extrinsic heart rate control mechanisms exist in vertebrates: 1) parasympathetic (vagal) innervation; 2) sympathetic (adrenergic) innervation; and 3) hormonal control (Nilsson, 1983). Vagal (cholinergic) and sympathetic (noradrenergic) controls are rapidly conducted by the autonomic nervous system, while hormonal control is mediated by catecholamines (noradrenaline and adrenaline) released from either adrenal or other chromaffin tissues. Autonomic neural and hormonal stimulation have been well studied in mammals and fishes, but to a lesser extent in hagfishes. These mechanisms act by stimulating or inhibiting cAMP production via transmembrane adenylyl cyclase (tmAC) increasing or decreasing the pacemaker potential respectively as described for each system below.

The vagus nerve innervates all vertebrate hearts, with cell bodies situated in the area of the primary pacemaker (Yamauchi and Burnstock, 1968; Burnstock, 1969; Santer, 1971; Santer and Cobb, 1972). Vagal stimulation is cardioinhibitory in all vertebrates from elasmobranchs to mammals, with acetylcholine universally acting on muscarinic receptors in the membrane of pacemaker cells (Randall, 1966; Cameron, 1979; Holmgren, 1981; Nilsson, 1983; Taylor, 1992;). Muscarinic receptors are linked to an inhibitory G-protein that when stimulated antagonizes tmAC, which decreases cAMP production, and subsequently, decreases the slope of the pacemaker potential and causes a hyperpolarization (Sorota et al., 1958; DiFrancesco, 2010).

In fishes, vagal stimulation of the heart is responsible for the well-documented hypoxic bradycardia, a response blocked by the muscarinic antagonist atropine (Nilsson, 1983; Farrell, 2007b, Gamperl and Driedzic, 2009). Interestingly, when mammals are exposed to hypoxia, they experience a tachycardia due to adrenergic stimulation (Lifson et al., 1977; Marshall and Metcalfe, 1988). Hagfishes do not have a cardiac branch of the vagus nerve and cannot respond in this manner. Also, injections of acetylcholine and atropine have no effect on hagfish heart rate, which means that the receptor system is likely absent (Axelsson et al., 1990; Forster et al., 1992).

Despite histological recordings of ganglion-like cells close to, and in, the epicardium of the ventricle, evidence from comprehensive dissections, histological and

functional physiological studies show that the hagfish heart is anatomically and functionally aneural (Greene, 1902; Augustinsson et al., 1956; Jensen, 1961; Hirsch et al., 1964; Jensen, 1965). Interestingly, embryonic mammalian hearts pass through an aneural stage; contractions start at embryonic day 9 in the rat but vagal control starts at day12 (Greene, 1902; Goss, 1938; Gómez, 1958; Ebert and Thompson, 2001). Also, following a surgical transplant, the human heart is also aneural. However, as shown below in hagfish, being aneural does not preclude effective modulation of heart rate.

Cardiac vagal innervation is first seen in the lamprey, a related cyclostome, but in this case acetylcholine is cardiostimulatory as a result of nicotinic receptors on the cardiac tissue (Greene, 1902; Augustinsson et al., 1956; Jensen, 1961; Jensen, 1965, Farrell, 2007a).

Catecholamine stimulation of the heart rate involves noradrenaline and adrenaline. Both can be released into the circulatory system from chromaffin tissue, while noradrenaline is also released from sympathetic nerve endings whenever they innervate the heart (Nilsson, 1983). Sympathetic nerves innervate the sinoatrial node of mammals, some teleosts, but not elasmobranchs (Gannon and Burnstock, 1969; Cameron, 1979; Donald and Campbell, 1982; Taylor, 1992; Zaccone et al., 2009). Thus, sympathetic cardiac innervation appeared even later than vagal innervation.

All fishes, instead, release noradrenaline and adrenaline into the blood in varying amounts and proportions from chromaffin tissue during stress and hypoxia when, arterial oxygen saturation reaches about 50% (Nandi, 1961; Nilsson, 1983; Farrell and Jones, 1992; Perry and Reid, 1992; Taylor, 1992; Zacone et al., 2009).

Hagfishes are among a small group of craniates, whose hearts contain chromaffin cells filled with catecholamines that show up as densely staining granules in electron micrographs (Johnels and Palmgren, 1960; Östlund, 1960; Bloom et al., 1961; von Euler and Fänge, 1961; Jensen, 1961; Perry et al., 1993). These local catecholamine stores may compensate or account for the much lower circulating catecholamine concentrations found in hagfish blood, even under very stressful conditions, when compared to teleosts (Perry et al., 1993; Farrell, 2007a). Indeed, there is clear evidence that the hagfish heart acts as a catecholamine-releasing gland exerting paracrine actions on the heart. For example, application of adrenaline to a perfused hagfish heart preparation and *in vivo* produced a very slight tachycardia (Fänge and Östlund, 1954; Forster et al., 1992). Blocking catecholamine action with dihydroergotamine during cardiac perfusion decreased heart rate, with heart rate being restored with subsequent adrenaline or noradrenaline administration (Fänge and Östlund, 1954).

Despite the various sources of noradrenaline and adrenaline, both humoral and neural stimulation of heart rate in most vertebrates is due to β-adrenoreceptor-mediated stimulation of tmAC via a stimulatory G-protein. This then increases cAMP

production, leading to an increase in I_f as described above (Brown et al., 1979a; Brown et al., 1979b; Brown and DiFrancesco, 1980; Kaumann et al., 1982, Waelbroeck et al., 1983, DiFrancesco, 1985; Ikezono et al., 1987, Lohse et al., 2003, Guo et al., 2004, DiFrancesco, 2010). However, in elasmobranchs, perch (*Perca fluviatilis*), eel (*Anguilla anguilla*) and possibly the winter flounder (*Pleuronectes americanus*) an α-adrenoreceptor-mediated bradycardia is found upon catecholamine stimulation (Capra and Satchell, 1977; Peyraud-Waitzenegger et al., 1980; Tirri and Ripatti, 1982; Mendonça and Gamperl, 2009).

1.5.3 The variable heart rate of the aneural Pacific hagfish heart

Given the aneural nature of the hagfish heart, it might be expected that the heart rate of the hagfish doesn't change much. However, this is not what is found in the laboratory. The most dramatic change in heart rate reported for hagfish occurs during recovery from prolonged anoxia. Pacific hagfish can survive, and fully recover from a 36-h anoxic exposure (lack of environmental oxygen; Cox et al., 2010). Several hours into this anoxic period, heart rate had slowed to approximately 5 bpm, a rate that was then sustained for the next 34 h. Yet within 1.5 h of reaerating the water, heart rate increased to approximately 17 bpm before returning to the routine rate. Thus, within 1.5 h, heart rate could approximately double the routine heart rate and quadruple the anoxic rate without any neural control. How such large, albeit slow, changes in hagfish heart rate are controlled is unknown. My thesis aims to answer how an aneural heart can control its rate.

Results from *in vivo* injections of the β-blockers sotalol or propranonol that produced marked but slowly developing bradycardia in the Atlantic hagfish, (*Myxine glutinosa*) and the New Zealand hagfish (*E. cirrhatus*) strongly suggest a tonic catecholaminergic cardiac stimulation via β-adrenergic receptors (Axelsson et al., 1990; Forster et al., 1992). Thus, my thesis will examine the effects of catecholamine stimulation on hagfish heart rate during normoxia, anoxia and subsequent recovery. My working hypothesis being, that there is a tonic, paracrine stimulation of the hagfish heart that is removed upon anoxic exposure, resulting in bradycardia due to reduced cAMP production. This stimulation is restored, and increased during recovery from anoxia, resulting in the observed tachycardia.

1.6 Overview of thesis questions and working hypotheses

My primary thesis question is: How is heart rate controlled in an aneural heart? My primary hypothesis is that the aneural heart of the Pacific hagfish is controlled by mechanisms only located within the heart and involving HCN channels.

In order to test my primary hypothesis, I will examine three specific aspects of cardiac control, each of which has their own testable hypotheses and experimental approach. These are summarized below. Subsequent chapters detail the approach, results and discussion. My thesis closes with overall conclusions addressing each hypothesis and an integrated model of hagfish cardiac control.

1.7 Initiation of the heartbeat

1.7.1 Does the membrane clock set intrinsic heart rate in Pacific hagfish?

The hagfish heart is myogenic, has pacemaker potentials, has a low power generating ability and has a low ryanodine receptor density. I propose that the membrane clock is responsible for the initiation of the hagfish heart rate. In order to address the hypothesis that "the heartbeat is initiated by I_f", I will examine whether or not HCN channels are expressed in hagfish hearts.

Pacemaker potentials are present throughout hagfish myocardium and isolated pieces of myocardium continue to spontaneously contract (Jensen 1965). Assuming I_f is responsible for the pacemaker potential, HCN channels must also exist in both myocardial chambers. Also, since multiple HCN isoforms have been found in both urochordates (*Ciona*) and throughout the vertebrates, it is likely that multiple isoforms exist in the hagfish. Given that the *Ciona* isoforms closely resemble HCN3, it is also likely that this is the ancestral isoform of HCN. I will test the following specific hypotheses concerning the initiation of the heartbeat.

Hypothesis: The heartbeat is initiated by HCN channels that generate I_f.

This hypothesis will be tested pharmacologically using ion channel blockers such as zatebradine, a specific HCN channel blocker. To assess the respective importance of the membrane clock and calcium clock models of pacemaker function the pharmacological agent ryanodine will be used. (See Chapter 2)

Hypothesis: Multiple isoforms of HCN exist in the hagfish heart, with HCN3 being the ancestral isoform.

This hypothesis will be addressed using a phylogenetic analysis built from the partially cloned hagfish genes and other craniate and *Ciona* HCN genes to confirm that HCN3 is the ancestral craniate HCN isoform as shown by Jackson et al. (2007). (See Chapter 3)

Hypothesis: HCN channel mRNA is expressed in both cardiac chambers of the hagfish heart.

This hypothesis will be tested using partial gene cloning using tissue from both the atrium and the ventricle. (See Chapter 3)

1.8 Synchronization of cardiac contraction

1.8.1 Are HCN channels involved in synchronous beating of the cardiac chambers in the Pacific hagfish?

If my results suggest HCN channels and I_f exist in both cardiac chambers, how can such a heart contract synchronously? Unlike in chambered hearts, *Ciona* can initiate cardiac contraction at either end of its contractile tube, causing blood flow to periodically reverse directions (Kriebel, 1970). Efficient cardiac contractions in a chambered heart require a completed atrial contraction before the ventricle, preferably with a small delay to allow for complete ventricular filling. While the

cardiac conduction system of the hagfish is unknown, pacemaker activity in mammalian and teleost hearts is not restricted solely to the primary pacemaker. The atrioventricular node of both taxa, as well as the Purkinje fibers and bundle of His in mammalian hearts, also express pacemaker potentials, and it is possible for cardiomyocytes to express pacemaker potentials under certain conditions. In mouse and rabbit, HCN gene expression per unit mass is greater in atria than ventricles, and is even higher in the sinoatrial node. This allows the complicated conduction system to function correctly, and the heart as a whole to contract synchronously (Shi et al., 1999; Marionneau et al., 2005). This is because a larger HCN channel density would allow a larger total current to flow, assuming gene expression is mirrored in protein expression. Therefore, the magnitude of I_f and, in turn, the gradient of the pacemaker potential would be greater.

In addition, or conversely, the electrophysiological properties of the four HCN isoforms might differ, allowing a further level of intrinsic control of cardiac conduction. For example, HCN1 has the shortest activation time constant, followed by HCN2, then HCN3 with HCN4 being the longest (Moosmang et al., 2001). This means that HCN1 channels open the fastest, with HCN4 opening slower. Evidence that HCN isoform expression influences cardiac conduction comes from work on mice and rabbits, where the dominant isoform in the sinoatrial node is HCN4, whereas in the ventricle, HCN2 dominates (Shi et al., 1999; Marionneau et al., 2005). Also, in rabbit Purkinje fibres, HCN1 is the dominant isoform, very closely followed by HCN4 (Shi et al., 1999). Given that HCN1 has the fastest time constant,

cardiac excitability is probably controlled by both HCN channel density and isoform expression in order to keep the sinoatrial node more excitable than the Purkinje fibres. If sinoatrial HCN4 was expressed at the same density as Purkinje fibre HCN1, the fibres would be more exciteable than the sinoatrial node, therefore, HCN4, and total HCN expression needs to be higher in the sinoatrial node.

Hypothesis: The intrinsic rate of atrial contraction is higher than that of the ventricle.
This hypothesis will be tested using an atrioventricular block to investigate conduction of the wave of excitation from the atrium to the ventricle and to compare atrial pacemaker function to that of the ventricle. (See Chapter 2)

Hypothesis: Higher HCN mRNA expression in the atrium compared to the ventricle supports a higher spontaneous atrial rate.
This hypothesis will be tested using quantitative real-time reverse transcriptase PCR to quantify and compare mRNA expression of HCN channels between the cardiac chambers. (See Chapter 3)

Hypothesis: Different HCN isoforms are expressed in the atrium compared to the ventricle.
This hypothesis will be tested by quantifying the mRNA of each isoform in each chamber to find out both, which channel is dominant, and if, and how, HCN isoform expression differs in each chamber. (See Chapter 3)

1.9 Control of heart rate

1.9.1 Does varying HCN mRNA expression during anoxia and subsequent recovery suggest that HCN channel expression may have a role in heart rate control under these conditions?

In the embryonic mouse heart, a developmental transition from HCN3 to HCN4 expression occurs when cardiac contractions first begin (Qu et al., 2008; Schweizer et al., 2009). Thus, there is clear evidence suggesting that changing HCN isoform expression, on a much slower time course than afforded by typical neural and humoral regulation, might change heart rate. It is possible that during prolonged anoxia and recovery, HCN gene expression could alter and change heart rate, as seen *in vivo* during anoxia (Cox et al., 2010).

Hypothesis: HCN mRNA expression changes in response to prolonged anoxia and subsequent recovery.

This hypothesis will be tested by comparing the mRNA expression of each HCN isoform in atria and ventricles of hagfish hearts from animals exposed to normoxia, anoxia and recovery from anoxia. While measuring mRNA expression does not give a direct measurement of protein expression in the membrane, it does possess some advantages over measuring protein expression. The CNBD of HCN is highly conserved; however, it differs slightly between isoforms. Therefore, it is possible to design and test neucliotide primers to search for HCN isoforms, and then measure them using real-time reverse transcriptase PCR (RT-PCR). Moreover, to measure

protein expression, antibodies that are specific to each isoform are needed, which has proven to be very difficult, especially for HCN3. Therefore, for this exploratory study of HCN expression in hagfishes, mRNA expression will be measured, leaving protein expression for future work. (See Chapter 3)

1.9.2 Does varying tmAC activation modulate heart rate?

Evidence suggests that the hagfish heart contains chromaffin tissue that exerts paracrine catecholamine stimulation of the heart itself. Via β-adrenoreceptors and tmAC, this would increase basal heart rate by stimulating cAMP production, removing inhibition of the CNBD on HCN channels.

Hypothesis: The heart receives tonic stimulation of tmAC by catecholamine stimulation of β-adrenoreceptors.

This hypothesis will be tested pharmacologically using agonists and antagonists of tmAC in isolated hearts in normoxia while monitoring heart rate. (See Chapter 4)

A related hypothesis is that:

Hypothesis: cAMP concentration in the heart decreases during prolonged anoxia.

This hypothesis will be tested by subjecting hagfish to prolonged anoxia and sampling their hearts. cAMP concentration in these hearts will then be determined using a chemiluminescent immunoassay kit. (See Chapter 4)

1.9.3 Are there other mechanisms of HCN gating with cAMP?

Since only modest increases in resting hagfish heart rate have been found in response to tmAC stimulation by adrenaline, it is unlikely that an increase in tmAC is responsible for the doubling of heart rate in recovery from anoxia when compared to the resting rates by Cox et al. (2010) (Axelsson et al., 1990). However, in addition to tmAC, a soluble counterpart has been recently discovered. Soluble adenylyl cyclase (sAC) was discovered in rat testes (Buck et al., 1999) and has since been discovered to play a role in kidney, eye, respiratory tract, digestive tract including the pancreas, bone, neural and immune function (see review by Tresguerres et al., 2011), as well as in animals as evolutionarily diverse as elasmobranchs and corals (Tresguerres, 2010; Barott et al., 2013). Unlike tmAC, sAC is activated by bicarbonate ions, not G-linked proteins. As for the heart, a small amount of work suggests that soluble adenylyl cyclases are involved in the apoptosis signaling cascade in epithelial cells and cardiac myocytes (Chen et al., 2012). To date, no one has considered the possibility that sAC is involved in heart rate control. These considerations lead to three testable hypotheses.

Hypothesis: sAC is present in hagfish hearts.

This hypothesis will be tested using a Western blotting and immunofluorescence to identify and localize sAC in hagfish hearts using an anti-sAC antibody. (See Chapter 4)

Hypothesis: sAC produces cAMP in response to bicarbonate stimulation.

A cAMP production assay using homogenized hagfish hearts will determine if cAMP is produced in response to sAC agonism by bicarbonate ions and blocked in response to sAC antagonism. (See Chapter 4)

Hypothesis: sAC stimulation is involved in hagfish cardiac control.
This hypothesis will be tested pharmacologically using bicarbonate ions and an antagonist of sAC in isolated hearts before, and during, anoxia while monitoring heart rate. (See Chapter 4)

1.10 My proposed model of heartbeat control in an aneural heart.

The results of testing my hypotheses will be used to generate a model to explain the 4-fold change in heart rate seen by Cox et al. (2010) implicating both tmAC and sAC regulation of cAMP in the control of hagfish heart rate (Fig. 1.4). The rate limiting step of catecholamine production, the conversion of tyrosine to 3,4-dihydroxyphenylalanine, is oxygen dependent (Levitt et al., 1965). Therefore, in anoxia, it makes sense that tonic catecholamine production, must eventually cease. This would reduce stimulation of tmAC and cAMP production, and subsequently heart rate. Concurrently, anoxia decreases bicarbonate ion concentration due to both a reduction in CO_2 production from aerobic respiration and an increase in H^+ production from metabolic acidosis. Therefore, bicarbonate stimulation of sAC would also decrease during anoxia, reducing heart rate alongside the actions of tmAC.

Upon reoxygenation, catecholamine production and tmAC stimulation would restart increasing cAMP production. Concurrently, an increase in bicarbonate ion concentration due to aerobic respiration being restored would increase cAMP production from sAC. This, together with tmAC stimulation will increase heart rate in the recovery phase. As bicarbonate levels return to normal, sAC stimulation would return to routine levels, as would heart rate, supported by tonic tmAC stimulation.

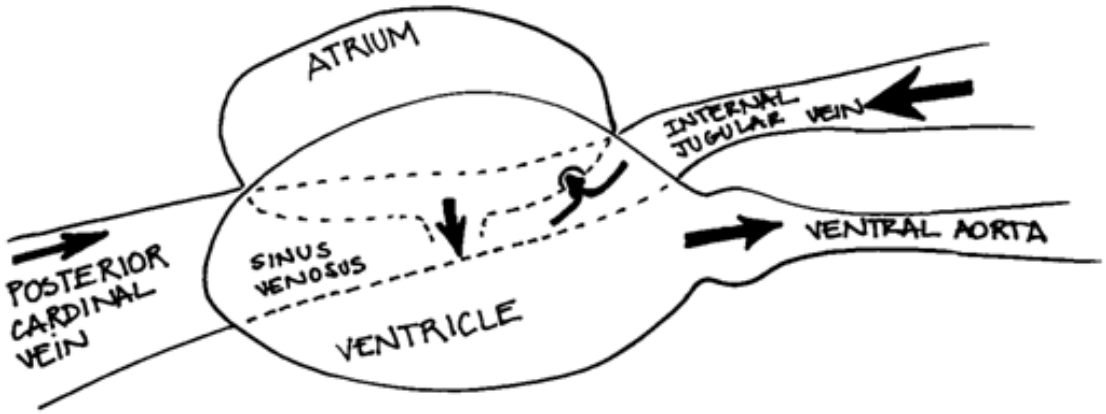

Figure 1.1. Diagram of the hagfish heart highlighting the cardiac chambers and the input and output vessels. Blood enters the sinus venosus from the internal jugular vein and posterior cardinal vein. From the sinus venosus, blood is pushed into the atrium, then the ventricle and exits through the ventral aorta. Arrows indicate blood flow direction.

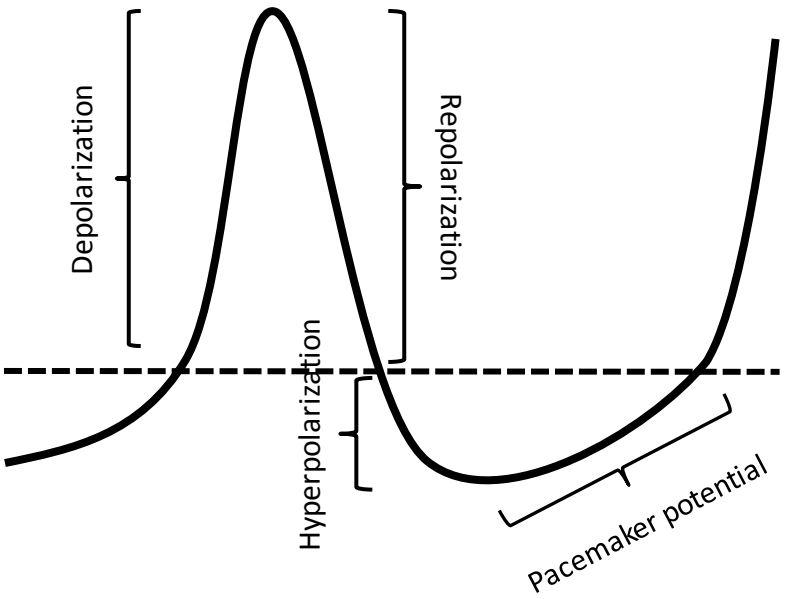

Figure 1.2. The major electrical stages of a cardiac pacemaker cell action potential. Upon the reaching of the T-type Ca^{2+} threshold potential (indicated by dashed line), an influx of Ca^{2+} depolarizes the cell. Efflux of potassium repolarizes the cell, and even hyperpolarizes it, activating the pacemaker potential. This steady influx of Na^{+} and K^{+} gently depolarizes the cell back to the threshold for T-type Ca^{2+}. See text for more details.

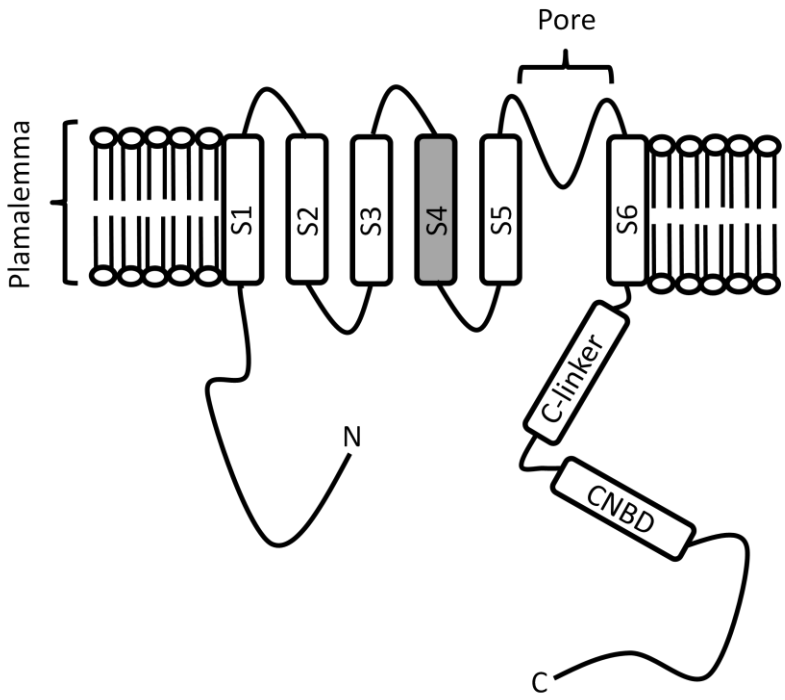

Figure 1.3. Schematic of a Hyperpolarization-activated cyclic nucleotide-gated (HCN) channel subunit showing the major protein segments. Four subunits come together to form a complete channel. Six transmembrane segments (S1 to S6) contain both the voltage sensor (gray shading) and the pore region. The C-linker joins the cyclic nucleotide binding domain (CNBD) to S6. Here cAMP binds to remove the auto-inhibition of the CNBD, allowing a greater current (I_f) to flow. N and C refer to their relative termini. Based on Figure 1 from Jackson et al., 2007.

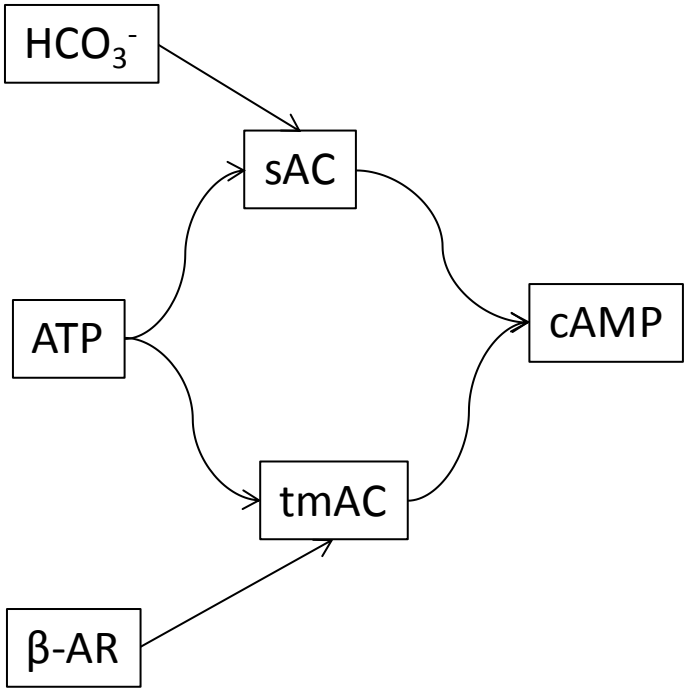

Figure 1.4. A proposed model of cAMP production in a hagfish cardiac pacemaker cell. ATP is converted to cAMP via adenylyl cyclases. Transmembrane adenylyl cyclase (tmAC) is agonized by stimulation from β-adrenoreceptors that are in turn stimulated by catecholamines. Soluble adenylyl cyclase (sAC) is agonized by bicarbonate ions (HCO_3^-). In turn, cAMP binds to hyperpolarization-activated cyclic nucleotide-gated channels to facilitate channel gating and increasing heart rate.

CAPTER 2: PHARMACOLOGICAL CHARACTERIZATION OF THE HEARTBEAT IN AN EXTANT VERTEBRATE ANCESTOR, THE PACIFIC HAGFISH, *EPTATRETUS STOUTII*

2.1 Introduction

Modern day hagfishes are extant representatives of the ancestral chordate. Their heart shares many features common to vertebrate hearts, such as valved atrial and ventricular chambers, myogenic contractions and a Frank-Starling response to filling pressure (reviewed by Farrell, 2007), but lacks autonomic nervous innervation (Greene, 1902; Augustinsson et al., 1956; Jensen, 1961; Jensen, 1965). Yet, hagfish can still vary *in vivo* heart rate considerably. For example, the heart rate of Pacific hagfish, *Eptatretus stoutii*, changes 4-fold (5 – 17 bpm) during an anoxic challenge and subsequent recovery phase (Cox et al., 2010). In vertebrates, heart rate is primarily controlled via the autonomic nervous system (vagal deceleration and sympathetic acceleration of the intrinsic pacemaker rate), mechanisms that are unavailable to modern day hagfishes (Nilsson, 1983). Therefore, to understand heart rate control in hagfishes, the present study aimed to discover what mechanisms for regulating intrinsic heart rate might be targets for control.

Electrophysiological studies of hagfish hearts are very limited (Jensen, 1965; Arlock, 1975; Satchell, 1986). They are distinguished from vertebrate hearts by having a prolonged atrioventricular delay and a more depolarized resting membrane potential,

as well as possessing pacemaker potentials in all areas of the atrium and ventricle, which suggests that the hagfish heart may be less dependent on its primary pacemaker cells. The action potential of the working myocardium of the vertebrate heart, has a Na^+-based upstroke, an L-type Ca^{2+} mediated plateau phase and a more stable resting membrane potential maintained by the inward rectifier K^+ current and a reduced pacemaker potential (Tomaselli and Marbán 1999; Bers 2002; Vornanen et al., 2002a). Specialized pacemaker cells set the spontaneous rhythm of the vertebrate heart with an action potential generated by a T-type Ca^{2+}-mediated upstroke with an L-type Ca^{2+} component. Two hypotheses exist for the rhythmicity of the pacemaker action potential; one is a slowly depolarizing K^+ and Na^+ current (I_f) between action potentials (termed the pacemaker potential) which is regulated by the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel (Brown et al., 1979b; Brown and DiFrancesco, 1980; DiFrancesco and Ojeda, 1980; Yanagihara and Irisawa, 1980; Hagiwara et al., 1988; Doerr et al., 1989; Zhou and Lipsius, 1994; DiFrancesco, 2010). The other is intracellular Ca^{2+} cycling through the sarcoplasmic reticulum (SR) that rhythmically activates an inward sodium/calcium exchanger current (I_{NCX}) as the membrane potential nears that of the activation potential for T-type I_{Ca} (reviewed by Maltsev and Lakatta, 2008). Irrespective of how the pacemaker potential depolarizes the cell membrane, once the activation potential for T-type I_{Ca} is reached, the rapid upstroke of the action potential is triggered, initiating the wave of contraction in the heart. To gain further insights into how the hagfish heartbeat is initiated and then spread throughout the myocardium to better direct

electrophysiological studies, the present study examined the effects of channel blockers on the contraction rate of spontaneously beating, isolated hagfish hearts.

2.2 Materials and methods

2.2.1 Animals

Pacific hagfish, *Eptatretus stoutii*, were caught off the coast of Bamfield Marine Sciences Centre, Bamfield, B.C. Canada using baited traps and transferred to flow-through seawater fibreglass tanks after capture. A total of 36 hagfish were used in the present study with a body mass averaging 94 ± 1 g. Animal care approval was provided by both the University of British Columbia and Bamfield Marine Sciences Centre in accordance with the Canadian Council on Animal Care. Fish were sacrificed by decapitation, after which the heart was rapidly excised by first removing the liver and gall-bladder to gain easier access to the heart. The heart was immediately placed in ice-chilled saline and experiments were conducted immediately.

In order to measure intrinsic atrial and ventricular contraction rates, six hearts had the atrioventricular canal crushed by tying a silk ligature tightly around the canal to create an atrioventricular conduction block. Spontaneous contraction rates of the atrium and ventricle were then measured every 5 min for the next 2 h to determine if they remained stable.

2.2.2 Heart rate observations

The spontaneously beating isolated heart was monitored on a microscope slide using a Digital Blue QX5 Microscope connected to a Sony Vaio FW Series Notebook running CamStudio Recorder. The heart was continually perfused with chilled saline that was delivered via a multi-barrelled solution exchanger allowing rapid (<30 s) fluid exchange for the drug treatments. Saline temperature was maintained at 10°C throughout the experiments using a dual in-line heater/cooler (Warner Instruments SC- 0 coupled with Warner Instruments Bipolar temperature controller CL-100, Hamden, Connecticut, USA). Heart rate was allowed to stabilize for 30 min prior to any experimental treatment, and heart rate at this time was taken as the control value. Increasing concentrations of the four channel blockers were then added to the saline every 30 min and only one blocker was tested on each preparation (n=6 per blocker). Recordings of contraction rates were recorded every 5 min to ensure a steady state had been reached with each concentration.

2.2.3 Solutions and pharmacological agents

I_f was blocked with zatebradine (0.005, 0.05, 0.5 and 5 mM; Tocris Bioscience, Minneapolis, Minnesota, USA). Calcium cycling through the SR was blocked with ryanodine (0.1, 1.0, 10 and 100 μM). Voltage-gated (VG) I_{Na} was blocked with tetrodotoxin (TTX: 0.01, 0.1, 1 and 10 μM; Tocris Bioscience, Minneapolis, Minnesota, USA). T-type I_{Ca} was blocked with $NiCl_2$ (0.01, 0.1, 1 and 10 mM; Sigma Aldrich, St. Louis, Missouri, USA). L-type I_{Ca} was blocked with nifedipine (0.1, 1, 10 and 100 μM; Sigma Aldrich, St. Louis, Missouri, USA) prepared in 95.5% DMSO

(Sigma Aldrich, St. Louis, Missouri, USA). All solutions were stored at 5°C and protected from light until used. Hagfish saline contained (mM): 450 NaCl, 5.4 KCl, 2 $CaCl_2$, 10 $MgSO_4$, 0.4 NaH_2PO_4, 20 glucose and10 HEPES, and pH was adjusted to 7.9 using NaOH.

2.2.4 Calculations and statistical analyses

Data are presented as means ± standard error of the mean (s.e.m., n=6 per blocker treatment). Normally, the atrium and ventricle contracted in synchrony and at the same rate. Some blockers created asynchrony and a Student's *t*-test was used to determine if a measured ventricular contraction rate remained above, at, or below (P<0.05) the rate determined for the atrioventricular block via ligature. The ventricular contraction rate following the atrioventricular block with a ligature was expressed as a percentage of the atrial rate, and is represented as a line of identity in graphical presentations of the effects of channel blockers. Statistically significant treatment effects (P<0.05) were determined using repeated measures two-way ANOVA, with a Holm-Sidak post-hoc test for comparisons among blocker concentrations and between contraction rates of the atrium and ventricle.

2.3 Results

2.3.1 Atrioventricular block

Heart rate for whole, isolated hagfish hearts perfused with saline at 10°C for 30 min was 21.1 ±1.1 bpm. The atrioventricular ligature had no significant effect on atrial

contraction rate (21.2 ± 0.4 bpm after 2 h), but the ventricle began contracting at a significantly slower and independent rate of 8.4 ± 0.3 bpm (Fig. 2.1), which is consistent with an atrioventricular conduction block. After the first 20 min, ventricular rate remained stable for the remainder of the 2 h period at less than half (41.3 ± 1.5%) of the atrial contraction rate (Fig. 2.1), which is the value used as a line of identity for intrinsic ventricular rate in subsequent figures.

2.3.2 Zatebradine

Zatebradine, an HCN channel blocker, decreased the atrial and ventricular contraction rates in a concentration-dependent manner. Atrial rate decreased to 23.5 ± 5.0% of the initial rate with 0.005 mM zatebradine (P<0.05, Fig. 2.2A). All but one heart ceased to contract with 5.0 mM, indicating the strong role of I_f in setting atrial rate. Ventricular rate followed the declining atrial rate, and decreased significantly below the intrinsic ventricular rate. Even so, the majority of ventricles contracted asynchronously with the atria with 0.5 mM zatebradine and, ultimately, significantly outpaced the atria with 5.0 mM zatebradine (P<0.05, Fig. 2.2A).

2.3.3 Ryanodine

Ryanodine, used to block calcium cycling through the SR, had no significant effects on atrial and ventricular contraction rates (Fig. 2.2B). No asynchronous beating was induced by ryanodine.

2.3.4 Tetrodotoxin

Tetrodotoxin (TTX), which blocks VG Na^+ channels, had different but concentration-dependent effects on atrial and ventricular contraction rates. At the highest TTX concentration, atrial rate was reduced to 64.9 ± 6.6% of the control rate, whereas ventricular rate was reduced to 44.6 ± 10.6% of the control rate, one that was not significantly different from intrinsic ventricular contraction rate (Fig. 2.2C). Thus, TTX had minimal effects on the atrial rate compared with zatebradine, despite the very high concentrations that were used. Instead, the major effect of TTX was apparently a chemically induced atrioventricular block, allowing the ventricle to beat at its own rate independent of the atrium.

2.3.5 Nickel

A T-type Ca^{2+} channel blocker, Ni^{2+}, had no significant effect on heart rate until a concentration of 1.0 mM, where there was a concentration-dependent decrease in contraction rates and contraction asynchrony (P<0.05, Fig. 2.2D). Atrial contractions almost ceased with 10.0 mM (6.1 ± 4.3% of the control rate), but ventricular rate continued at 30.5 ± 10.7% of the control rate, a level not significantly different from intrinsic ventricular rate (Fig. 2.2D). Thus, the atrium was more sensitive to a T-type Ca^{2+} channel blocker than the ventricle, which may have been insensitive since it contracted at its intrinsic rate.

2.3.6 Nifedipine

Nifedipine, an L-type Ca^{2+} channel blocker, significantly reduced atrial and ventricular contraction rates in a similar concentration-dependent manner at concentrations of 1.0 µM and higher (P<0.05, Fig. 2.2E). At the highest nifedipine concentration tested, atrial rate was 36.4 ± 28.7% and ventricular rate 41.1 ± 9.5% of the control rate, with occasional asynchrony (Fig. 2.2E). Thus, an L-type calcium channel blocker had a major effect on atrial rate compared to the ventricular rate, but independent effects on ventricle were not resolved as ventricular rate did not fall below the line of identity (Fig. 2.2E).

2.4 Discussion

The present study is the first to investigate the mechanisms underlying the heartbeat of the Pacific hagfish, an extant representative of an ancestral chordate and one devoid of autonomic cardiac innervation. The intrinsic heart rate at 10°C was 21.1 ± 1.1 bpm in the present study, which is slightly lower than that reported for isolated *E. stoutii* hearts at 15°C (26 ± 1.4; Jensen, 1965). This earlier study showed that the heart rate of *in situ E. stoutii* hearts increased with temperature, perhaps accounting for the difference between studies. Axelsson et al. (1990), showed the intrinsic heart rate of the perfused, working *Myxine glutinosa* heart was 22.3 ± 1.0 at 10-11°C, a similar rate to that reported here for *E. stoutii*. The stability of both the atrial and ventricular contraction rates for up to 2 h following application of an atrioventricular

ligature allowed me to be confident that the observed reductions in contraction rates seen after blocker applications were not run down of the heart.

An overriding caveat to the conclusions made below is the requirement for confirmatory studies using cellular electrophysiological techniques on hagfish cardiomyocytes. However, to date, cardiac myocytes have never been successfully isolated from any hagfish species, preventing ionic currents to be measured from single cells. Considering the present findings from whole hagfish hearts, and their evolutionary importance, electrophysiological recordings from isolated hagfish cardiac myocytes are worth doing. I also add a note of caution regarding the large concentration of some channel blockers that were required to elicit a reduction in contraction rates since there may have been diffusion issues in the present whole heart preparation when compared to single myocyte studies. However, high concentrations introduce the possibility of non-specific inhibition of other ion channels. In whole-cell patch-clamp studies a single current can be measured by controlling the voltage conditions of the sarcolemma. Therefore, I focused on how particular ion channel blockers altered contraction rate and chamber synchrony of the whole heart, something not possible with single myocytes. Pharmacological studies rely on concentration-dependent responses, which proved to be the case for the four blockers that showed significant results. In addition, asynchronous atrial and ventricular contractions with some blockers and comparisons with the intrinsic atrial and ventricular contraction rates helped distinguish independent atrial and ventricular effects of the blockers.

Zatebradine is a potent blocker of the HCN channel that has been used to reduce I_f in both vertebrate and urochordate hearts by binding to the pore of the HCN channel when it is in its open state, from within the cell, blocking Na^+ and K^+ entrance to the cell (I_f) (DiFrancesco, 1981; Van Bogaert and Goethais, 1992; Van Bogaert and Pittoors 2003; Stieber et al., 2006; Hellbach et al., 2011). A reduction in I_f causes an increased time for threshold to be reached in pacing cells due to a reduction in the gradient of the pacemaker potential, which then decreases intrinsic heart rate. Zatebradine significantly reduced ventricular contraction rate and essentially stopped atrial beating, whereas ryanodine had no significant effect on either chamber. These results suggest a vital role for the HCN channels in establishing the intrinsic heart rate of *E. stoutii*, contrasted with the lack of support for the calcium clock, as discussed further below.

In the urochordate, *Botryllus schlosseri*, whose heart consists of a single layer of myocytes and lacks valves (Kriebel, 1968; Delsuc et al., 2006), zatebradine reduced heart rate by 31.4% and 46.6% at 0.005 mM and 0.05 mM, respectively, compared to 23.5% and 60.7% in the present study with hagfish (Hellbach et al., 2011). The asynchrony seen here at higher zatebradine concentrations has also been reported in both *B. schlosseri* and mammals, and adds support to previous findings of different HCN channel expression among cardiac chambers in mammalian hearts (Biel et al., 2002; Marionneau et al., 2005; Stieber et al., 2006; Hellbach et al., 2011). However, zatebradine has been shown to have a small effect on delayed rectifier K^+ channels,

which are involved in repolarization of both pacemaker and cardiac myocytes (Bois et al., 1996). Therefore, a portion of reduction in contraction rates seen in the current and previous studies with zatebradine additions may be due to an extension of action potential duration prior to I_f activation upon hyperpolarization.

Ryanodine had little to no effect on contraction rate of the hagfish atria and ventricles, therefore the role of SR-calcium-cycling may be minor in terms of rate control for the hagfish. This result is not surprising considering the results of Thomas and colleagues (1996) suggesting extremely low ryanodine receptor density in hagfish hearts. The role of the SR in contributing Ca^{2+} during contraction has not been recorded, however the above results suggest it is minimal and, like other sluggish fishes such as carp, most of the Ca^{2+} influx occurs across the sarcolemma (Vornanen, 1989). It would be of interest to examine the role of SR-calcium-cycling in setting the heart rate of more active fishes known to have a larger SR dependence such as tuna, mackerel and trout (Shiels et al., 1998; Shiels et al., 1999; Shiels and Farrell, 2000). Finally, it would be of interest to use Ca^{2+} imaging of isolated pacemaker cells to track Ca^{2+} movements into the cytoplasm. This is because while the above results suggest that the calcium clock is not important for pacing in hagfish hearts, without further work, it is not possible to say more than ryanodine has no effect on hagfish hearts. It is possible that hagfish hearts posses a SR-calcium-cycling mechanism, but why it would be insensitive to ryanodine is unclear.

The relatively modest effect of TTX on hagfish heart rate was a surprising discovery. Tetrodotoxin, a potent neurotoxin naturally present in pufferfish (*Fugu rubripes*), binds to site 1 on the extracellular surface of the α-subunits of VG Na^+ channels occluding the pore and preventing influx of Na^+ (Scheib et al., 2006). Since a rapid I_{Na} through VG Na^+ channels is the initial phase of non-pacing action potentials, TTX prevents cardiomyocytes in some species from firing and contracting. Indeed, TTX is a potent inhibitor of I_{Na} in both chick and fish cardiomyocytes (Prakash and Tripathi, 1998; Haverinen et al., 2007). However, in contrast, hagfish atria continued to beat at 65% of the control rate with a pharmacological dose of 10 μM TTX. By comparison, the EC50 of TTX is orders of magnitude lower for chick hearts strips (4 nM), and even lower for trout atrial (1.8 nM) and ventricular (2.0 nM) myocytes (Prakash and Tripathi, 1998; Haverinen et al., 2007). Consequently, the hagfish heart is remarkably resistant to TTX, and since contraction rates of neither cardiac chamber decrease further with TTX from 1 μM to 10 μM, it is unlikely that the observation for the atrium is due to limited access of the toxin to the cells by the thickness of the preparation since the atrial wall is extremely thin.

Resistance to TTX is reported for both invertebrate and vertebrate species, including species that either accumulate TTX, or consume TTX-containing animals (reviewed by Soong and Venkatesh, 2006). TTX resistance is brought about by mutations to the TTX binding site in the VG-Na^+ channel protein. For example, in the TTX-resistant mammalian cardiac isoform of the VG-Na^+ channel (Na_v 1.4), there is a substitution of an aromatic tyrosine with a cysteine residue (Satin et al., 1992). The

same substitution of the aromatic amino acid, but with a serine, is also found in TTX-resistant mammalian Na_v 1.8 and Na_v 1.9 isoforms (Sivilotti et al., 1997).

Tetrodotoxin produced asynchronous as well as slower contractions. Even so, the atrial contraction rate outpaced the ventricular contraction rate, which remained at that of the atrioventricular block. The most parsimonious explanation for these results is that I_{Na} has a small role in setting the intrinsic atrial contraction rate in addition to the role of I_f. In contrast, I_f dominates in setting the intrinsic ventricular contraction rate with perhaps no contribution of I_{Na}. In the toad, *Bufo marinus*, TTX reduces the firing rate of isolated pacemaker cells by reducing the gradient of the pacemaker potential, especially at membrane potentials closer to the threshold for stimulation of T-Type I_{Ca} (Ju et al., 1995; Ju et al., 1996). It is thought that this sodium current arises via TTX-sensitive inactivation-resistant Na^+ channels (henceforth called I_{NaTTX}) that remain open following an action potential, letting in a small amount of depolarizing Na^+ during the pacemaker potential (Ju et al., 1996). Another possibility for TTX-mediated reductions in contraction rate in both hagfish and toads is that TTX may be blocking T-type I_{Ca}, a secondary effect of TTX reported by Sun et al. (2008). This would cause a slight lengthening of the action potential duration, leading to a reduction in contraction rate. Regardless of this, the TTX-induced atrioventricular block suggests that I_{NaTTX} channels are vital for proper atrioventricular conduction and synchrony of atrial and ventricular contractions given their different intrinsic rates. Perhaps hagfish have a higher proportion of I_{NaTTX} channels to HCN channels in the ventricle compared with the sinoatrial node and this

arrangement may be the precursor of the conduction system required for a multi-chambered heart to contract synchronously.

The contributions of both T-type I_{Ca} and L-type I_{Ca} to pacemaker cell action potentials is well established, with T-type I_{Ca} predominating for the upstroke and L-type I_{Ca} acting to increase the overall depolarization and slightly prolonging the action potential (Hagiwara et al., 1988; Doerr et al., 1989; Zhou and Lipsius, 1994). Ni^{2+} and nifedipine block T-type I_{Ca} and L-type I_{Ca}, respectively, and it is expected that Ni^{2+} would have a stronger effect on heart rate due to the greater role of the T-type I_{Ca} in pacing cells. However, distinguishing between T-type I_{Ca} and L-type I_{Ca} in a whole heart preparation is difficult due to the poor selectivity of Ca^{2+} channel blockers and the multitude of Ca^{2+} currents that may compensate for any inhibited current. Ni^{2+} has been shown to block T-type I_{Ca} and the cardiac Na^{+}/Ca^{2+} exchanger at higher concentrations, while higher concentrations of nifedipine have been shown to block the transient outward K^{+} current with other dihydropyridines blocking I_{Na} (Yatani and Brown, 1985; Gotoh et al., 1991; Hobai et al., 2000; Uehara et al., 2005). Both Ca^{2+} channel blockers had an effect on heart rate, with 1 μM nifedipine and 1 mM Ni^{2+} significantly reducing rates. These concentrations are fairly high, especially for Ni^{2+} since usually the most Ni^{2+} resistant subtype of T-type Ca^{2+} channel, α1G has an IC_{50} of 0.25 mM (Lee et al. 1999). This may be due to a limitation of the drugs diffusing to the channels in the thick preparation used compared to single myocyte studies. Nevertheless, the results do suggest a minor role of Ca^{2+} currents in setting the heart rate. A minor role is to be expected since T-

type I_{Ca} and L-type I_{Ca} contribute to the upstroke of the action potential in pacing cells, therefore, inhibition of one of these currents would be expected to extend the action potential duration, but have little effect on the pacemaker potential itself. Also, since only one blocker was used at a time, Ca^{2+} may have entered via another pathway such as the Na^{+}/Ca^{2+} exchanger. Clearly, electrophysiological work on single myocytes are required to dissect the role of Ca^{2+} currents in the hagfish heart since it will allow fine control of the voltage environment of the cells.

2.4.1 Summary

To summarize, the novel results in this chapter for the mechanistic underpinnings of intrinsic heart rate in hagfish have revealed important similarities and dissimilarities when compared with hearts in the craniate lineage. Pacemaker potentials have been recorded in all areas of the hagfish atrium and ventricle (Jensen, 1965; Arlock, 1975) and the intrinsic beating rates of these chambers were characterized here. Like urochordates (Jackson et al., 2007), atrial and ventricular beating rates in hagfish appear to be established with HCN channels and the presumed I_f flowing through them, providing further evidence for HCN presence being the ancestral mechanism of cardiac pacing compared to Ca^{2+}-cycling through the SR. However, whereas the tunicate heart consists of a simple contractile tube in which, the wave of contraction can be initiated at either end (Kriebel, 1968; Delsuc et al., 2006), the synchronous and rectified contractions of the hagfish atrium and ventricle is similar to that of vertebrate hearts. A relatively small I_{NaTTX} current contribution to the pacemaker potential, which is indicated by the present result, contrasts with findings for adult

vertebrates, except for some amphibians (Ju et al., 1995; Ju et al., 1996). Different expression ratios of HCN versus I_{NaTTX} channels and a large role of I_{NaTTX} in spreading the wave of excitation from the atria to the ventricles are putative mechanisms to control the spread of excitation sequentially from the atrium to the ventricle despite pacemaker activity being present throughout all cardiac chambers of the hagfish. This confirms hagfish as the most ancestral representative of a craniate-type chambered heart with a rectifying conduction system. While HCN blockers revealed that atrial rate typically outpaces ventricular rate over twofold, further study is needed to determine how intrinsic ventricular rate is held lower than the atrial rate should heart rate fall as low as it does in anoxic hagfish (5 bpm *in vivo* (Cox et al. 2010)), a value below the intrinsic ventricular contraction rate of 8.4 bpm determined here. In order to obtain more direct information on the contributions of different ionic currents to the control of heart rate in the hagfish, electrophysiological measurements on isolated cells will be required, enabling single currents to be isolated but at the cost of not being able to see the effects on the heart as an intact organ, as presented in the present chapter.

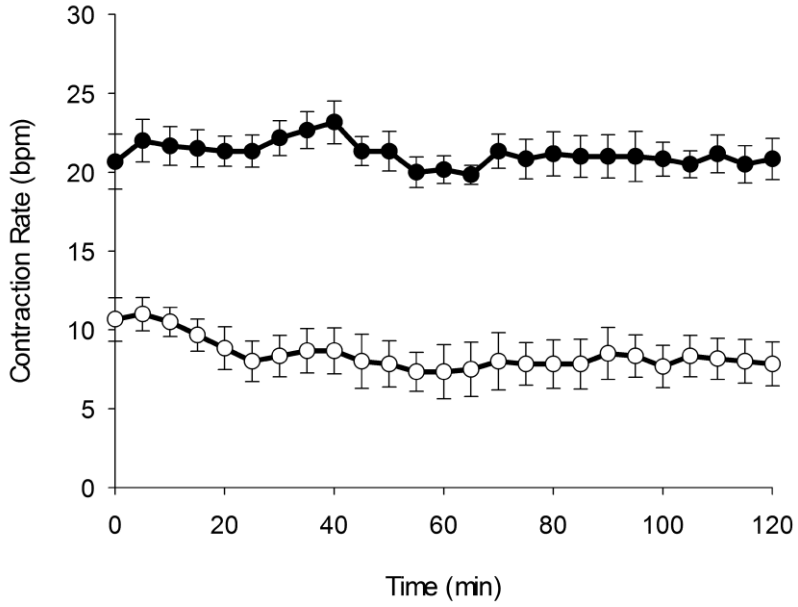

Figure 2.1. Effects of a ligature being tied around the atrioventricular canal on the contraction rates of atria (●) and ventricles (○) of intact, isolated hagfish hearts. The atrioventricular block produced an immediate decrease in the ventricular contraction rate to 8.4 ± 0.3 bpm, which averaged 41.3 ± 1.5% that of atrial contraction rate. Error bars show s.e.m. for n=6 preparations.

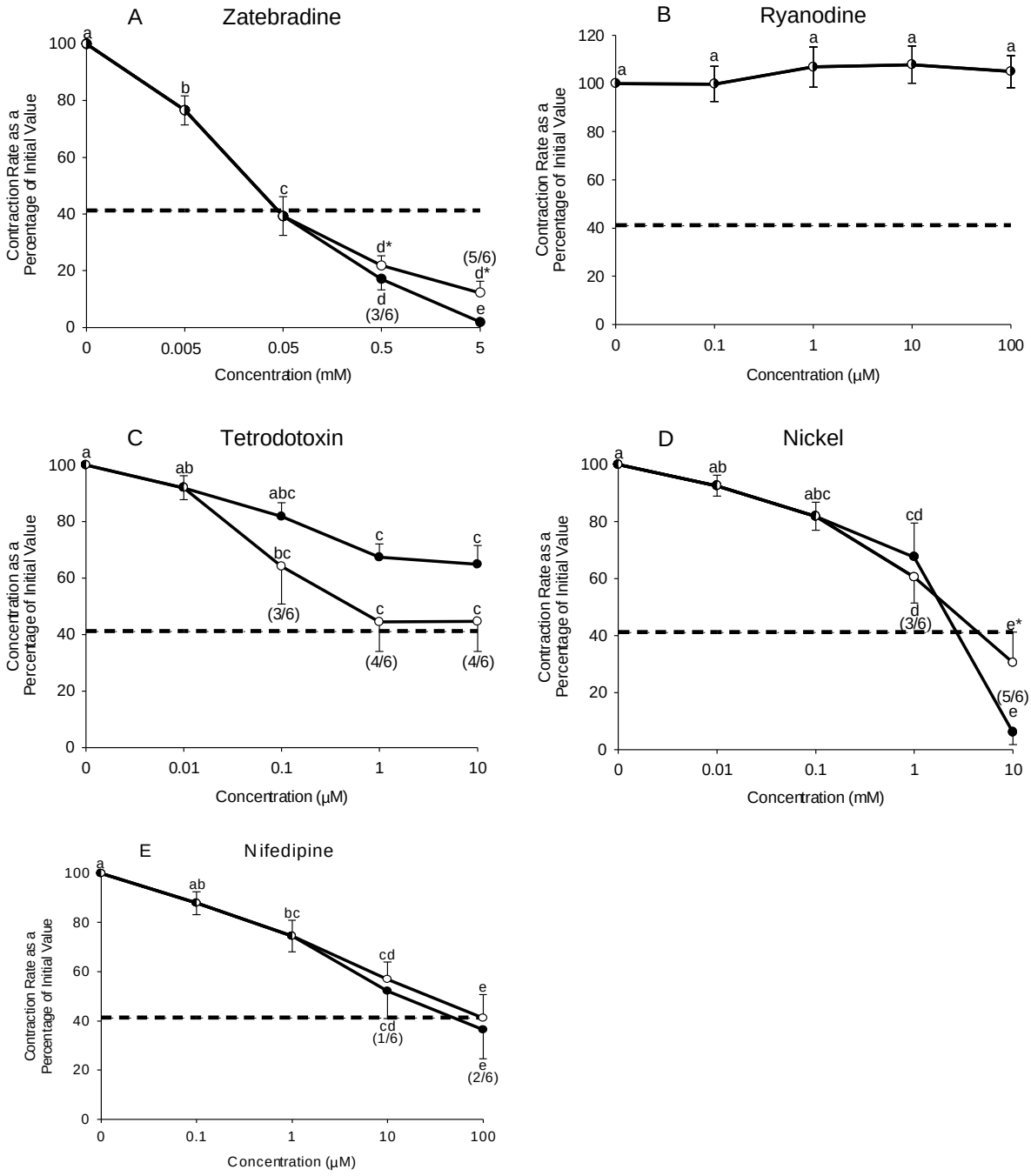
A
Zatebradine
Contraction Rate as a Percentage of Initial Value
Concentration (mM)
B
Ryanodine
Contraction Rate as a Percentage of Initial Value
Concentration (μM)
C
Tetrodotoxin
Concentration as a Percentage of Initial Value
Concentration (μM)
D
Nickel
Contraction Rate as a Percentage of Initial Value
Concentration (mM)
E
Nifedipine
Contraction Rate as a Percentage of Initial Value
Concentration (μM)

Figure 2.2. Effects of increasing concentrations of channel blockers on the contraction rate of atria (●) and ventricles (○) from isolated hagfish hearts. **A**. The effect of the hyperpolarization-activated cyclic-nucleotide-gated channel blocker zatebradine. **B**. The effects of ryanodine receptor blocker ryanodine. **C**. The effects of the voltage-gated sodium channel blocker tetrodotoxin. **D**. The effects of the T-type calcium channel blocker nickel. **E**. The effects of the L-type calcium channel blocker nifedipine. Dissimilar letters indicate statistically significant differences in contraction rate among concentrations calculated from raw, non-normalized data (repeated measures two-way ANOVA, $P<0.05$). * indicates significant differences between atrial and ventricular contraction rate at a given concentration. Numbers in parentheses indicate the fraction of hearts with asynchronous atrial and ventricular contraction rates. The dashed line shows the line of identity for the atrioventricular block with a ligature, see Fig. 1. Error bars show s.e.m. for n=6 preparations for each panel.

CHAPTER 3: PHYLOGENY AND EFFECTS OF ANOXIA ON HYPERPOLARIZATION-ACTIVATED, CYCLIC NUCLEOTIDE-GATED CHANNEL GENE EXPRESSION IN THE HEART OF A PRIMITIVE CHORDATE, THE PACIFIC HAGFISH, *EPTATRETUS STOUTII*

3.1 Introduction

The regular vertebrate heartbeat requires no external input and is initiated by specialized pacemaker cells located in the sinoatrial node (DiFrancesco, 1993). These pacemaker cells have a Ca^{2+}-mediated upstroke to the action potential rather than a Na^{+}-mediated one that characterizes contractile cardiac myocytes. In addition, pacemaker cells have a slowly depolarizing membrane potential, known as the pacemaker potential, rather than the stable membrane potential present between action potentials characteristic of the working myocardium (Brown and DiFrancesco, 1980; Doerr et al., 1989; Baker et al., 1997; DiFrancesco, 2010). In mammals, the pacemaker potential is a result of inward cation currents. These are a mixed K^{+} and Na^{+} current (I_f) flowing through hyperpolarization-activated cyclic nucleotide-gated (HCN) protein channels in the cell membrane, and an inward sodium/calcium exchange current (I_{NCX}) activated by rhythmic cycling of Ca^{2+} through the sarcoplasmic reticulum (DiFrancesco and Ojeda, 1980; Yanagihara and Irisawa, 1980; Dobrzynski et al., 2007; Maltsev and Lakatta, 2008; Monfredi et al., 2013). The magnitude of these inward currents directly affects the slope of the pacemaker potential, which, in turn, sets heart rate by determining the time taken to reach the

threshold voltage needed to open the Ca^{2+} channel (DiFrancesco and Tortora, 1991; Wainger et al., 2001).

The ancestral hagfishes are believed to have diverged from the vertebrate lineage over 500 million years ago and prior to the genome duplications postulated for the vertebrate lineage (Ota and Kuratani, 2007). Despite their evolutionary importance, knowledge on cardiac pacemaker mechanisms in hagfishes is very limited (Farrell, 2007a). The hagfish heart is unique by being the only craniate, myogenic heart that is anatomically aneural. However, despite aneural control, heart rate can vary four-fold during recovery from prolonged anoxia (Greene, 1902; Augustinsson et al., 1956; Jensen, 1961; Hirsch et al., 1964; Jensen, 1965; Farrell, 2007a; Cox et al., 2010). Recent work suggests that the sarcoplasmic reticulum plays a very minor, if any, role in hagfish cardiac pacemaking, whereas the central importance of HCN channels in setting the intrinsic heart rate is implied by the finding that the HCN channel antagonist zatebradine almost stops the heartbeat (Chapter 2). Therefore, this chapter examines more closely the HCN isoforms and their expression in the Pacific hagfish heart.

HCN channels are closely related to the ether-a-go-go and cyclic nucleotide-gated K^+ channels. It is thought that HCN channels consist of four protein subunits, each with 6 transmembrane segments, a voltage sensor, an ion conducting pore and an evolutionarily-conserved cyclic nucleotide-binding domain (CNBD) (Doyle et al., 1998; Ishii et al., 1999; Jiang et al., 2003). CNBD inhibition of I_f is inversely related

to the concentration of cAMP (Wang et al., 2001; Biel et al., 2002). Neural and humoral stimulation can modulate adenylyl cyclase activity, changing cytosolic cAMP concentration, which then alters the inhibition imposed by the CNBD (Brown et al., 1979a; Brown et al., 1979b; Brown and DiFrancesco, 1980; DiFrancesco, 1985; DiFrancesco, 2010). Furthermore, in mammals, the relative expression of the four HCN isoforms (HCN's 1-4), each of which has different electrophysiological properties, can vary among tissues. For example, expression of HCN4 is dominant in adult mammalian atria, whereas HCN2 is dominant in the ventricle (Moosmang et al., 2001; Qu et al., 2002; Shi et al., 2002), and this differential expression is associated with a faster intrinsic atrial beating rate.

The genes encoding the four mammalian HCN isoforms are highly conserved among other vertebrate hearts. A large-scale phylogenetic study by Jackson et al. (2007) suggested that the four HCN isoforms arose from duplications of a single HCN ancestral gene most similar to HCN3, with some teleost fishes having extra isoforms that likely arose from lineage-specific gene or genome duplications. Outside the vertebrates, in *Ciona*, lineage specific duplications of the ancestral gene have resulted in three *Ciona*-specific HCN isoforms (Jackson et al., 2007). Despite occupying the basal position in craniate evolution and possessing a craniate-type, myogenic heart with valved chambers, unlike *Ciona*, nothing is known about the quantity of or regulation of HCN in hagfishes, Here, I postulate that modulation of HCN channel mRNA is central to the control of hagfish heart rate, in that the decrease in heart rate seen by Cox et al. (2010) in anoxia results from a decrease in

HCN channel mRNA expression, and the following increase in heart rate upon reoxygenation is coupled with an increase in HCN channel mRNA expression.

To test this hypothesis, I partially cloned hagfish cardiac HCN isoforms from atria and ventricles and compared them to known vertebrate isoforms to establish where the hagfish fits within the chordate phylogeny. In addition, real-time reverse transcriptase-PCR (RT-PCR) was utilized to examine the mRNA expression of HCN isoforms in the hagfish heart before, during and after an anoxic challenge to test for plasticity in the expression of HCN. Both cardiac chambers were examined because pacemaker activity has been recorded from multiple areas of the atrium and ventricle in hagfishes, and both chambers continue beating following an atrioventricular ligature, albeit with the atrium outpacing the ventricle (Jensen, 1965; Chapter 2).

3.2 Methods

3.2.1 Animal husbandry

All experiments were carried out in accordance with animal care policies of the University of British Columbia, the Bamfield Marine Sciences Center, and the Department of Fisheries and Oceans Canada. Pacific hagfish (*Eptatretus stoutii*) were caught near Bamfield Marine Sciences Centre, Bamfield, B.C. Canada using baited traps. Hagfish (100 ± 1 g; mean ± s.e.m.) were transported to the Department of Fisheries and Oceans Canada, West Vancouver Laboratory, B.C. Canada, where they were housed in a 4000 l fibreglass tank supplied with flow-

through seawater (10±1°C) and fed squid weekly. Hagfish were fasted a minimum of 48 h prior to any experimental treatment.

3.2.2 Anoxia Exposure

Individual hagfish were placed in darkened 2.5 l chambers that were continuously flushed with aerated seawater (10±1°C) at a flow rate of 0.5 l min^{-1} that was maintained throughout the experiments. Hagfish were left undisturbed for 24 h to habituate to the chamber, during which time they readopted their typical curled posture, as observed in the holding aquarium (see Cox et al., 2010). Anoxia was achieved in the same manner as in Cox et al. (2010) by supplying the chambers with N_2-saturated seawater from two gas-exchange columns placed in series. The transition period to anoxia lasted less than 1 h (water oxygen concentration was measured continuously with an OxyGuard Handy MkIII, Birkerød, Denmark, accuracy ±0.1 mg l^{-1}). During the transition to anoxia, hagfish became slightly agitated before assuming a straightened position, as previously reported (Cox et al., 2010). Hagfish were maintained in flow-through anoxic seawater for 24 h, before quickly (under 30 min) restoring normoxic conditions for up to 36 h. Hagfish were sampled at the end of the 24-h anoxic period (no normoxic recovery, termed 0 h group), 2 h into normoxic recovery and 36 h into normoxic recovery (2 h and 36 h group respectively) (N=10 for each group). Control animals (N=10) were sampled after 84 h in the chamber in normoxic water. At each sample time, hagfish were rapidly removed from their chamber, sacrificed by decapitation and the branchial heart was dissected, freeing the atrium (7.7±1.2 mg) and ventricle (59.2±4.4 mg),

prior to freeze-clamping each separately and storing them in liquid N_2. Tissues were stored at -80°C. The precise location of the hagfish sinoatrial node is unknown; therefore, it could not be reliably isolated.

3.2.3 RNA Extraction

Total RNA was extracted from tissues using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). Extractions followed a modified protocol outlined by Ellefsen et al. (2008). Briefly, each frozen tissue was weighed (atria 17.2±1.2 mg, ventricles 59.3±4.4 mg mean ± S.E.M.) and immersed in 60 µl TRIzol per mg tissue. Then, 0.2 µl per mg tissue of an external RNA control gene (mw2060) was added and the tissue/TRIzol/mw2060 mixture homogenized for 1 min using a T-25 Basic homogenizer (IKA Works, Inc., Wilmington, NC, USA). The use of an external RNA control has been argued to be the most accurate method for the normalization of real-time RT-PCR data (Huggett et al., 2005). mw2060, a 2060 base pair long mRNA species from the cyanobacterium *Microcystis cf. wesenbergi* that shows no sequence homology to known vertebrate mRNA species, was recently developed as a more reliable method for the normalization of real-time RT-PCR data from tissues of anoxia-tolerant species than other commonly employed normalization techniques (Ellefsen et al., 2008). Additionally, the methodology allows for inter-tissue comparisons of target gene expression (Stecyk et al., 2012). Samples were then chilled on ice, vortexed, incubated at room temperature for 15 min and vortexed again. The maximum amount of tissue/TRIzol homogenate (up to 1 ml) was transferred to an Eppendorf tube before adding chloroform (20% of the volume of

tissue/TRIzol homogenate). The mixture was incubated at room temperature for 3 min, vortexed, centrifuged at 10,000 rpm for 15 min at 4°C and placed on ice. A volume corresponding to 40% of the tissue/TRIzol homogenate of the upper aqueous phase was then transferred to a new Eppendorf tube before adding an equal amount of ice-chilled isopropanol. The mixture was vortexed, incubated at -20°C overnight, incubated at room temperature for 10 min and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was then discarded and the pellet washed twice with ice-chilled 75% ethanol. Each ethanol wash was followed by centrifugation at 14,000 rpm for 10 min at 4°C. After the final ethanol wash, the pellet was air dried and eluted in 30 μl of nuclease-free water (Ambion, Austin, TX, USA). The mixture was then incubated at 65°C for 5 min to ensure the pellet was completely dissolved, and stored at -80°C.

Care was taken to avoid systematic errors introduced by sample processing during RNA extraction. All samples were handled without intermission and in a systematic, random order. Samples were processed in groups, wherein each group consisted of samples of a single tissue type and representing each of the treatment groups. The samples within each group were processed at random. Similar procedures were also employed for cDNA synthesis.

3.2.4 cDNA synthesis

The concentration of total RNA in every sample was determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). One μg of total RNA from each sample was then treated with DNase I (DNA-free; Invitrogen, Carlsbad, CA, USA) and subsequently reverse transcribed using Random Hexamers (50 ng μl^{-1}) and Superscript III (both from Invitrogen, Carlsbad, CA, USA) in reaction volumes of 20 μl and in accordance with the manufacturer's protocol. cDNA solutions were diluted 1:30 with nuclease-free water and stored at -20°C. Duplicate cDNA syntheses were performed on RNA samples when possible.

3.2.5 Cloning of HCN genes

The HCN isoforms were partially cloned using PCR primers designed to recognize gene regions conserved among vertebrate species. The regions were located using GeneDoc (version 2.6.0.2, http://www.psc.edu/biomed/genedoc/) and ClustalX (version 1.81), while primers were designed using Primer3 (Nicholas et al., 1997; Thompson et al., 1997; Rozen and Skaletsky, 2000). Primers used for cloning are listed in Table 3.1.

PCR was performed on a mixture of 1:30 diluted cDNA from atrial and ventricular tissues and all treatment groups using Platinum®*Taq* DNA Polymerase (Invitrogen, Carlsbad, CA, USA; 94°C for 10 min, 94°C for 30 s, 48°C for 1 min, 72°C for 1 min, repeat steps 2-4 44x, 72°C for 10 min, hold 4°C). Resulting dsDNA fragments were ligated into pGEM®-T Easy Vector System I (Promega, Madison, WI, USA). Ligation

reactions were transformed into $CaCl_2$-competent cells (TOP10 F'; Invitrogen, Carlsbad, CA, USA) and positive colonies were checked for inserts of a correct size via agarose gel electrophoresis. PCR products from up to eight colonies were sequenced using T7 primers (ABI-lab, University of Oslo, Oslo, Norway). All procedures were carried out according to the manufacturer's protocol.

3.2.6 Analysis of partially cloned HCN genes

The partial sequences of cardiac HCN for hagfish were compared with known vertebrate HCN genes using ClustalX and NCBI Blast (Altschul et al., 1990; Thompson et al., 1997). A phylogeny was created by running a neighbour joining phylogenetic analysis with the translated amino acid products from the hagfish HCN isoforms, plus those of vertebrate species downloaded from Ensembl and GenBank. The closely related human ether-à-go-go-related gene (hERG) and *Drosophila* cyclic-nucleotide-gated gene (dCNG) were added to the sequence alignment as out-groups. A 37 amino acid segment was used since all partially cloned isoform protein segments overlapped in this area. 1000 bootstrap replications were conducted on the amino acid sequences and a consensus tree was produced. The phylogeny was completed using the Seqboot, Protdist, Neighbor, Consense and Drawgram programs of PHYLIP 3.65, and viewed using TreeView 1.6.6 and formatted using Adobe Illustrator (Page, 1996).

3.2.7 Real-time RT-PCR primer design and protocol

All real-time RT-PCR primer pairs were designed using Primer3. The small differences between the paralogs of HCN2 and HCN3b prevented distinctions between paralogs using the tested primers, therefore both paralogs of HCN3b and HCN2 are compared collectively as HCN3b and HCN2, respectively. Forward and reverse primers were targeted to either side of an exon-exon overlap as a further precaution against amplifying genomic DNA (i.e., in addition to the extraction of total RNA and DNAse treatment). Amplification of the desired cDNA species by the primer pairs was verified by melting curve analyses (Lightcycler® 480 software) and cloning and sequencing of all primer pair products (performed as described above for the cloning of HCN genes). Real-time RT-PCR primer sequences are summarized in Table 3.2. To find primers that worked well with the real-time RT-PCR protocol (primer concentration of 1 mM and annealing temperature of 60°C), a minimum of 4 primer pairs were tested for each gene. As an alternative to primer concentration/annealing temperature optimization, primer pairs that displayed distinct melting curves, the highest efficiency (calculated as described below for RT-PCR analyses) and the lowest Cq (crossing point or threshold cycle) values were chosen. All procedures followed manufacturer's protocols.

Real-time RT-PCR for HCN isoforms 2, 3a, 3b and 3c were performed using Lightcycler® 480 (Roche Diagnostics, Basel, Switzerland). All real-time RT-PCR reactions were performed with a reaction volume of 10 μl that contained 5 μl of Lightcycler® 480 SYBR Green I Master (Roche Diagnostics, Basel, Switzerland), 3 μl

of 1:15 diluted cDNA as the template, 1 μl of 5 mM gene-specific forward primer and 1 μl of 5 mM gene-specific reverse primer (i.e., a final primer concentration of 1 mM). The real-time RT-PCR program was: 95°C for 10 min, 95°C for 10 s and 60°C for 10 s, 72°C for 13 s, repeating steps 2-4 42 times. Two real-time RT-PCR reactions were performed on each gene for each cDNA synthesis. The replicates were conducted on different plates and days. As a result, four real-time RT-PCR reactions were performed on each gene for each RNA sample.

For HCN4, real-time RT-PCR was conducted using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, USA). Each real-time RT-PCR reaction contained 10 μl SYBR Green Master Mix (Applied Biosystems), 4 μl of 1:15 diluted cDNA as the template, 0.8 μl of 5 mM gene-specific forward primer and 0.8 μl of 5 mM gene-specific reverse primer, and 4.4 μl of double-processed tissue-culture water (Sigma-Aldrich, Ayrshire, UK) bringing the total reaction volume to 20 μl. The real-time RT-PCR program was: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Following the real-time RT-PCR, a melt curve analysis was performed. As for the other isoforms, two real-time RT-PCR reactions were performed on each cDNA synthesis, resulting in four real-time RT-PCR reactions for each mRNA sample.

3.2.8 Real-time RT-PCR analysis

For HCN2, HCN3a, HCN3b and HCN3c, Cq values were obtained for each reaction using the Lightcycler® 480 software and were defined according to the second

derivative maximum (Luu-The et al., 2005). Priming efficiencies were calculated for each real-time RT-PCR reaction using LinRegPCR software (Ruijter, 2009). For HCN4, each plate was run with a standard dilution of a mixture of randomly chosen cDNA samples from atrial and ventricular tissues. The primer efficiencies were then determined for each plate from the slope of the standard curve using Applied Biosystems 7000 System Sequence Detection Software 1.2.3. This software was also used to obtain the Cq values for each reaction.

Average priming efficiencies (E_{mean}) were used for the final calculations, which were calculated separately for each tissue and primer pair from all real-time RT-PCR reactions (Čikoš et al., 2007). Then, to normalize HCN isoform gene expression to the expression of the external RNA control mw2060, E_{mean}^{Cq} was calculated for every reaction, as well as the ratio (R1) between ${}_{mw2060}E_{mean}^{Cq}$ and ${}_{Tar}E_{mean}^{Cq}$ (where Tar = target gene, E = priming efficiency and Cq = quantification cycle). To compare the expression of each HCN isoform among the different treatments, R1 was referenced to the mean expression of the control, normoxic samples.

3.2.9 Calculations and statistical analyses

Results are expressed as means ±s.e.m. Statistical analyses were performed using SigmaPlot for Windows 11.0 (Systat Sotfware, Inc. Chicago, USA). Differences in HCN isoform expression within a tissue were assessed using a one-way ANOVA followed by a Tukey *post hoc* test. A two-way ANOVA, followed by a Tukey *post hoc* test, was used to determine statistically significant effects of anoxia and recovery on

the expression of HCN gene isoforms. Differences in atrial and ventricular HCN isoform expression among each sampling time were assessed using a Student's *t*-test. Significance was accepted when $P<0.05$.

3.3 Results

3.3.1 Phylogenetics

A total of 6 isoforms of HCN mRNA were found in hagfish cardiac tissue, with all 6 isoforms being represented at varying levels in both atrial and ventricular chambers and one isoform existing with two paralogs for a total of 7 partially cloned genes. The amino acid sequences aligned with mouse HCN3 are shown in Figure 3.1 with the lengths of the partially cloned sequences shown in Table 3.1. Two isoforms of a hagfish representative of vertebrate HCN2, termed HCN2a and HCN2b, differed by 5 amino acid substitutions (P-T at 16, H-N at 56, P-H at 111, S-P at 253, S-G at 261) and 1 deletion of a G at location 280 in HCN2a. Three isoforms of HCN3 were found, HCN3a, HCN3b and HCN3c, with HCN3b existing as two paralogs. These two paralogs, termed HCN3bi and HCN3bii, differed by 2 substitutions (G-E) at amino acid locations 148 and 262. One hagfish representative of HCN4 was also found.

Neighbour-joining phylogenetic analysis (Fig. 3.2) was conducted on a 37 amino acid sequence from the CNBD of multiple vertebrate and urochordate HCN genes where 6 of the 7 hagfish sequences overlapped, as shown in Figure 3.3 (HCN3bi

and HCN3bii paralogs are included as HCN3b on the tree because the G-E substitutions of HCN3bi and HCN3bii occurred outside of this segment).

To summarise the phylogenetic tree, HCN3b and HCN3c cluster together, and along with HCN3a form a cluster with the HCN3 of teleosts. The HCN3 of mammals cluster together on the other side of the outgroup, which contains both the HCN-related human ERG and *Drosophila* CNG proteins, as well as the *Ciona* HCN isoforms. The HCN4 isoforms, including the hagfish sequence, all cluster together apart from the pufferfish HCN4a isoforms. The hagfish HCN2 sequences cluster with the mammalian HCN2 and pufferfish HCN2b isoforms, while the other teleost HCN2 sequences cluster in a second group either side of the HCN1 cluster. The splitting of HCN isoforms in the tree may be a consequence of using short sequences for the analysis. Despite the lack of a hagfish HCN1 sequence in the phylogeny, it is noted that the 2 teleost HCN1 isoforms cluster as a separate branch of the HCN1 cluster.

3.3.2 HCN gene expression

Of the five measured hagfish HCN isoforms, HCN3a had the highest expression in both the atrium and ventricle in normoxia. HCN3a was expressed over 17-times more than HCN4 in the atrium and 25-times more than HCN4 in the ventricle (Fig. 3.4). Expression of the other isoforms followed the order of HCN4, HCN3b, HCN2 and HCN3c for both the atrium and the ventricle. Due to the similarity of the two HCN2 and HCN3b mRNA segments, it was not possible to distinguish between them

using the real-time RT-PCR primers, and both segments would have been duplicated together by their respective primers.

3.3.3 Effects of anoxia on HCN gene expression

The effects of 24 h of anoxia and subsequent recovery on HCN isoform gene expression are shown in Figure 3.5. In the atrium, the only statistically significant change was an increase in HCN3a at 2 h of normoxic recovery from anoxia, which returned to control expression levels by 36 h of recovery. In contrast, anoxia induced a significant decrease of HCN3a expression in the ventricle, which again progressively recovered back to the control level within the 36-h recovery period. The trends in HCN3b, HCN3c, and HCN2 expression during the anoxia, while not reaching statistical significance (Fig. 3.5), did return to the control values with normoxic recovery.

3.4 Discussion

The present study documents for the first time HCN expression in a representative of the basal chordates and discovers changes in HCN expression in anoxic hagfish that were different in the atrium and ventricle. Specifically: 1) six isoforms of HCN were described from seven partial HCN amino acid sequences in the cardiac tissues of Pacific hagfish; 2) a phylogenetic analysis was performed on six of these sequences; 3) a quantitative comparison was made of the mRNA expression of five

HCN isoforms for the atrium and ventricle; and 4) changes in HCN isoform expression were discovered in response to chronic anoxia that were reversed following normoxic recovery.

Six HCN isoforms were identified, HCN2a, HCN2b, HCN3a, HCN3b, HCN3c and HCN4, a finding that differs from the adult mammalian heart where four isoforms, HCN1-4, are expressed. Contrary to HCN4 dominance in mammalian pacemaker tissue (Shi et al., 1999; Marionneau et al., 2005), HCN3a was the dominantly expressed isoform in the hagfish atrium and ventricle. In addition, hagfish cardiac HCN3b existed as two paralogs, designated here as HCN3bi and HCN3bii. These findings have important implications for the phylogenetic appearance of pacemaker channels in the chordate lineage, as summarised in Figure 3.6. Moreover, given what is known of the biophysical properties of the mammalian HCN isoforms, and assuming that mRNA expression reflects protein presence (see below), the findings lend new, but preliminary, insights into the electrophysiological properties of the hagfish heart and possibly how heart rate is modulated during and following prolonged anoxia.

Based on the neighbour-joining phylogenetic analysis, the vertebrate HCN2, HCN3 and HCN4 isoforms had already appeared in cardiac tissues of the chordate lineage prior to the hagfish/gnathostome divergence. This conclusion is in accordance with previous work that compared HCN of *Ciona* with vertebrate species that included modern teleost species (Jackson et al., 2007). Jackson et al. (2007) proposed that

HCN3 is ancestral, and was followed by the appearance of HCN4 and then HCN2. As shown here, all HCN3 isoforms cluster together and therefore appear ancestral. The two isoforms of HCN2 (2a and 2b) cluster together with the mammalian and avian forms of HCN2. Clearly, limitations do exist when using partial genes to create a phylogeny. Potentially, minor mutations of the CNBD during the long period between the appearance of hagfish and vertebrates may make ancestral genes appear more derived, something which will not be revealed with partial cloning and sequencing. However, a more robust phylogenetic analysis will require full hagfish HCN sequences. Nevertheless, the CNBD has been highly conserved throughout evolution (Jackson et al., 2007), likely due to its important regulatory function. Indeed, changing a single amino acid in this region is known to appreciably alter the effect of cAMP binding to the CNBD (Tibbs et al., 1998). Also, HCN2 and HCN4 are strongly gated by cAMP, whereas HCN1 is not (Wang et al., 2001). Moreover, since three lineage-specific HCN isoforms have been reported previously in urochordates (Jackson et al., 2007), it was previously hypothesized that, prior to the divergence of the deuterostomes, duplication of the ancestral HCN gene may have given rise to two HCN isoforms in the sea urchin and, due to a further duplication, three HCN isoforms were found in the urochordates (Gauss et al., 1998; Galindo et al., 2005; Jackson et al., 2007). A further possibility is that the sea urchin also had three HCN isoforms, but subsequently lost one. Jackson et al. (2007) suggested HCNa, HCNb and HCNc emerged early in *Ciona*, before *C*. *intestinalis* and *C*. *savignyi* had diverged, therefore these three HCN isoforms may have been present in the urochordate ancestor (Jackson et al., 2007). Since the three hagfish HCN3 isoforms

do not align with the *Ciona* isoforms, it is unlikely that the hagfish HCN3a, HCN3b and HCN3c isoforms represent an ancestral condition prior to the urochordate/chordate divergence, and more likely represent HCN3 duplication events following the hagfish/vertebrate split.

What is apparent from the available information is that lineage-specific HCN gene duplications are relatively regular evolutionary events, being previously identified in the urochordates and some teleost fishes (Gauss et al., 1998; Galindo et al., 2005; Jackson et al., 2007), and now in hagfishes. Having many isoforms of these genes could provide flexibility and fine-tuning of pacemaker activity, perhaps providing an evolutionary advantage, which I speculate on below.

Many primers were used without success to target conserved regions of the vertebrate HCN1 isoform in the hagfish heart. I propose three possible explanations for the absence of HCN1 in the hagfish heart. First, HCN1 is commonly associated with central neural tissue in addition to the heart (Franz et al., 2000; Nolan et al., 2003). Therefore, HCN1 expression in hagfish may be limited to non-cardiac tissues. This absence may then be related to the normally important role of catecholamine modulation of heart rate through cAMP effects on the pacemaker channel since HCN1 is fairly weakly affected by cAMP compared to the other isoforms (Nilsson, 1983; DiFrancesco and Tortora, 1991; Wainger et al., 2001; Wang et al., 2001). Another explanation could be related to a very low expression of HCN1 that was below detection level. Finally, HCN 1 may not have appeared in the

vertebrate lineage until after the hagfish/vertebrate split. Failure to find HCN1 in hagfish would suggest that duplication of the ancestral HCN2-like gene likely produced HCN1 following the hagfish/vertebrate divergence. Indeed, in other phylogenetic studies, HCN1 is suggested as the most recent HCN gene, although the exact timing of its appearance is unknown (Jackson et al., 2007). Data on HCN expression in elasmobranchs and basal teleosts would be particularly informative in this regard. All the same, conclusive knowledge on the exact number and type of HCN isoforms will not be possible until the entire hagfish genome is known.

The proceeding speculations are based on the assumption that altered mRNA expression leads to an increase in protein expression. This is a large assumption, and major caveat to these suggestions. There is very little information on HCN translation from mRNA, however, the work that has been done imlies a heavy role of TRIP8b, a protein that colocalizes with HCN1 in the brain (Santoro et al., 2004). Different splice varients of TRIP8b can increase or decrease HCN1 expression depending on the mix of varients (Santoro et al., 2009). Due to the the interactions of multiple proteins in the translocation of HCN channels, it is unlikely that a 1:1 mRNA:protein expression ratio exists. However, in other systems, an increase in mRNA expression does lead to an increase in protein expression, for example, in breast carcinomas, an increase in epidermal growth factor receptor mRNA is correlated with an increase in receptor protein expression, and hypoxia has been shown to upregulate both bone morphogenic protein-2 mRNA and protein expression in endothelial cells (Bouletreau et al, 2002; Bhargava et al., 2005).

However, in lieu of HCN electrophysiological data from hagfish, and with a lack of reliable HCN antibodies for Western bloting, these speculations are meant to ispire further research in this area.

In mammalian studies, HCN4 is by far the most dominantly expressed HCN isoform in the heart, with expression in the sinoatrial node dwarfing that of other HCN channels (Shi et al., 1999; Marionneau et al., 2005). Contrary to this, I discovered that, in hagfish, HCN3a mRNA expression in both the atrium and ventricle dominates HCN4 by at least an order of magnitude, similar to what has been reported for the turtle (*Trachemys scripta*) ventricle (J. A. W. Stecyk, personal communication) (Fig. 3.4). In this regard, the hagfish heart bears some resemblance to mammalian embryonic stem cells where, in early stage mouse embryonic stem cells, the dominant HCN isoform is 3 (Qu et al., 2008). Throughout development in the mouse, expression of HCN3 decreases and HCN4 becomes dominant in association with the onset of cardiac contractions (Qu et al., 2008; Schweizer et al., 2009). While I am not prescribing Lamarkian recapitulation of phylogeny, the similarity points to a functional role of variations in cardiac HCN isoform expression. Nevertheless, care must be taken when speculating on the physiological implications of differential HCN isoform expression in the hagfish since the electrophysiological and other functional properties of these new isoforms have yet to be examined. Therefore, the electrophysiological ramifications of the present results that are proposed below are based on the rich knowledge of mammalian HCN electrophysiology.

In lieu of electrophysiological evidence on the activation dynamics of the hagfish HCN isoforms, it is not possible to know exactly what the effect of HCN3 mRNA dominance over that of HCN4 might be on hagfish cardiac excitation-contraction-coupling. However, Moosmang et al. (2001) showed that HCN3 activates at a similar voltage to HCN4, but has activation kinetics more similar to the faster HCN2 isoform, allowing a larger current to flow at lower membrane potentials. Therefore, a large proportion of HCN3 may allow the hagfish heart to become easily activated and then allow the spread of excitation throughout the myocardium to occur rapidly. Indeed, evidence exists for the presence of pacemaker potentials throughout the hagfish myocardium, and may be a requirement to allow enough current to flow in order to activate contraction in a heart known to have a low diastolic membrane potential (Jensen, 1965).

A higher HCN mRNA expression in the hagfish atrium compared with the ventricle for all isoforms of HCN (apart from the similar expression of HCN3b) was to be expected for synchronous cardiac contraction, because, if HCN protein expression is increased as a result of higher the mRNA expression, the atrial pacemaker potential would be be shorter than that of the ventricle. Indeed, HCN gene expression per unit mass in rabbit atria and especially the sinoatrial node are much higher than that in the ventricles (Shi et al., 1999; Moosmang et al., 2001; Marionneau et al., 2005). In turn, a larger sarcolemmal ion channel density allows a larger current to pass when the membrane channels are open (Mukherjee et al., 1998). Thus, a higher

atrial HCN channel expression leads to a larger I_f and a faster depolarisation to the threshold potential between action potentials, which ensures atrial contraction precedes ventricular contraction. Although, expression of 4 out of 5 isoforms was roughly twice as high in the atrium compared to the ventricle, I have no information on sarcolemmal protein expression. However, it seems that relative excitation, and therefore synchrony of contraction, of hagfish cardiac chambers is controlled primarily by HCN3a channel density rather than up regulation and down regulation of specific isoforms in the chambers, however this needs to be investigated directly. Indeed, it is possible that regulation of HCN channel density throughout the myocardium may be a precursor of the cardiac conduction system seen in other craniate hearts.

I postulated, and my data support the idea that alterations in HCN channel mRNA expression could play a role in controlling heart rate during prolonged anoxia and subsequent recovery. Consistent with the 50% decrease in heart rate during anoxia, an increased heart rate during the initial phase of recovery and the return of heart rate to the control normoxic rate after 36 h of recovery (Cox et al., 2010), I discovered significant changes in HCN mRNA expression with anoxia and recovery. Ventricular HCN3a mRNA expression decreased significantly to only 7 times that of HCN4 compared to 25 times in controls. Even though, in mammals, HCN4 allows a smaller current to flow compared to HCN3, and even if there is a concurrent decrease in ventricular HCN3a protein, this result will have no effect on heart rate since there is no decrease in the atrium which is the driver of heart rate.

The immediate response to restoration of normoxic conditions for the hagfish heart is a quadrupling of the anoxia-induced heart rate after 2 h (Cox et al., 2010), which correlates with the increase in the mRNA expression of atrial HCN3a and a restored level of ventricular HCN3a. An increase in HCN3a mRNA could increase I_f if it is matched by rising protein levels. In turn, this would increase the pacemaker potential, and therefore, increase heart rate since the action potential upstroke threshold would be reached earlier. HCN3a mRNA expression returned to control levels by the end of the 36h recovery period in both cardiac chambers. Therefore, it is possible that regulation of HCN3a may play a role in controlling heart rate during prolonged anoxia and subsequent recovery; however, the time course for HCN protein translation and insertion in to the cell membrane has not been measured. The only studies that have looked at the relationship between HCN mRNA production and HCN protein function measured the protein function 3-5 days following the increase in mRNA expression (Santoro et al., 2004; Santoro et al., 2005). Therefore, these hypotheses need to be tested using protein expression and electophysiological studies.

The above discussion considered the HCN channel as the primary craniate cardiac pacemaker, yet a recent alternative hypothesis suggests that pacemaker activity involves a calcium clock (Rubenstein and Lipsius, 1987; Huser et al., 2000; Ju and Allen, 2000; Dobrzynski et al., 2007; Maltsev and Lakatta, 2008; Monfredi et al., 2013). In brief, this hypothesis proposes that spontaneous Ca^{2+} sparks released

from the sarcoplasmic reticulum via ryanodine receptors interact with local sodium-calcium exchanger proteins to cause an overall inward depolarizing current. The Ca^{2+} is then pumped back into the sarcoplasmic reticulum, thus resetting the clock. The most recent synthesis of experimental evidence in support of the calcium clock hypothesis includes HCN as a part, albeit a minor one, of the inter-related cycling of ions in pacemaker tissue (Monfredi et al., 2013). That said, this model does recognize that future work is still needed to completely explain how HCN blockers such as ZD7288 exert their effect within the calcium clock paradigm. Here, in chapter 2, I found no experimental support for a calcium clock in the hagfish atrium because I used another HCN blocker, zatebradine, which completely abolished the intrinsic heart beat of atrial tissue and nearly abolished it in ventricular tissue of hagfish, and revealed that ryanodine, a blocker of ryanodine channels, preventing calcium cycling through the sarcoplasmic reticulum, was without effect on both atrial and ventricular tissues. Moreover, future calcium clock studies will have to resolve why zatebradine slows pacemaker activity in rainbow trout hearts (Gamperl et al., 2011) and how recent work in mice has shown that an increase in heart rate during pregnancy is coupled with an increase in HCN2 expression (Khoury et al., 2013).

3.4.1 Summary

In summary, I have provided novel insights into cardiac pacemaker channel mRNA expression and their evolution using real-time RT-PCR of partially cloned HCN mRNA expressed in hagfish hearts under normoxic and anoxic conditions. Of the six isoforms, HCN3a dominated in atrium and ventricle. The presence of HCN2,

HCN3 and HCN4 in the hagfish heart suggests their presence prior to the hagfish/vertebrate divergence and an importance in cardiac pacemaker activity at the time of the emergence of the chambered myogenic heart. HCNa and HCNb either appeared as hagfish-specific duplications or remnants of the forms previously identified in ancestral deuterostomes and urochodates. My postulate that altered HCN expression is involved in the control of heart rate in the aneural hagfish heart was supported by several observations, including a decrease in the ventricular HCN3 mRNA expression during anoxia and an increase in atrial HCN3 mRNA expression shortly after return to normoxia. Functional studies on the activation and cAMP gating of each isoform, and their respective roles in cardiac pacing will require electrophysiological study, which, when coupled with HCN protein expression studies, will provide even further insight into regulation of heart rate in the aneural hagfish heart.

Table 3.1. Primers used for, and lengths of, partially cloned hagfish HCN genes.

Gene		Primer for Cloning	Amino acid length of partially cloned segment
HCN2a	F	TTCCTCATTGTGGAGAAGGG	291
HCN2b	R	GATTTCCCCGAAGTAGGAGC	367
HCN3a	F	GGGGAGCTGAGTGAGCCTCTCAAA	78
	R	TGTTGCCCTTGGTGAGGATGCTG	
HCN3bi	F	GCTGAGGTCTACAAGACGGC	283
HCN3bii	R	TAAAGGCGGCAGTAGGTGTC	304
HCN3c	F	CATGCCGCTGTTTGCCAAT	48
	R	CAAAGTAGGAGCCATCTGCCAGTTTAG	
HCN4	F	TCCCAACTTTGTGACATCCA	65
	R	GGCAGTAGGTATCAGCTCGC	

HCN, Hyperpolarization-activated cyclic nucleotide-gated channel
F, Forward primer
R, Reverse primer

Table 3.2. Primers used for real-time RT-PCR.

Gene		Primer for Cloning
HCN2	F	GCATCCTGGGCGAGCTGAAC
	R	CCCGGCTGGAAGACCTCGAACTT
HCN3a	F	GGGGAGCTGAGTGAGCCTCTCAAA
	R	TGTTGCCCTTGGTGAGGATGCTG
HCN3b	F	CGTGCTATGCCATGTTCATC
	R	CCCCAAGAATGCTATCCTCA
HCN3c	F	CATGCCGCTGTTTGCCAAT
	R	CAAAGTAGGAGCCATCTGCCAGTTTAG
HCN4	F	AGGGCACCATTGGCAAGAAA
	R	CAAAGTAGGAGCCATCTGCCAGTTAG

HCN, Hyperpolarization-activated cyclic nucleotide-gated channel; F, Forward primer; R, Reverse primer

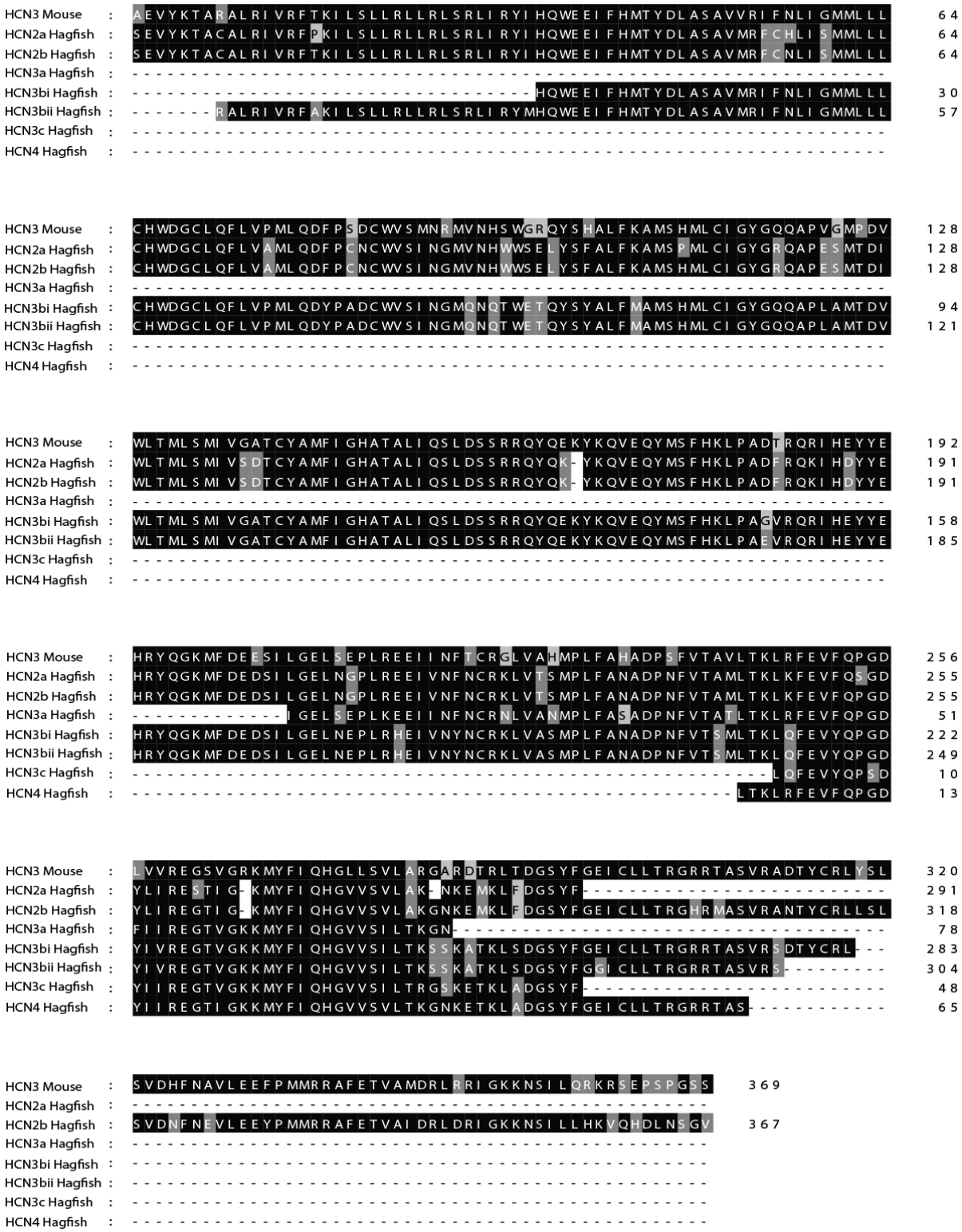
HCN3 Mouse : AEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRIFNLIGMMLL 64
HCN2a Hagfish : SEVYKTACALRIVRFPKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVMRFCHLISMMLL 64
HCN2b Hagfish : SEVYKTACALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVMRFCNLISMMLL 64
HCN3a Hagfish : ---------- 0
HCN3bi Hagfish : ---------- HQWEEIFHMTYDLASAVMRIFNLIGMMLL 30
HCN3bii Hagfish : -------RALRIVRFAKILSLLRLLRLSRLIRYMHQWEEIFHMTYDLASAVMRIFNLIGMMLL 57
HCN3c Hagfish : ---------- 0
HCN4 Hagfish : ---------- 0
HCN3 Mouse : CHWDGCLQFLVPMLQDFPSDCWVSMNRMVNHSWGRQYSHALFKAMSHMLCIGYGQQAPVGMPDV 128
HCN2a Hagfish : CHWDGCLQFLVAMLQDFPCNCWVSINGMVNHWWSELYSFALFKAMSPMLCIGYGRQAPESMTDI 128
HCN2b Hagfish : CHWDGCLQFLVAMLQDFPCNCWVSINGMVNHWWSELYSFALFKAMSHMLCIGYGRQAPESMTDI 128
HCN3a Hagfish : ---------- 0
HCN3bi Hagfish : CHWDGCLQFLVPMLQDYPADCWVSINGMQNQTWETQYSYALFMAMSHMLCIGYGQQAPLAMTDV 94
HCN3bii Hagfish : CHWDGCLQFLVPMLQDYPADCWVSINGMQNQTWETQYSYALFMAMSHMLCIGYGQQAPLAMTDV 121
HCN3c Hagfish : ---------- 0
HCN4 Hagfish : ---------- 0
HCN3 Mouse : WLTMLSMIVGATCYAMFIGHATALIQSLDSSRRQYQEKYKQVEQYMSFHKLPADTRQRIHEYYE 192
HCN2a Hagfish : WLTMLSMIVSDTCYAMFIGHATALIQSLDSSRRQYQK-YKQVEQYMSFHKLPADFRQKIHDYYE 191
HCN2b Hagfish : WLTMLSMIVSDTCYAMFIGHATALIQSLDSSRRQYQK-YKQVEQYMSFHKLPADFRQKIHDYYE 191
HCN3a Hagfish : ---------- 0
HCN3bi Hagfish : WLTMLSMIVGATCYAMFIGHATALIQSLDSSRRQYQEKYKQVEQYMSFHKLPAGVRQRIHEYYE 158
HCN3bii Hagfish : WLTMLSMIVGATCYAMFIGHATALIQSLDSSRRQYQEKYKQVEQYMSFHKLPAEVRQRIHEYYE 185
HCN3c Hagfish : ---------- 0
HCN4 Hagfish : ---------- 0
HCN3 Mouse : HRYQGKMFDEESILGELSEPLREEIINFTCRGLVAHMPLFAHADPSFVTAVLTKLRFEVFQPGD 256
HCN2a Hagfish : HRYQGKMFDEDSILGELNGPLREEIVNFNCRKLVTSMPLFANADPNFVTAMLTKLKFEVFQSGD 255
HCN2b Hagfish : HRYQGKMFDEDSILGELNGPLREEIVNFNCRKLVTSMPLFANADPNFVTAMLTKLKFEVFQPGD 255
HCN3a Hagfish : ----------IGELSEPLKEEIINFNCRNLVANMPLFASADPNFVTATLTKLRFEVFQPGD 51
HCN3bi Hagfish : HRYQGKMFDEDSILGELNEPLRHEIVNYNCRKLVASMPLFANADPNFVTSMLTKLQFEVYQPGD 222
HCN3bii Hagfish : HRYQGKMFDEDSILGELNEPLRHEIVNYNCRKLVASMPLFANADPNFVTSMLTKLQFEVYQPGD 249
HCN3c Hagfish : ----------LQFEVYQPSD 10
HCN4 Hagfish : ----------LTKLRFEVFQPGD 13
HCN3 Mouse : LVVREGSVGRKMYFIQHGLLSVLARGARDTRLTDGSYFGEICLLTRGRRTASVRADTYCRLYSL 320
HCN2a Hagfish : YLIRESTIG-KMYFIQHGVVSVLAK-NKEMKLFDGSYF---------- 291
HCN2b Hagfish : YLIREGTIG-KMYFIQHGVVSVLAKGNKEMKLFDGSYFGEICLLTRGHRMASVRANTYCRLLSL 318
HCN3a Hagfish : FIIREGTVGKKMYFIQHGVVSILTKGN---------- 78
HCN3bi Hagfish : YIVREGTVGKKMYFIQHGVVSILTKSSKATKLSDGSYFGEICLLTRGRRTASVRSDTYCRL--- 283
HCN3bii Hagfish : YIVREGTVGKKMYFIQHGVVSILTKSSKATKLSDGSYFGGICLLTRGRRTASVRS--------- 304
HCN3c Hagfish : YIIREGTVGKKMYFIQHGVVSILTRGSKETKLADGSYF---------- 48
HCN4 Hagfish : YIIREGTIGKKMYFIQHGVVSVLTKGNKETKLADGSYFGEICLLTRGRRTAS---------- 65
HCN3 Mouse : SVDHFNAVLEEFPMMRRAFETVAMDRLRRIGKKNSILQRKRSEPSPGSS 369
HCN2a Hagfish : ---------- 0
HCN2b Hagfish : SVDNFNEVLEEYPMMRRAFETVAIDRLDRIGKKNSILLHKVQHDLNSGV 367
HCN3a Hagfish : ---------- 0
HCN3bi Hagfish : ---------- 0
HCN3bii Hagfish : ---------- 0
HCN3c Hagfish : ---------- 0
HCN4 Hagfish : ---------- 0

Figure 3.1. Amino acid sequence comparison of partially cloned hagfish HCN isoforms. The segment of mouse HCN3 that aligns with hagfish sequences is also shown to highlight the large degree of sequence identity between the two species. Shading indicates 4 levels of sequence conservation with darker shading corresponding to higher conservation. Genes aligned using ClustalX, compared using GeneDoc and edited using Adobe Illustrator CS5. HCN, hyperpolarization-activated cyclic nucleotide-gated channel.


HCN1
HCN2
HCN3
HCN4
Outgroup
HCN2a Hagfish
HCN2b Hagfish
HCN2b Green puffer
HCN2 Human
HCN2b Fugu
HCN2 Rat
HCN2 Mouse
HCN4a Green puffer
HCN2 Hen
HCN4a Fugu
HCN4 Human
HCN4 Mouse
HCN4 Rat
HCN4 Chimpanzee
HCN4b Fugu
HCN4 Rabbit
HCN4b Green puffer
HCN4 Hen
HCN4 Hagfish
HCN3c Hagfish
HCN3a Hagfish
HCN3b Hagfish
HCN3 Green puffer
HCN3 Fugu
HCNb Ciona
HCNa Ciona
HCNc Ciona
ERG Human
CNG Drosophila
HCN3 Opossum
HCN3 Rat
HCN3 Dog
HCN3 Human
HCN3 Cow
HCN3 Mouse
HCN2 Zebrafish
HCN2a Fugu
HCN2 Killifish
HCN2a Green puffer
HCN2 Trout
HCN1 Trout
HCN1 Green puffer
HCN1 Hen
HCN1 Mouse
HCN1 Chimpanzee
HCN1 Rabbit
HCN1 Human
HCN1 Opossum
HCN1 Rat
826
141
109
972
288
31
342
237
80
851
111
118
118
121
303
224
604
988
442
430
667
930
644
829
409
329
974
400
442
866
681
300
158
91
110
670
82
95
55
130
0.01

Figure 3.2. Bootstrapped Neighbor-Joining phylogeny of HCN genes including hagfish HCN isoforms. Hagfish genes were partially cloned and sequenced from hagfish hearts. *Drosophila*, *Ciona* and vertebrate sequences downloaded from GenBank and Ensembl. Sequences aligned in ClustalX, edited and translated to amino acids in GeneDoc. Phylogeny compiled using Phylip-3.69 and edited using Adobe Illustrator CS5. Tree run with 1000 bootstrap datasets, low bootstrap values are likely a consequence of both the short 37 amino acid sequence used and a high sequence identity as seen in Jackson et al. (2007). HCN, Hyperpolarization-activated cyclic nucleotide-gated channel, CNG, cyclic nucleotide gated channel, ERG, Ether-á-go-go related gene. Nomenclature used by Jackson et al. (2007) is used with chicken abbreviated to Hen for clarity. Scale bar represents 1% residue substitutions per site.

Name		Amino acid sequence	Identifier
HCN2a Green puffer	:	LRFEVFQPHDYIIREGTIGKKMYFIQHGVCSVITKGT	ENSTNIG00000015314
HCN2a Fugu	:	LRFEVFQPHDYIIREGTIGKKMYFIQHGVCSVITKGT	ENSTRUG00000009472
HCN2 Killifish	:	LRFEVFQPHDYIIREGTIGKKMYFIQHGVCSVITKGT	NA
HCN2 Zebrafish	:	LRFEVFQPGDYIIREGTIGKKMYFIQHGVVNVITKGT	ENSDARG00000090115
HCN2b Green puffer	:	LRFEVFQPKDYIVREGTIGKKMFFIQHGVVSILTKGN	ENSTNIG00000010643
HCN2b Fugu	:	LRFEVFQPKDYIVREGTIGKKMFFIQHGVVSILTKGN	ENSTNIG00000018063
HCN2 Mouse	:	LKFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKGN	ENSMUSG00000020331
HCN2 Rat	:	LKFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKGN	ENSRNOG00000008831
HCN2 Human	:	LKFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKGN	ENSG00000099822
HCN2a Hagfish	:	LKFEVFQSGDYLIRESTIG-KMYFIQHGVVSVLAK-N	
HCN2b Hagfish	:	LKFEVFQPGDYLIREGTIG-KMYFIQHGVVSVLAKGN	
HCN2 Trout	:	LRFEVFQPGDYIIREGTIGKKMYFIQHGVTSVLTKGT	NA
HCN1 Mouse	:	LRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSS	ENSMUSG00000021730
HCN1 Rat	:	LRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSS	ENSRNOG00000011522
HCN1 Opossum	:	LRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSS	ENSMODG00000020267
HCN1 Chimpanzee	:	LRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSS	ENSPTRG00000016855
HCN1 Human	:	LRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSS	ENSG00000164588
HCN1 Rabbit	:	LRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSS	GI:38605639
HCN1 Hen	:	LRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSN	ENSGALG00000014875
HCN1 Green puffer	:	LKFEVFQPNDYIIREGTVGKKMYFIQHGVASVITKSN	ENSTNIG00000007528
HCN1 Trout	:	LKFEVFQPNDYIIREGTVGKKMYFIQHGVASVITKLN	NA
HCN3b Hagfish	:	LQFEVYQPGDYIVREGTVGKKMYFIQHGVVSILTKSS	
HCN2 Hen	:	LKFEVFQPGDYIIREGTIGKKMYFIQHGVVSILTKGN	ENSGALG00000001342
HCN4 Hagfish	:	LRFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKGN	
HCN4 Mouse	:	LRFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKGN	ENSMUSG00000032338
HCN4 Rat	:	LRFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKGN	ENSRNOG00000009450
HCN4 Human	:	LRFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKGN	ENSG00000138622
HCN4 Chimpanzee	:	LRFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKGN	ENSPTRG00000007258
HCN4 Rabbit	:	LRFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKGN	GI:38605640
HCN3c Hagfish	:	LQFEVYQPSDYIIREGTVGKKMYFIQHGVVSILTRGS	
HCN4 Hen	:	LRFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKGN	ENSGALG00000001764
HCN3a Hagfish	:	LRFEVFQPGDFIIREGTVGKKMYFIQHGVVSILTKGN	
HCN4b Green puffer	:	LRFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKGN	ENSTNIG00000011958
HCN4b Fugu	:	LRFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKGN	ENSTRUG00000013040
HCN4a Green puffer	:	LRFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKSS	NA
HCN4a Fugu	:	LRFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKSS	ENSTRUG00000005413
HCN3 Green Puffer	:	LRFEVFQPSDFIIREGTVGRKMYFIQHGRVSVLTRGN	ENSTNIG00000008070
HCN3 Fugu	:	LRFEVFQPSDFIIREGTVGRKMYFIQHGRVSVLTRGN	ENSTRUG00000015016
HCN3 Mouse	:	LRFEVFQPGDLVVREGSVGRKMYFIQHGLLSVLARGA	ENSMUSG00000028051
HCN3 Rat	:	LRFEVFQPGDLVVREGSVGRKMYFIQHGLLSVLARGA	ENSRNOG00000020444
HCN3 Human	:	LRFEVFQPGDLVVREGSVGRKMYFIQHGLLSVLARGA	ENSG00000143630
HCN3 Cow	:	LRFEVFQPGDLVVREGSVGRKMYFIQHGLLSVLARGA	ENSBTAG00000017055
HCN3 Opossum	:	LRFEVFQPGDLVVREGSVGRKMYFIQHGLLSVLTRGA	ENSMODG00000017060
HCN3 Dog	:	LRFEVFQPGDLVVREGSVGRKMYFIQHGLLSVLARGA	ENSCAFG00000016963
HCNb Ciona	:	LKYEVFQPKDVIVREGEIGKKMYFIQHGLVEVKNSHR	NA
HCNa Ciona	:	LNFEVFLNDEVIVKEGTEGKKMYFINRGTVTIKSAQH	NA
HCNc Ciona	:	MVFTVYTADAEVVSEGEKAFHMFFILRGELVIEASDG	NA
ERG Human	:	FKTTHAPPGDTLVHAGDLLTALYFISRGSIEILRGDV	ENSG00000143473
CNG Drosophila	:	LKLQVFSPGDYICRKGDVGKEMYIVKRGKLSVVGDDG	FBgn0261612

Figure 3.3. Aligniment of the overlapping, thirty seven amino acid long sequences from *Ciona*, hagfish and vertebrate HCN cyclic-nucleotide binding domain utilized in the phylogenetic analysis. Shading indicates 4 levels of sequence conservation with darker shading corresponding to higher conservation. Genes aligned using ClustalX, compared using GeneDoc and edited using Adobe Illustrator CS5. CNG, cyclic nucleotide gated channel, ERG, Ether-á-go-go related gene. Hen = Chicken. HCN, Hyperpolarization-activated cyclic nucleotide-gated channel.

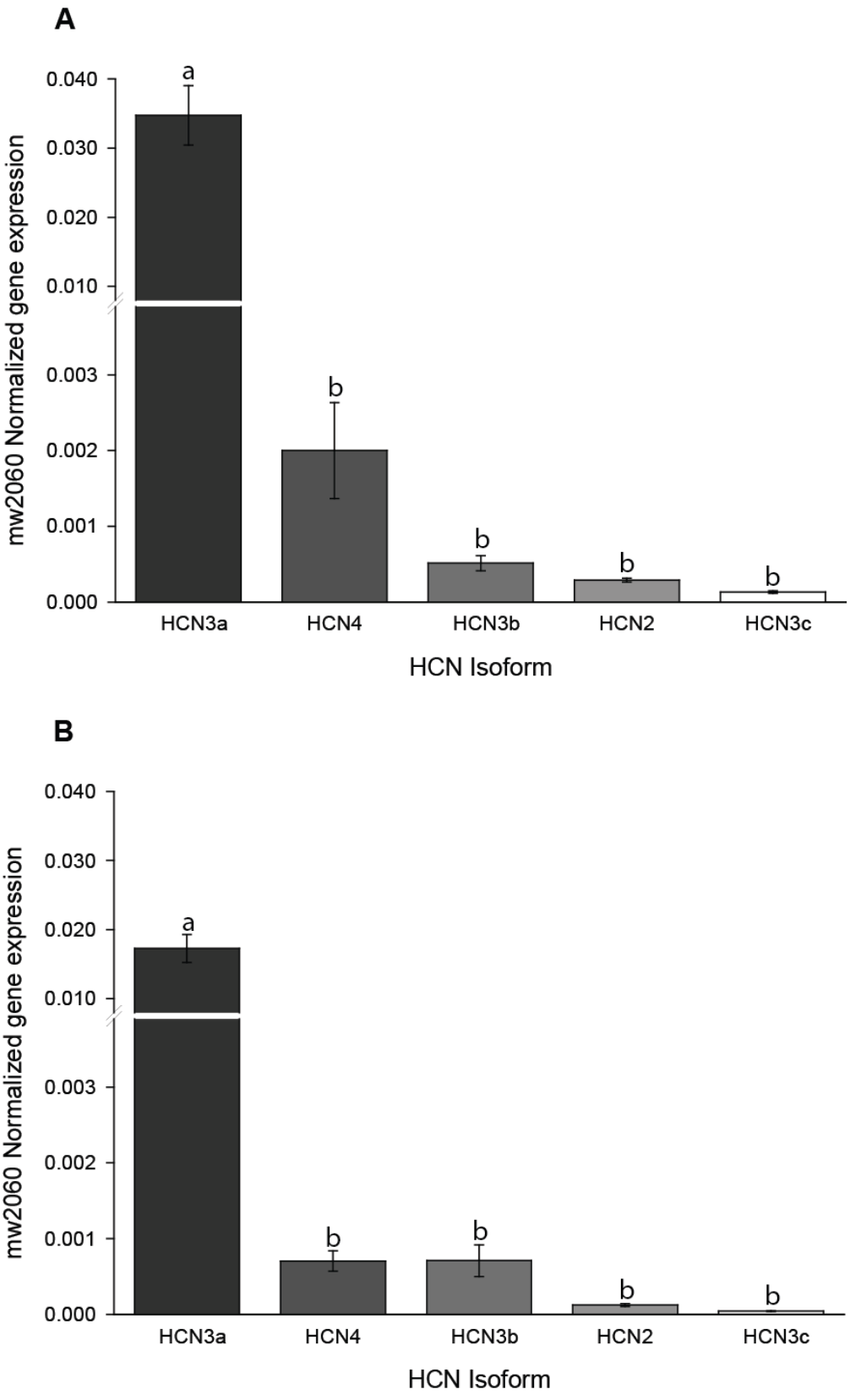
A
mw2060 Normalized gene expression
0.040
0.030
0.020
0.010
0.003
0.002
0.001
0.000
a
b
b
b
b
HCN3a
HCN4
HCN3b
HCN2
HCN3c
HCN Isoform
B
mw2060 Normalized gene expression
0.040
0.030
0.020
0.010
0.003
0.002
0.001
0.000
a
b
b
b
b
HCN3a
HCN4
HCN3b
HCN2
HCN3c
HCN Isoform

Figure 3.4. A comparison of HCN gene isoform expression in hagfish atrium (**A**) and ventricle (**B**) under normoxic control conditions. Gene expression measured by real-time RT-PCR and normalized to mw2060 control gene expression. Note the change in scale on the y-axis following the break. Dissimilar letters denote significant differences between gene expression (1-way ANOVA, $P<0.05$). N = 10. Error bars show s.e.m. HCN, hyperpolarization-activated cyclic nucleotide-gated channel.

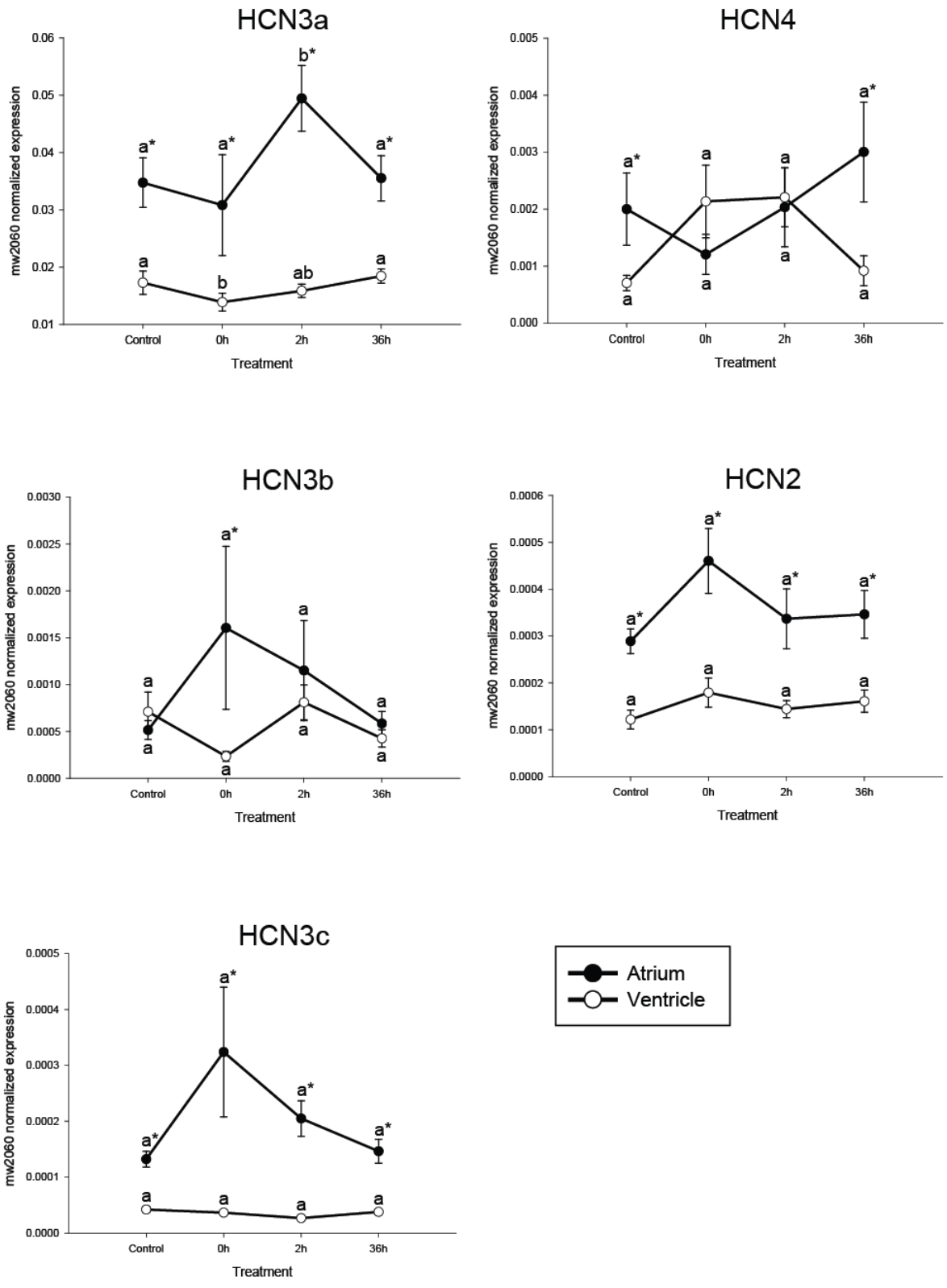
HCN3a
mw2060 normalized expression
0.06
0.05
0.04
0.03
0.02
0.01
a*
a*
b*
a*
a
b
ab
a
Control
0h
2h
36h
Treatment
HCN4
mw2060 normalized expression
0.005
0.004
0.003
0.002
0.001
0.000
a*
a
a
a*
a
a
a
a
Control
0h
2h
36h
Treatment
HCN3b
mw2060 normalized expression
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
a
a*
a
a
a
a
a
a
Control
0h
2h
36h
Treatment
HCN2
mw2060 normalized expression
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
a*
a*
a*
a*
a
a
a
a
Control
0h
2h
36h
Treatment
HCN3c
mw2060 normalized expression
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
a*
a*
a*
a*
a
a
a
a
Control
0h
2h
36h
Treatment
Atrium
Ventricle

Figure 3.5. Effect of 24 h anoxia (0h) and normoxic recovery following 24 h anoxia (2h and 36h) on HCN isoform gene expression in atrium and ventricle of hagfish hearts. Gene expression measured by real-time RT-PCR and normalized to mw2060 control gene expression. Dissimilar letters denote significant differences within a tissue among treatment times (2-way ANOVA, $P<0.05$) and an asterisk denotes significantly higher gene expression in the atrium compared to the ventricle within at a specific treatment time (*t*-test, $P<0.05$). N = 10 for all groups bar N = 9 for 0 h ventricles. Error bars show s.e.m. HCN, Hyperpolarization-activated cyclic nucleotide-gated channel.

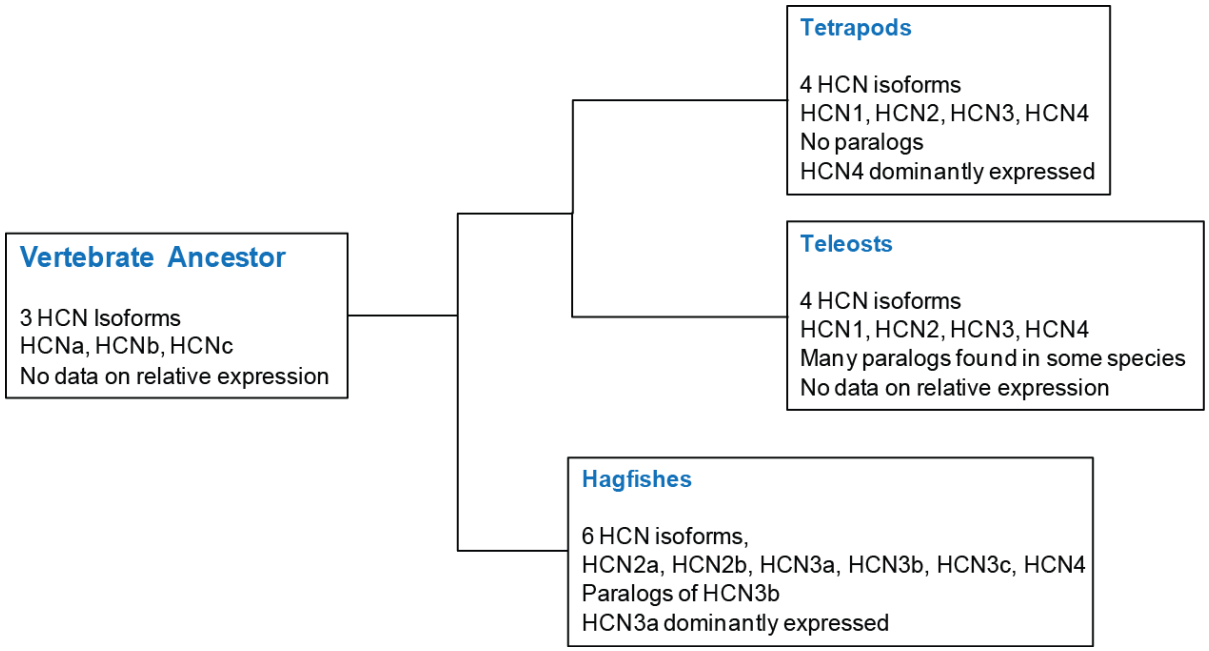

Figure 3.6. Summary of phylogenetics and HCN isoform expression in Chordates showing known presence of HCN isoforms, paralogs resulting from duplications of a HCN isoform and dominantly expressed HCN isoforms. Prior to the angnathan/gnathostome split, at least 3 of the 4 vertebrate HCN isoforms had appeared. Since this split, likely through gene specific duplications and mutations, at least 5 isoforms, plus two paralogs emerged in the hagfishes. The emergence of paralogs also took place in the teleost lineage. Following the emergence of the gnathostomes, a reliance on HCN4 as the dominantly expressed HCN isoform occurred. When this happened is presently unknown due to the lack of HCN expression data from teleost fishes and ancestral tetrapods. HCN, Hyperpolarization-activated cyclic nucleotide-gated channel.

CHAPTER 4: THE ROLES OF TRANSMEMBRANE AND SOLUBLE ADENYLYL CYCLASE-PRODUCED cAMP ON THE HEART RATE OF THE PACIFIC HAGFISH, *EPTATRETUS STOUTII*: THE DISCOVERY OF A NOVEL CARDIAC CONTROL MECHANISM

4.1 Introduction

Hagfishes are ancestral craniates, whose origin dates back 0.5 billion years. Their chambered heart is structurally and functionally similar to the vertebrate heart by possessing a sinus venosus, atrium and ventricle (all guarded by valves) that contract myogenically and by increasing stroke volume with increased cardiac filling thereby following the Frank-Starling mechanism (Satchell, 1991; Farrell, 2007b). Moreover, neural control of heart rate has progressively evolved in the vertebrate lineage towards a dual sympathetic (stimulatory β-adrenergic) and parasympathetic (inhibitory cholinergic) control. Thus, the hagfish heart offers a fascinating model for the study of early cardiac control mechanisms because it is anatomically and functionally aneural, but is known to include conventional adrenergic mechanisms, albeit from catecholamines stored in the heart itself (Greene, 1902; Augustinsson, 1956; Jensen, 1961; Jensen, 1965; Nilsson, 1983; Farrell, 2007a). In addition, like other unusual vertebrates, such as crucian carp and freshwater turtles, hagfish tolerate prolonged anoxia by greatly slowing heart rate (Vornanen and Tuomennoro, 1999; Hicks and Farrell 2000 a,b; Stecyk et al., 2004; Stecyk et al., 2007; Cox et al., 2010). In fact, whereas the hagfish heart beats rhythmically at half its normal rate

during prolonged (36 h) anoxia, heart rate can then increase almost four-fold during a 1.5 h normoxic recovery without cardiac innervation (Cox et al., 2010).

The role of catecholaminergic controls of heart rate are presumed to be similar in hagfishes as to vertebrate hearts due to the role of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels in setting basal pacemaker rate (Chapters 2 and 3). In hearts from all vertebrate classes from sharks to mammals, catecholamine-stimulated production of cAMP by membrane-bound adenylyl cyclase (tmAC) and binding of this cAMP to the cyclic nucleotide binding domain (CNBD) of the HCN channel subunits increases the formation of gating rings, the open probability of the channel, I_f (a gently depolarizing influx of Na^+ and K^+ ions into the pacemaker cells) and heart rate (DiFrancesco and Tortora, 1991; Wainger et al., 2001; Chow et al., 2011). This effect can be produced through humoral (noradrenaline or adrenaline) or neural (noradrenaline) in certain teleosts and more derived vertebrates (Nilsson, 1983). In hagfish, the β-adrenoreceptor antagonist sotalol also slows heart rate, suggesting tonic stimulation of pacemaker rate, but most likely through paracrine actions of noradrenaline and adrenaline, which are released from chromaffin tissue located within the cardiac chambers themselves (von Euler and Fänge, 1961; Bloom et al., 1963; Axelsson et al., 1990; Perry et al., 1993; Farrell, 2007a). Therefore, I hypothesized that the changes in hagfish heart rate seen during an anoxic challenge were primarily due to catecholamine stimulation, with adrenergic stimulation ceasing during anoxia and being restored during recovery to produce the observed tachycardia.

In addition, given the recent discovery of a soluble form of adenylyl cyclase (sAC) that increases cAMP concentration in response to HCO_3^- stimulation in a variety of organs and organisms ranging from corals to elasmobranchs (Buck et al., 1999; Tresguerres et al., 2010; Tresguerres et al., 2011; Barott et al., 2013), I hypothesized that a sAC-mediated pathway of cAMP production and HCN gating might represent a novel, but ancient heart rate control mechanism in hagfish.

4.2 Materials and methods

4.2.1 Animal husbandry

Pacific hagfish (112 ± 1 g; mean ± s.e.m.; n =110), *Eptatretus stoutii* (Lockington 1878), were collected off the coast of Bamfield Marine Sciences Centre (BMSC), Bamfield, BC, Canada, using baited traps. Hagfish were held either at BMSC or transported to the Vancouver area where they were housed in 4,000 l tanks supplied with flow-through seawater (10±1°C) and fed squid weekly at the West Vancouver Laboratory (WVL), Department of Fisheries and Oceans Canada, West Vancouver, BC, Canada. Animals were fasted for a minimum of one week prior to experimental treatment, and all experiments were conducted in accordance with the animal care policies of the University of British Columbia, BMSC and WVL. *In vivo* anoxia exposures and subsequent sampling were carried out at BMSC, cAMP measurements, Western blotting and immunoflourescence were conducted at the Scripps Institute of Oceanography, University of California, San Diego, CA, USA,

while isolated heart experiments took place at the University of British Columbia, Vancouver, BC, Canada.

4.2.2 Isolated heart experiments

Hagfish were sacrificed by a blow to the head followed by immediate decapitation. The heart was rapidly excised and immersed in 25 ml chilled (10°C) hagfish saline (mM: 410 NaCl, 10 KCl, 14, $MgSO_4$, 4 urea, 20 glucose, 10 HEPES, 4 $CaCl_2$, pH 7.9. Chemicals sourced from Sigma Aldrich, St. Louis, MO, USA) within a plastic basket containing two electrocardiogram (ECG) electrodes. ECG was continuously monitored, stored and analyzed using Biopac and AcqKnowledge software (Goleta, CA, USA). Heart rate was allowed to reach a stable rate, which took <1 h, prior to any measurement (an average over 10 beats). Trial runs (data not shown) were used to establish that heart rate would remain stable under control conditions for >24 h and dose-response curves established the minimum dose required for the maximum effect for each treatment.

For transmembrane adenylyl cyclase (tmAC) inhibition studies, the saline was changed every 30 min with those containing 1.0 pM to 1 mM nadolol (Tocris Bioscience, Minneapolis, MN, USA) in increments. Following the maximal response with 1 mM nadolol, anoxia was induced by bubbling with N_2 for 2 h before recovering the heart in aerated saline. To assess the role of tmAC in increasing heart rate during recovery, additional hearts were subjected to anoxia for 2 h prior to the addition of increasing doses (1.0 pM to 100 µM) of the tmAC agonist forskolin

(Tocris Bioscience) every 30 min. Forskolin was dissolved in DMSO (Sigma Aldrich) for a final DMSO concentration of up to 0.01 µl DMSO.ml saline^{-1} in the final preparation. Controls showed that hagfish heart rate was unaffected by up to 10 µl DMSO.ml saline^{-1}.

For soluble adenylyl cyclase (sAC) investigations, hearts were first exposed to 2 h anoxia prior addition of 20 to 60 mM $NaHCO_3$ (Sigma Aldrich) every 30 min. The sAC antagonist KH7 (Tocris Bioscience) was used to show that effects of HCO_3^- were mediated by sAC in separate experiments by adding 50 µM KH7 dissolved in DMSO (as above) with 60 mM HCO_3^- during anoxia. In order to asses the effects of added HCO_3^- solely, as opposed to in addition to HCO_3^- in the perfusate, CO_2 was not added to the saline. Some of the HCO_3^- added would have converted to CO_2 in the saline, meaning the HCO_3^- concentration seen by the heart would have been lower than that added. However, all experiments were run identically, therefore, each heart would have seen the same HCO_3^- concentration.

4.2.3 Anoxic exposure in vivo

Batches of four hagfish were individually held overnight in darkened 2.5 litre chambers that were continuously flushed at 0.5 l.min^{-1} with aerated seawater (10 ± 1°C), during which they adopted their stereotypical curled-up posture (Cox et al., 2010, Chapter 3). Oxygen concentration was monitored throughout the experiment using MINI-DO probes (Loligo Systems, Tjele, Denmark) every 1 s. Anoxia was generated in <1 h by supplying N_2-saturated seawater to the chambers from two

gas-exchange columns placed in-series. Hagfish were sampled at 3 h, 6 h, 12 h and 24 h into anoxic exposure, as well as following rapid recovery of normoxic conditions (less than 30 min) at 1 h and 6 h recovery. Control animals remained in normoxia for 42 h. At their sampling time, animals were sacrificed by a blow to the head, followed by decapitation. Blood was taken from the subcutaneous sinus and the plasma separated by centrifugation for plasma total CO_2 measurement. Following this, the heart was rapidly excised and freeze clamped in liquid N_2 for Western blots, cAMP assays and sAC activity assays. Blood and tissue samples were stored at -80°C until analysis. As with my other chapters, animals here were not anaesthetized, as a result, fish struggled, knotted and produced slime. Movements were particularly forceful from control and recovery fish. Additionally, a caveat with taking blood from the sinus is that it is a low-turnover blood compartment. During the prolonged anoxia period, sinus blood may equilibrate with the venous circulation, however, during the more frequent normoxic measurements, the sinus and venous blood compartments may not have been equilibrated during blood sampling.

4.2.4 cAMP measurements

cAMP concentration was measured in control hearts and those exposed to anoxia for 3, 6, 12 and 24 h (n=5 per treatment, 89 ± 8 mg, mean ± s.e.m.) using a DirectX® high sensitivity direct cyclic AMP chemiluminescent immunoassay kit (Arbor Assays, Ann Arbor, MI, USA) and a slightly modified version of their acetylation protocol. Samples were homogenized under liquid N_2 using a pestle and mortar, followed by a glass homogenizer filled with a volume (µl) of simple diluents (SD) equal to sample

mass (mg) x 10, and were incubated on ice for 10 min. Samples were then spun down at 600 g for 15 min at 4°C, the supernatant collected and stored at -80°C for further analysis. An acetylation reagent (AR) mix was produced by mixing acetic anhydride with triethylamine in a ratio of 1:2. An aliquot of 6 µl AR was added to 12 µl sample supernatant and 108 µl SD in a 96-well plate. In each well of a separate 96 well plate, 50 µl plate primer, 50 µl acetylated sample, 25 µl DirectX® cAMP conjugate and 25 µl DirectX® cAMP antibody were added and the plate was shaken for 2 h at room temperature. Following this, the wells were aspirated 4 times with 300 µl wash buffer and tapped dry prior to the addition of 100 µl chemiluminescent substrate. The luminescence at 450 nm was then read using a 96-well plate reader with cAMP concentration being calculated against a concurrently produced cAMP standard curve using the plate reader's built-in software.

4.2.5 Western blots

Hearts exposed to 24 h anoxia and controls (n=5 per treatment, 100 ± 17 mg, mean ± s.e.m) were homogenized under liquid N_2 using a pestle and mortar, followed by a glass homogenizer filled with 500 µl homogenization buffer. Samples were then spun down at 500 g for 15 min at 4°C, the supernatant collected and stored at -80°C until further analysis. The supernatant was then mixed with equal volumes of Laemmli buffer with 5% β-mercaptoethanol and heated at 95°C for 5 min. 13 µg of total protein from each sample (estimated by Bradford assay) was separated by SDS/PAGE and transferred to PVDF membranes. Transfer was blocked in 10% (w/v) milk-TBST for 30 min and incubated with anti-dfsAC (Tresguerres et al., 2010)

overnight at 4°C, followed by 1 h incubation with HRP-conjugated goat anti-rabbit antibodies. Membranes were washed 3 times in TBST for 20 min between antibody treatments. Bands were detected by chemiluminescence with sAC being quantified using densitometry normalizing to the most intense band.

4.2.6 cAMP production assay

Hagfish heart homogenate was incubated for 45 min at room temperature in an orbital shaker (300 rpm) in 100 mM Tris (pH 7.5), 5 mM ATP, 10 mM $MgCl_2$, 0.1 mM $MnCl_2$, 0.5 mM isobutylmethylxanthine (IBMX), 1 mM dithiothreitol (DTT), 20 mM creatine phosphate, and 100 $U.mL^{-1}$ creatine phosphokinase. Homogenates were incubated in the indicated concentrations of HCO_3^- or KH7 and cAMP production was determined using DetectX Direct Cyclic AMP Enzyme Immunoassay (Arbor Assays, Ann Arbor, MI, USA).

4.2.7 Plasma [HCO_3^-] determination

Plasma HCO_3^- concentration was calculated from total CO_2 and pH measured from plasma samples taken from the subcutaneous sinus from control fish, 3, 6, 12 and 24 h following the onset of anoxia, and at 0.5 and 1 h following the onset of recovery from 24 h anoxic exposure. Total CO_2 was measured using a Cornel TCO_2 Analyzer Model 965 and pH was measured using a thermostated capillary pH electrode (Radiometer, BMS 3 MkII). Using these values, [HCO_3^-] was calculated using the Henderson-Hasselbach equation and values of the CO_2 solubility coefficient and pK' as described previously (Boutilier et al., 1984).

4.2.8 Calculations and statistical analyses

For all statistical analyses, p-values of < 0.005 were considered significant. The effects of nadolol and forskolin on heart rate of isolated hearts were assessed using repeated measures one-way ANOVA followed by a Holm-Sidak test, while HCO_3^- and KH7 were assessed using a one-way ANOVA also followed by a Holm-Sidak test. In order to confirm that changes in cAMP concentration were significant, a one-way ANOVA was run, however, due to low n values, significance was only found using a Student-Newman-Keuls test. To test for differences in sAC abundance between control and 24 h, a one way ANOVA was conducted with no post-hoc test due to the lack of significance. A one-way ANOVA followed by a Holm-Sidak test was used to monitor the effects of anoxic exposure and subsequent recovery on HCO_3^- plasma concentrations. Differences in bicarbonate-stimulated cAMP production in hagfish heart crude homogenate were assessed using repeated measures one-way ANOVA.

4.3 Results

4.3.1 Transmembrane adenylyl cyclase

Isolated hagfish hearts slowed from 13.4 ± 1.1 bpm to 8.6 ± 1.1 bpm upon the addition of 1 mM nadolol, a β-adrenoreceptor antagonist, and then to 5.1 ± 0.5 bpm after a 2-h anoxic exposure (Fig. 4.1A). *In vivo*, cardiac cytosolic cAMP concentration was reduced from 8.4 ± 0.7 pmol.mg protein^{-1} to 5.6 ± 0.5 pmol.mg protein^{-1} following a 3-h anoxic exposure, a decrease of 33%. Cardiac cAMP

concentrations then remained stable and low throughout a 24-h anoxic period, consistent with heart rate (Fig. 4.1B). Forskolin, a tmAC agonist, was applied to isolated hearts during anoxia to assess the role of tmAC. Forskolin (1 and 10 µM) significantly increased the intrinsic heart rate but to a level that was not statistically different from the normoxic level (Fig. 4.1C). However, 100 µM forskolin reduced heart rate back to the anoxic rate. When tested on normoxic, control hearts, forskolin failed to increase heart rate at concentrations of up to 100 µM.

4.3.2 Soluble adenylyl cyclase

Immuno-fluorescence of hagfish hearts with anti-dfsAC identified sAC throughout atrial and ventricular myocardial cells of hagfish, with strong staining evident in cardiac trabeculae (Fig. 4.3A,B). sAC protein expression was clearly demonstrated in Western blotting analysis of crude homogenate cardiac cell suspensions from hagfish. The anticipated band was at 112 kDa, and did not show up when the antibody was pre-incubated with oversaturating concentrations of purified sAC peptide prior to application to the hagfish transfer membrane (Fig. 4.2B). Moreover, sAC abundance was unchanged following a 24-h anoxic challenge (Fig. 4.2C,D).

To confirm the role of sAC in the cAMP synthesis pathway in hagfish hearts, cAMP production was measured in homogenized hagfish hearts in the presence of HCO_3^- and KH7. A 2-fold increase in cAMP production occurred with 40 mM HCO_3^- and was completely blocked by KH7 (Fig. 4.4).

Addition of HCO_3^- to anoxic isolated hearts beating at 7.9 ± 1.2 bpm produced a significant tachycardia (Fig. 4.2A.). 20 mM HCO_3^- significantly increased heart rate from the anoxic value back to control levels, and increasing [HCO_3^-] to 60 mM increased heart rate to a level significantly beyond (146%) the intrinsic normoxic heart rate (23.1 ±2.6 bpm compared to 16.0 ±1.0 bpm; Fig. 4.2A). In the presence of HCO_3^-, this tachycardia was completely blocked by the sAC-specific antagonist KH7 (50 µM; Fig. 4.2A). In normoxic, control hearts, HCO_3^- and KH7 did not affect heart rate.

In anoxia, plasma [HCO_3^-] significantly fell from 8.5 ± 0.9 mM to 2.3 ± 0.5 mM (Fig 4.5). The decrease in [HCO_3^-] was gradual during the 24-h anoxic exposure becoming significant at 6 h. Following 0.5 h recovery, plasma [HCO_3^-] returned to levels statistically not different from controls and remained at this level after 1 h recovery.

4.4 Discussion

The present study documents the discovery of a novel cardiac control pathway in addition to that provided by catecholamine stimulation of tmAC. HCO_3^- can stimulate intracellular sAC to produce cAMP from ATP. This cAMP can then increase I_f by binding to HCN channels. sAC was shown to be present in throughout the myocardium by both immunefluorescence and Western blots, and was shown to

produce cAMP in response to HCO_3^- simulation, a response that could be blocked by the sAC agonist, KH7.

In hagfishes, the heart acts as a paracrine gland releasing both noradrenaline and adrenaline from chromaffin tissue located within the cardiac chambers (von Euler and Fänge, 1961; Bloom et al., 1963; Perry et al., 1993; Farrell, 2007). A tonic β-adrenergic cardiac stimulation in hagfish would explain the bradycardic effects of sotalol and nadolol *in vivo* and *in vitro* (Axelsson et al., 1990). Since oxygen is required to convert tyrosine to 3,4-dihydroxyphenylalanine (the rate-limiting step of catecholamine synthesis) and produce noradrenaline from dopamine, anoxia should prevent catecholamine production through these reactions, removing any tonic paracrine β-adrenergic stimulation of heart rate by reducing tmAC activity and decreasing cAMP production (Levitt, 1965). Thus, together with the discoveries in the previous two chapters, a parsimonious mechanistic explanation of the bradycardic effect of anoxia in hagfish (as well sotalol and nadolol effects) is a cessation of tonic β-adrenergic stimulation of HCN channels by cAMP (Axelsson et al., 1990; Cox et al., 2010). Thus, gating of HCN channels may become minimal during anoxia. This leaves unexplained the modest additional decline in heart rate during anoxia following β-adrenergic blockade (Fig. 4.1B). One possibility is that the acidotic conditions associated with anoxia have a bradycardic effect. In the Pacific hagfish, blood pH falls to 7.0 from 7.9 following 36 h anoxia (Cox et al., 2011). While the effects of acidosis has not been tested in hagfishes, extracellular acidosis has been shown to reduce heart rate in the anoxic turtle and normoxic fish heart, and

therefore testing the effects of acidosis on hagfish heart rate would be of interest (Farrell et al., 1983; Stecyk and Farrell, 2006). A second possibility is that sAC mediated cAMP production is inhibited in a way that was not assessed in the present study. During a prolonged anoxic exposure, many variables have been shown to change in hagfish blood and heart including decreasing concentrations of O_2, CO_2, ATP and glycogen, while lactate concentration in the blood increases (Cox et al., 2011). It is possible that one or more of these changes acts as an inhibitor of sAC in anoxic conditions.

The findings that forskolin failed to increase heart rate in normoxia, or beyond routine rates in anoxia, suggests that the tonic catecholamine stimulation was routinely maximal under the normoxic experimental conditions used here. The inhibition of heart rate (back to anoxic levels) with higher forskolin doses is possibly because cardiac ATP demand during anoxia out-stripped ATP production and ATP availability limited tmAC stimulation.

My results clearly suggest that maximal tmAC stimulation cannot increase heart rate beyond routine normoxic values, possibly because tmAC is tonically fully activated. Thus, while the at-best modest increases in heart rate in hagfish with injections of β-adrenergic agonists (Fänge and Östlund, 1954; Chapman et al., 1963; Axelsson et al., 1990; Johnsson and Axelsson 1996) can now be explained mechanistically, tachycardia during anoxic recovery is only partially explained.

A role of sAC in heart rate control has not been previously considered even though sAC has been implicated in the apoptosis signaling pathway of mammalian cardiac myocytes (Chen et al., 2010). I discovered sAC protein expression throughout the hagfish myocardium and that the sAC modulator, HCO_3^-, increased heart rate of anoxic isolated hearts to well beyond the routine heart rate achieved with adrenergic stimulation of tmAC. Indeed, heart rate (23.1 bpm) surpassed the maximum observed *in vivo* (approximately 17 bpm) during anoxic recovery. To confirm the specificity of the HCO_3^-stimulation through sAC, the increase was fully blocked by the sAC antagonist KH7, which also blocked the HCO_3^--stimulated increase in cAMP production by crude cardiac homogenates. Therefore, I have discovered a novel mechanism to control heart rate in a craniate. Thus, I propose that cAMP is produced via a HCO_3^--stimulated, sAC-mediated pathway in addition to the well characterized catecholamine-stimulated tmAC-mediated production of cAMP. This cAMP is then able to bind to HCN to increase heart rate.

Both mechanisms lead to HCN gating by cAMP, presumably increased I_f, and therefore heart rate, with tmAC being tonically and maximally active during normoxia and sAC stimulation increasing during recovery from anoxia to increase heart rate beyond that possible with tmAC alone. This hypothesis is supported by the cAMP measurements recorded in this chapter. During anoxia, cAMP concentration falls by one third similar to the fall in ATP following 36 h anoxia *in vivo* (Cox et al., 2011) When sAC is stimulated by HCO_3^- in homogenized heart tissue, cAMP concentration doubles from control levels. This correlates with the changes in heart rate measured

in the present investigations and those seen *in vivo* where heart rate falls in anoxia, and subsequently increases four-fold upon recovery (Cox et al., 2010).

This discovery, and a more detailed analysis of catecholamine control, allows me to propose a more complete hypothetical model of the control mechanisms of heart rate in the ancestral hagfish (summarized in Fig. 4.6). During anoxia, and with tmAC no longer tonically stimulating heart rate, CO_2 production from aerobic metabolism ceases and the metabolic acidosis produced by glycolytic ATP production (Cox et al., 2011) drives down plasma HCO_3^- concentration. During normoxic recovery, aerobic metabolism resumes and O_2 consumption is rapidly elevated, previously described as post-anoxic oxygen consumption (Cox et al., 2011). With the reinstation of aerobic reaspiration, CO_2 production will also increase at the same time as anaerobically produced lactate will fall. This increased CO_2 production will cause the production of HCO_3^-. This HCO_3^- production will also be stimulated by the reduction of the metabolic acidosis during recovery resulting in a spike in HCO_3^- concentration. Any increase in HCO_3^- concentration will be especially high in the venous circulation prior to branchial CO_2 removal from the blood, and it is this venous, CO_2-rich blood that is seen by the heart due to its lack of a coronoary circulation. The increase in HCO_3^- will increase sAC stimulation and cAMP production that, together with reinstated catecholamine-stimulated tmAC cAMP production, increases heart rate during recovery from anoxia until HCO_3^- falls.

A caveat to this hypothesis is that even though calculated HCO_3^- concentrations from sub-cutaneous sinus blood decresed during anoxia, they did not increase beyond the normoxic control value during recovery. One possible explanation for this is that the blood-chemistry of the sinus blood measured here does not match that of the venous blood seen by the heart. Forster et al. (1989) showed that it takes between 8 and 18 h for the blood of the sinus to equilibriate with the venous circulation. Therefore, getting a good estimate of the HCO_3^- concentration in venous blood may be possible by taking samples from the sinus following 24 h of anoxia, but a 1 h time difference between onset of recovery and blood sampling may not be long enough for CO_2 and HCO_3^- to equilibriate between the primary circulation and the central sinus blood. If this is the case, HCO_3^- concentration of the sinus blood would be below that of the venous blood during recovery. Hagfish plasma HCO_3^- concentrations can certainly be much higher than the present values (up to 70 mM during hypercapnia; Brauner, Personal Communication) and well beyond the concentration required for maximal sAC stimulation. However, hypercapnia will generate higher HCO_3^- concentrations than during recovery from anoxia. What is needed, are rapid measurements of blood sampled from the heart during recovery as well as measurements of cardiac intracellular HCO_3^- to confirm that sAC activity has functional *in vivo* relevance.

During recovery, i*n vivo* intracellular HCO_3^- is likely to be higher than in venous blood because as cardiac aerobic respiration restarts CO_2 produced by mitochondria would be rapidly converted to HCO_3^- by intracellular carbonic anhydrase. Thus,

sAC can be stimulated by an elevation in intracellular HCO_3^- that is greater than seen in venous blood. Also, because the $NaHCO_3$ was added to the saline in the isolated heart experiments, an increase in intracellular HCO_3^- would require the conversion of HCO_3^- to CO_2 in the saline, CO_2 diffusion into the heart and CO_2 conversion back to HCO_3^- by carbonic anhydrase inside the cell, where it would stimulate sAC. Thus, the intracellular HCO_3^- needed to stimulate sAC will be lower than the extracellular concentrations used here to maximally stimulate sAC.

In embryonic zebrafish, hypercapnia results in an increase in heart rate via a catecholaminergic pathway as the tachycardia was prevented using atenolol (Miller et al., 2014). This tachycardia is also reduced following block of carbonic anhydrase suggesting a role of HCO_3^- produced during the increase in CO_2 in hypercapnia in increasing heart rate. It is possible that both tmAC and sAC mediated cAMP production are increasing heart rate in this case, however, sAC effects on heart rate during hypercapnia have not been assessed. It would be interesting to see if a KH7 block of sAC would further reduce heart rate in these conditions following addition of atenolol.

The roles of cAMP as a second messenger are many, especially via cAMP-dependent protein kinase A (PKA), and likewise the possible roles of sAC as a metabolic indicator in the heart may not be limited to HCN channel gating and heart rate control. For example, the omnipresence of sAC throughout the atrial and ventricular myocardia indicates otherwise because sAC might be concentrated only

in the primary pacemaker area if heart rate was the sole cardiac process regulated by sAC. Instead, sAC expression throughout myocardial trabeculae of the hagfish might implicate a role in increased cardiac contractility by increasing cytosolic Ca^{2+} influx through PKA phosphorylation of L-type Ca^{2+} channels (Sculptoreanu et al., 1993; Gao et al., 1997). The roles of sAC produced cAMP in other cardiac mechanisms require testing, therefore, in addition to discovering a novel mechanism of cardiac control, the implications of this fascinating discovery may be very far reaching and opens up a new field of cardiovascular research, possibly stretching into the medical field.

4.4.1 Summary

The discovery of a novel cardiac control mechanism and a more detailed analysis of catecholamine control have provided a more complete picture of the control mechanisms for heart rate in ancestral hagfish. I confirmed tonic and possibly near maximal β-adrenoreceptor stimulation of pacemaker activity under normoxic conditions through production of cAMP by tmAC. This stimulatory activity is lost in anoxia and heart rate is halved. During normoxic recovery, heart rate can quadruple from the anoxic rate and exceed the normoxic rate by a combination of cAMP production through sAC and restored cAMP production by tmAC.

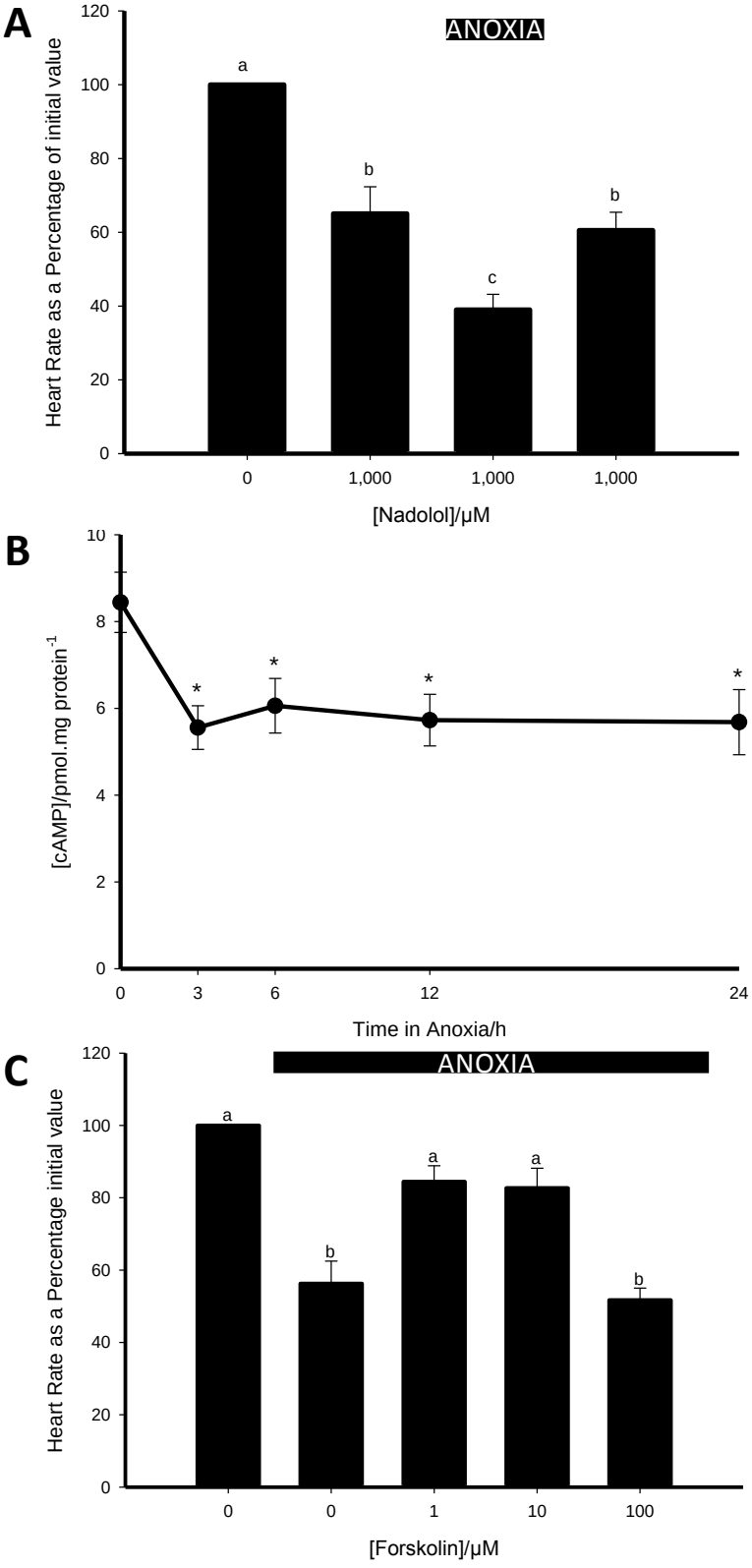
A
ANOXIA
Heart Rate as a Percentage of initial value
120
100
80
60
40
20
0
a
b
c
b
0
1,000
1,000
1,000
[Nadolol]/μM
B
[cAMP]/pmol.mg protein-1
10
8
6
4
2
0
*
*
*
*
0
3
6
12
24
Time in Anoxia/h
C
ANOXIA
Heart Rate as a Percentage initial value
120
100
80
60
40
20
0
a
b
a
a
b
0
0
1
10
100
[Forskolin]/μM

Figure 4.1. Effects of anoxia and β-adrenoreceptor stimulation and blockade on hagfish heart rate and cAMP. **A**. Isolated, spontaneously beating hearts (n = 8) slowed significantly when exposed to the β-adrenoreceptor antagonist nadolol and further still with a N_2-induced anoxia (indicated by thick black bar), recovering to pre-anoxic levels upon removal of N_2. Dissimilar letters indicate statistical differences between treatments calculated from raw, non-normalized data (P<0.005, repeated measures one-way analysis of variance (ANOVA)). **B**. cAMP concentrations fall in hearts of intact animals (n = 5) exposed to prolonged anoxia. Asterisks indicate significant differences from control group (P<0.005, one-way ANOVA). **C**. Increasing concentrations of the β-adrenoreceptor agonist, forskolin, failed to increase anoxic (indicated by thick black bar) heart rate of isolated hearts (n = 6) beyond routine rates. Dissimilar letters indicate statistical differences between treatments calculated from raw, non-normalized data (P< 0.005, repeated measures one-way ANOVA).

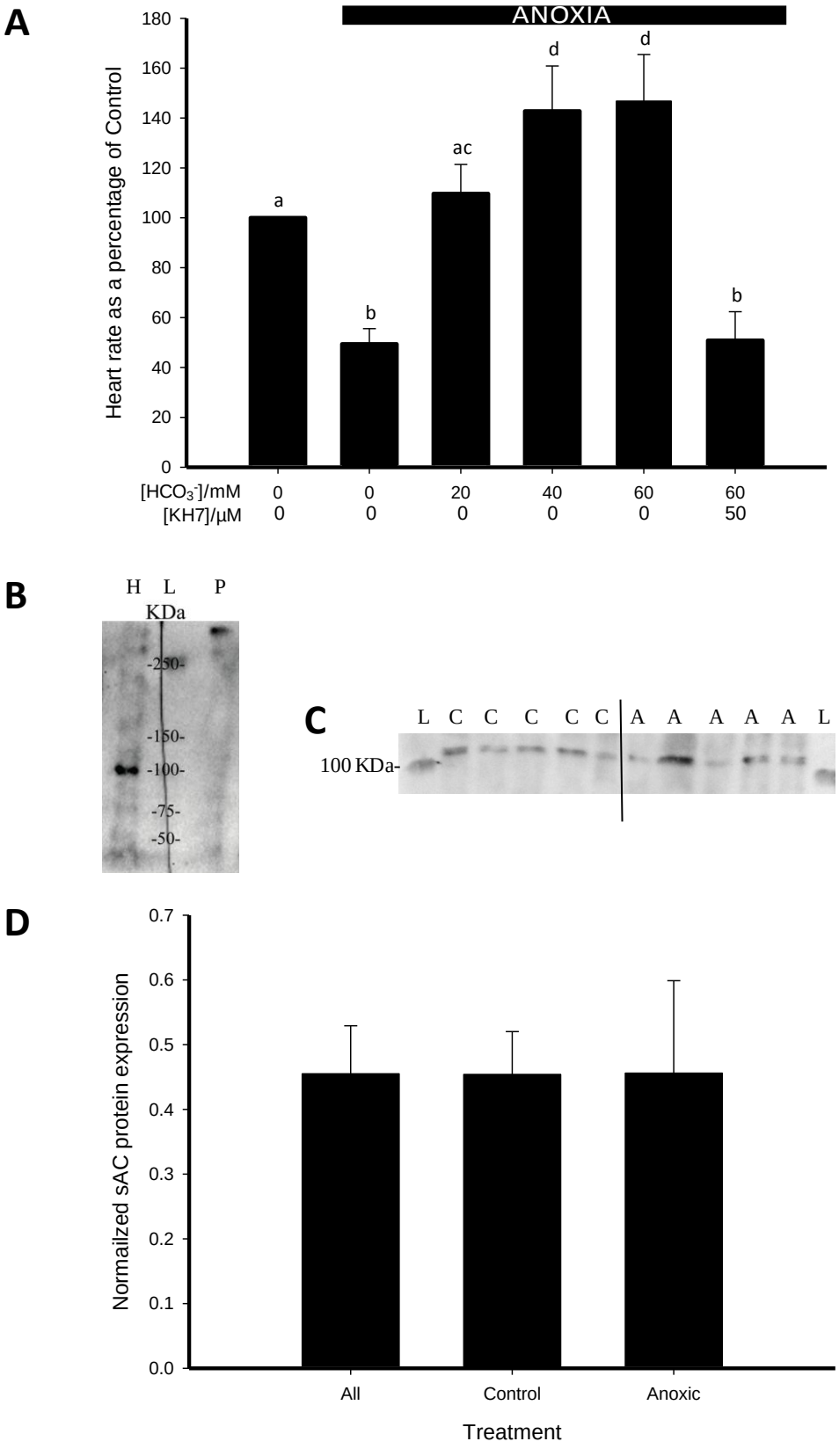
A
ANOXIA
Heart rate as a percentage of Control
180
160
140
120
100
80
60
40
20
0
a
b
ac
d
d
b
[HCO3-]/mM 0 0 20 40 60 60
[KH7]/µM 0 0 0 0 0 50
B
H L P
KDa
-250-
-150-
-100-
-75-
-50-
C
L C C C C C A A A A A L
100 KDa-
D
Normailzed sAC protein expression
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
All
Control
Anoxic
Treatment

Figure 4.2. The role of HCO_3^--stimulated soluble adenylyl cyclase (sAC) in the hagfish heart. **A**. Effects of sAC stimulation with HCO_3^- and blockade with KH7 on hagfish heart rate under anoxic conditions (n = 6). Bicarbonate caused a tachycardia in anoxic hagfish hearts in anoxic beyond the intrinsic normoxic pacemaker rate (Control), which could be completed reversed by the antagonist KH7. Dissimilar letters indicate statistical differences between treatments calculated from raw, non-normalized data (*P*<0.005, 1-way ANOVA). **B**. Western blot of hagfish heart crude homogenate using anti-dfsAC antibody in the absence (H) or presence (P) of purified sAC peptide, ladder indicated by L. **C + D**. Expression of sAC in hagfish hearts exposed to 24 h anoxia (A) compared to controls (C) shown by Western blot (**C**) and graphically, represented by measurement of the membrane shown in C by densitometry in **D**. Ladder indicated by L. The average band weight of hagfish sAC was 112 KDa. sAC expression in **D** normalized to the highest expression.

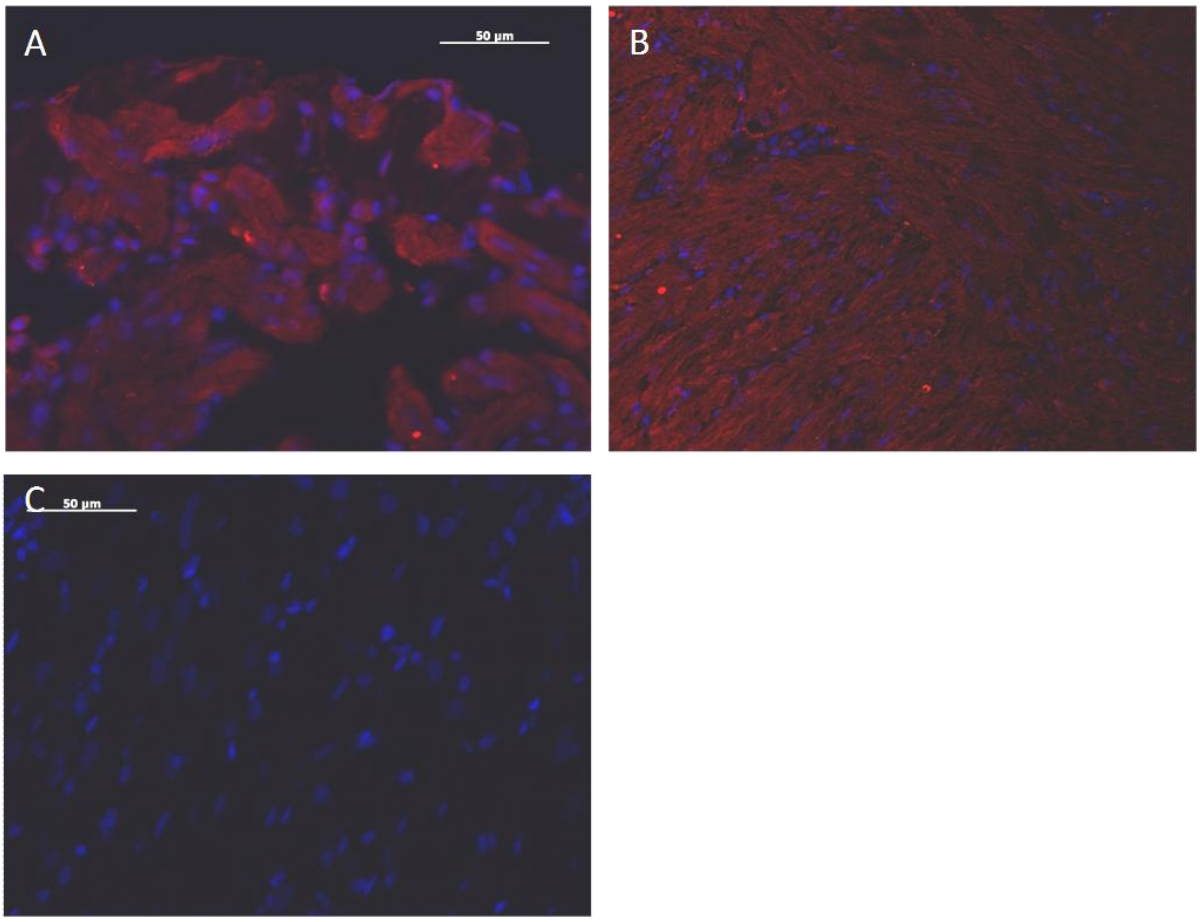

Figure 4.3. Immunoreactivity of soluble adenylyl cyclase (sAC) in hagfish hearts. **A** + **B**. Immunofluorescence staining of sAC (red) in hagfish atrium and ventricle respectively. Co-staining of nuclei shown in blue. **C**. Control showing staining of nuclei (blue) only.

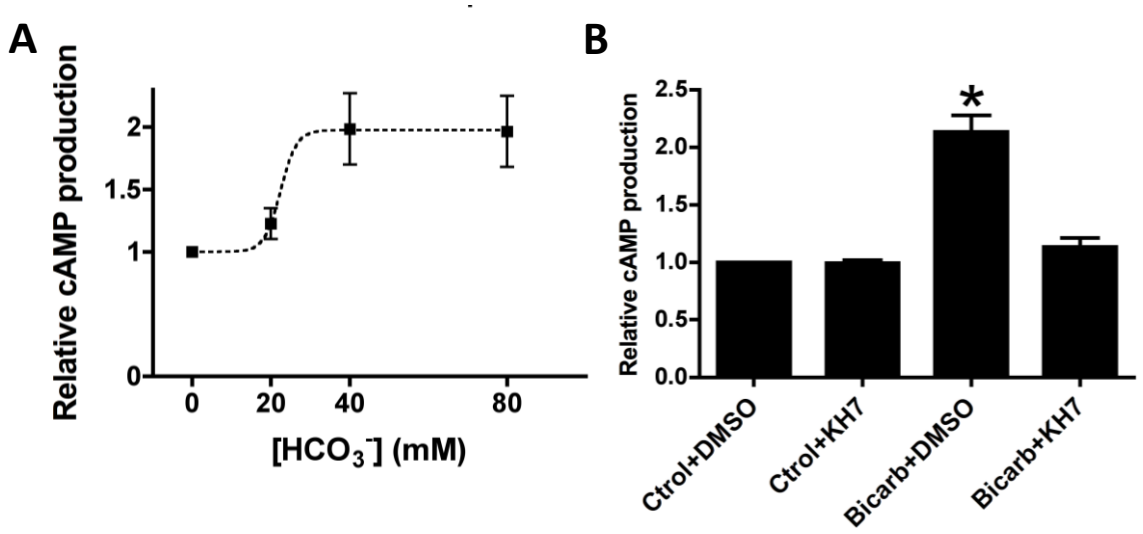

Figure 4.4. cAMP production by sAC in hagfish hearts. **A**. cAMP production in increasing HCO_3^- concentrations normalized to 0 mM HCO_3^-. At 0 mM, cAMP production equaled 18.65 pmol.mg^{-1}.min^{-1}, increasing 2 fold at 40 mM HCO_3^-. n = 4. **B**. Relative cAMP production in the presence of DMSO, KH7, HCO_3^- and DMSO and HCO_3^- with KH7 dissolved in cAMP. The increase in cAMP concentration by HCO_3^- is blocked by the sAC agonist KH7 dissolved in DMSO while showing that the effect is not due to the presence of DMSO. n = 6, significant increases from control shown by asterisk *(P*<0.005, 1-way ANOVA).

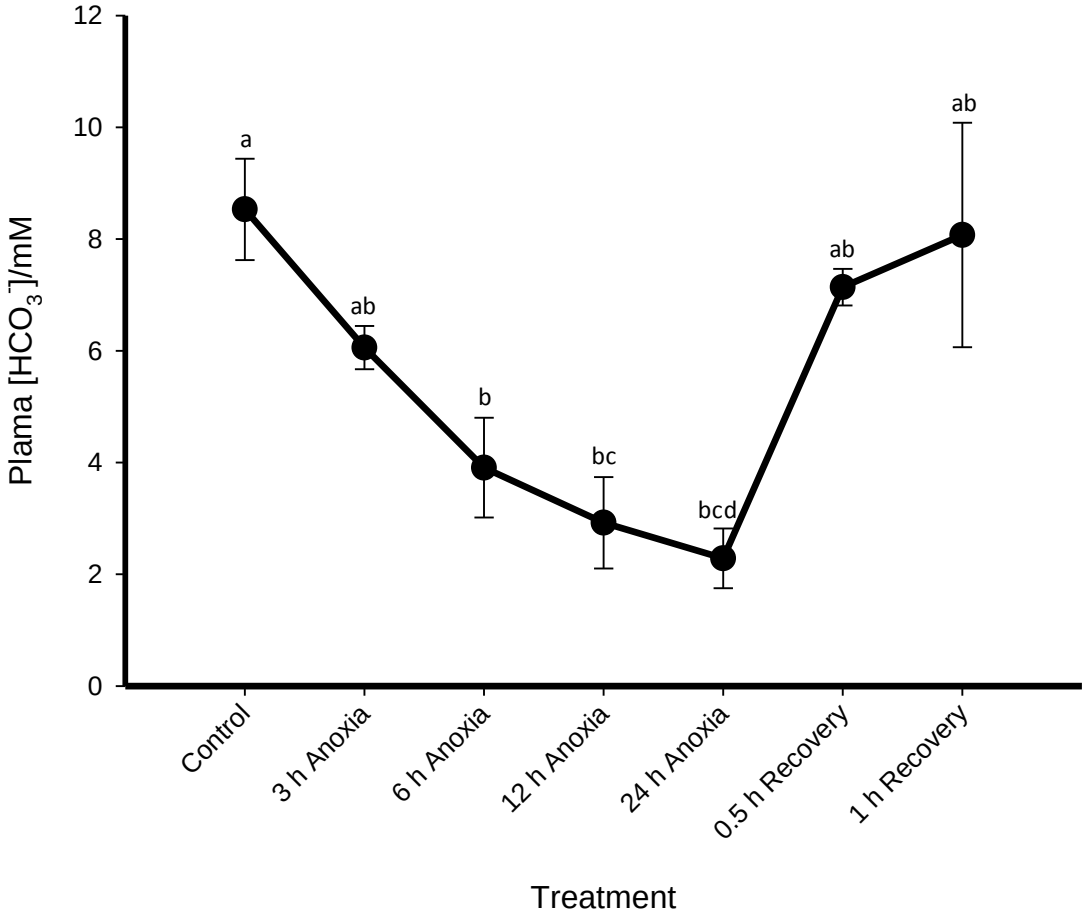

Figure 4.5. The effects of 24 h anoxia and subsequent normoxic recovery on plasma [HCO_3^-] in hagfish subcutaneous sinus blood. Plasma [HCO_3^-] gradually decreased during anoxia, becoming significant following 6 h. During recovery, plasma [HCO_3^-] returned to control levels. n = 5, Dissimilar letters indicate statistical differences between treatments (P<0.005, 1-way ANOVA)

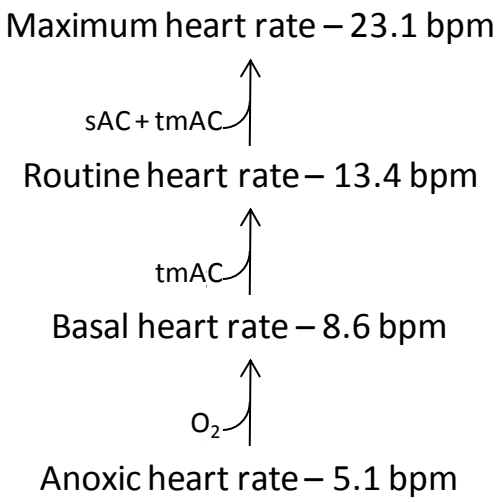

Figure 4.6. A summary of the proposed control of heart rate in hagfish. With a basal heart rate possibly representing minimal HCN gating, removal of oxygen reduces heart rate from 8.6 bpm to 5.1 bpm. Stimulation of tmAC by paracrine catecholamine release increases heart rate to 13.4 and represents the routine heart rate. During recovery from prolonged anoxia, cAMP produced from tmAC and HCO_3^- stimulated sAC increases heart rate to 23.1 bpm. Heart rates shown are averages from 6 animals.

CHAPTER 5: CONCLUSIONS

5.1 Introduction

The preceding data chapters detailed how I have explored three main aspects of cardiac control in the Pacific hagfish, *Eptatretus stoutii*: 1) Initiation of the heartbeat; 2) Synchronization of cardiac contraction; and 3) Control of heart rate. The main findings of my studies are outlined below, followed by a summary and general discussion of my proposed model of cardiac control in this ancestral craniate heart. Finally, the implications of my discovery of a novel cardiac control pathway are suggested.

5.2 Initiation and synchronization of the heartbeat

5.2.1 Does the membrane clock set intrinsic heart rate in Pacific hagfish, and, are HCN channels involved in synchronous beating of the cardiac chambers in the Pacific hagfish?

Hypothesis: The intrinsic rate of atrial contraction is higher than that of the ventricle. (Chapter 2)

Support for this hypothesis was provided by the atrium and ventricle beating spontaneously in isolation following destruction of the atrioventricular canal. Additionally, atrial rate was over twice that of the ventricule. Therefore, I confirmed

Jensen's (1965) discovery that both cardiac chambers have a spontaneous contraction rate. I have also characterized, for the first time, the contraction rates of the atrium and the ventricle as independent chambers. Atrial contraction must occur prior to the initiation of ventricular contraction. This would be ensured by an upstream location of cardiac pacemaker cells as well as the intrinsic atrial beating rate being greater than that of the ventricle.

All the same, atrial and ventricular contractions must be synchronized, which is achieved via a cardiac conduction system through the atrioventricular canal (a delay in electrical conduction to allow blood to be pumped from the atrium into the ventricle). In this regard, a novel and unexpected discovery was that tetrodotoxin (TTX), a potent voltage-gated (VG) Na^+ channel blocker, produced an atrioventricular block in the isolated heart that was equivalent to physical crushing of the atrioventricular canal with a ligature. These results suggest an integral role of the VG Na^+ channel in atrioventricular conduction in the hagfish, which may exist as primitive conduction system. Remarkably, the intrinsic atrial and ventricular contractions themselves were surprisingly resistant to TTX compared to the chick, *Gallus gallus*, and the rainbow trout, *Oncorhynchus mykiss*, with the heart continuing to rhythmically contract with 10 µM TTX in the perfusate, albeit at a reduced (65%) rate. As a potent I_{Na} antagonist, TTX is one of the most deadly neurotoxins known to man. The EC_{50} of TTX for chick, and rainbow trout, hearts are 4 nM and 1.8 nM respectively (Parakash and Tripathi, 1998; Haverinen et al., 2007). The reduction in heart rate may be due to a role of TTX-sensitive inactivation-resistant Na^+ (I_{NaTTX})

channels that allow some extra Na^+ to enter the cell during the pacemaker potential, allowing the Ca^{2+} threshold to be reached earlier (Ju et al., 1995).

Hypothesis: The heart beat is initiated by I_f. (Chapter 2)

Support for this hypothesis was provided by zatebradine, a potent HCN channel antagonist, which completely eliminated spontaneous contractions in isolated atria consistent with the membrane clock hypothesis. When coupled with the I_{NaTTX} data of the preceeding paragraph, it would appear that I_f is necessary to produce rhythmic contractions, while I_{NaTTX} is additive, increasing the pacemaker potential already established by I_f. Furthermore, ryanodine, which blocks Ca^{2+} cycling through the sarcoplasmic reticulum (SR), had no discernible effect on heart rate of isolated, spontaneously beating atria. This finding is inconsistent with the calcium clock hypothesis because ryanodine antagonism should prevent Ca^{2+} cycling through the SR. Thus, HCN channels set the intrinsic heart rate in the Pacific hagfish, with spontaneous heart rate being regulated by a pacemaker potential caused by I_f, a slowly depolarizing K^+ and Na^+ current.

In the ventricle, zatebradine greatly reduced, but did not stop, spontaneous contractions of the ventricle. In all other vertebrate hearts investigated, HCN channel antagonists exhibit a dose-dependent bradycardia but do not eliminate contraction (Steiber et al., 2006). This finding is consistent with a dual role of membrane and calcium clocks in setting the ventricular rate. Nevertheless,

ryanodine had no discernible effect on the heart rate of isolated, spontaneously beating ventricles. Why may zatebradine have had a lower effect in the ventricle compared to that of the atria? One simple explanation is that following cessation of atrial contractions in the isolated heart preparation, the perfusate containing the blocker was no longer forced into the ventricle, reducing the amount of the antagonist in this chamber. A second hypothesis is that I_{NaTTX} has a larger role in the ventricle, or possibly, another leak current yet to be discovered. Further work on isolated ventricular cells would be particularly enlightening here. In addition, work on physically (as opposed to atrioventricular blocked) isolated ventricles would be informative.

Hypothesis: Multiple isoforms of HCN exist in the hagfish heart, with HCN3 being the ancestral isoform. (Chapter 3)

Support for this hypothesis was provided by sequencing partial genes from mRNA expressed in hagfish hearts. When compared to known HCN genes from other species using the Blast engine, I was surprised to discover that the hagfish genome encodes at least six HCN isoforms compared to the four HCN isoforms discovered in a wide variety of vertebrate hearts. Even so, HCN isoforms similar to vertebrate HCN2, HCN3 and HCN4 were found: 2 versions of HCN2, 3 versions of HCN3 and 1 version of HCN4. No HCN1 isoform was found despite extensive searching, which may mean one of several possibilities: 1) that HCN1 evolved in the vertebrate linage following their split from the agnathans; 2) that HCN1 is expressed in tissues other

than the heart; 3) that the primers tested were not sufficient to find HCN1 expressed in the heart; 4) HCN1 has changed so much following the split from the vertebrate linage that it more closely resembles one of the other isoforms and is no longer identifiable as HCN1. Ultimately, both full HCN sequences and the total number of HCN isoforms present in the hagfish will only be confirmed following the publication of the hagfish genome. However, the finding of so many isoforms indicates that HCN gene duplications exist in the hagfishes similar to what has been observed in tunicates and some teleost fishes (Jackson et al. 2007).

Given that the three hagfish HCN3 isoforms cluster around the ancestral urochordate HCN genes and the outgroup of the phylogeny, the present finding supports the conclusion of Jackson et al. (2007) that HCN3 appears to be the ancestral isoform. It is most that in the tunicates, a single HCN3-like isoform duplicated twice to produce the three isoforms that appear following the tunicate/craniate split. The least parsimonious explanation for the findings of my thesis regarding HCN isoform evolution in the craniates is that HCN3 existed in the craniate ancestor. Between the first emergence of the craniates and the emergence of the gnathostomes, HCN2, 3 and 4 had evolved from gene or genome specific duplication events. If HCN1 is present in the hagfishes, HCN1 can be added to the previous list. However, if not present, HCN1 most likely evolved following the hagfish/vertebrate divergence. Following this split, HCN2 and HCN3 were duplicated in the hagfishes. The duplicated HCN isoforms found in the teleosts probably evolved following the actinopterygii/sarcopterygii split, leaving the tetrapods

with only single copies of each isoform. Another possible explanation for the multitude of HCN isoforms found in my work is that some of the isoforms are splice variants. Indeed, splice variants of HCN3 have been reported in liver and kidney (Santoro et al., 1998). Nevertheless, the findings presented here show a snapshot of HCN gene evolution at the same time as the emergence of the craniate heart.

Additionally, HCN3a mRNA expression in both cardiac chambers was over 10-times that of HCN4, the second most expressed isoform. The dominance of HCN3 mRNA in hagfish hearts differs from adult mammalian heart cells where HCN4 is the most prevalent. However, HCN4 dominance replaces early embryonic dominance of HCN3 in the heart (Qu et al., 2008). This discovery suggests that the ancestral condition is HCN3 dominance and at some point in the vertebrate linage, that changed to HCN4. The embryonic HCN3 dominance in mammals may be a relic of the ancestral condition, or it may be beneficial to the developing embryonic heart.

Hypothesis: HCN channel mRNA is expressed in both cardiac chambers of the hagfish heart. (Chapter 2 and 3)

Support for this hypothesis was provided by finding expression of all 6 HCN isoform genes in both atria and ventricles of hagfish hearts in chapter 3, and the reduction of both atrial and ventricular rates upon zatebradine antagonism of HCN channels in Chapter 2. These findings support my earlier conclusions regarding the membrane clock, and pacemaker activity in both cardiac chambers. In order for the membrane

clock model of cardiac pacemaking to be true, HCN channels must be present in order for I_f to flow through them. Finding HCN channel mRNA in the atrium supports my previous conclusion that I_f is responsible for the pacemaker potential, and allows a more complete picture of hagfish cardiac pacing to be produced. These data indicate that zatebradine may reduce heart rate by decreasing I_f by antagonizing HCN channels. Additionally, assuming that the protein is expressed, HCN channel mRNA expression in the ventricle provides a mechanism for spontaneous ventricular contractions when it is isolated from the atrium, contractions whose rate decreases upon addition of zatebradine.

Hypothesis: Higher HCN mRNA expression in the atrium compared to the ventricle supports a higher spontaneous atrial rate. (Chapter 3)

Support for this hypothesis was provided by the finding that measured mRNA expression of all HCN isoforms bar HCN3b was at least twice as high in the atrium compared to the ventricle. As noted above, in order for a multi-chambered heart to contract efficiently, the atrium must contract prior to the ventricle, which requires the atrium to be more exciteable. Albeit in the absence of protein expression data, my experimental findings suggest that HCN channel density may be higher in the atrium compared to the ventricle. Having a higher membrane density of HCN channels in the atrium will allow for a larger I_f and therefore, greater atrial excitability. Indeed, this discovery of higher atrial HCN expression in hagfish hearts is similar to HCN expression mammalian in cardiac chambers where HCN mRNA is much higher in

the sinoatrial node than in the ventricle (Shi et al., 1999; Marionneau et al., 2005). This presumably increases HCN protein density and therefore provides greater excitability in those areas that need to contract first.

Hypothesis: Different HCN isoforms were expressed in the atrium compared to the ventricle. (Chapter 3)

Support for this hypothesis was not provided by my experimental findings because the expression patterns of both cardiac chambers was remarkably similar, with both showing a dominance of HCN3a. Therefore, it seems unlikely that chamber excitability is controlled by upregulation the expression of, for example, faster opening HCN isoforms in the atrium compared to the ventricle. Instead, perhaps overall upregulation of HCN mRNA, as discussed above, is more important. Even though it would be an incredibly difficult task, electrophysiological studies of the individual hagfish HCN isoforms expressed in a model cell system, such as *Xenopus* oocytes, would be of particular interest.

5.3 Control of heart rate

5.3.1 Does varying HCN mRNA expression during anoxia and subsequent recovery suggest that HCN channel expression may have a role in heart rate control under these conditions?

Hypothesis: HCN mRNA expression changes in response to prolonged anoxia and subsequent recovery. (Chapter 3)

This hypothesis was supported by my discovery that HCN3a, the most highly expressed HCN isoform in both cardiac chambers, was altered by prolonged anoxia and recovery in the ventricle and atrium respectively. HCN3a expression decreased during prolonged anoxia in the ventricle, while remaining the same in the atrium. Following complete recovery from anoxia, both ventricular and atrial HCN3a mRNA expression levels had returned to control levels. Assuming that mRNA expression reflects actual channel density, a reduction in ventricular HCN3a compared to HCN4 during anoxia would be consistent with a decrease in ventricular excitability. This is because, in mammalian hearts, HCN3 allows a larger I_f to flow through the channel compared to HCN4 (Moosmang et al., 2001). However, since the ventricle follows the atrium, this would have no effect on heart rate. In the atrium, during normoxic recovery from prolonged anoxia, the increase in HCN3a mRNA would be consistent with an increase in atrial excitability. However, these inferences are made assuming a correlation between mRNA and protein expression. This is an important caveat. Protein expression and electrophysiological measurements will have to be made

before it is possible to form firm conclusions. Additionally, an alternative possible explanation for increased HCN3a mRNA expression during recovery is that since protein synthesis would stop during anoxia, mRNA expression in the atrium increases to replenish HCN3a channels lost during anoxia due to the natural removal of proteins from the SL. This is unlikely however, as if this were the case, expression of all the isoforms would be expected to increase in addition to solely HCN3a.

5.3.2 Does varying tmAC activation modulate heart rate?

Hypothesis: The heart receives tonic stimulation of tmAC by catecholamine stimulation of β-adrenoreceptors. (Chapter 4)

This hypothesis was supported by the reduction in heart rate in isolated hearts upon the addition of the β-adrenoreceptor antagonist nadolol under normoxic conditions. Nadolol more than halved heart rate. This finding supports the discovery of Axelsson et al. (1990) where the β-adrenoreceptor antagonist sotalol also caused a pronounced (over 50%) bradycardia in the Pacific hagfish. Surprisingly, the tmAC agonist forskolin failed to increase heart rate above routine rates showing that this tonic tmAC stimulation is also maximal. The cause of the further decrease in heart rate in anoxia following nadolol addition is presently unknown. However, it may be due to acidosis (as discussed in Chapter 4), an effect on sAC mediated cAMP production, or current flow through I_{NaTTX} channels. These hypotheses require futher testing using isolated hearts and electrophysiology.

Hypothesis: cAMP concentration in the heart decreases during prolonged anoxia. (Chapter 4)

This hypothesis was supported by the 33% reduction in cAMP concentration following a 3 h anoxic challenge, after which, cAMP concentrations remained stable for a further 21 h anoxic period. This discovery is consistent with a reduction in tmAC activity due to the removal of catecholamine stimulation in anoxia. *In vivo* heart rate halves in hagfish following 2 h anoxic exposure and then remains stable throughout prolonged (36 h) anoxic exposure (Cox et al., 2010). The above results also provide indirect support for the notion that heart rate is controlled by HCN channels. HCN channels are stimulated in the presence of cAMP, stimulation that would decrease in response to an anoxia-mediated reduction in cAMP.

5.3.3 Do other mechanisms gate HCN with cAMP?

Hypothesis: Soluble adenylyl cyclase (sAC) is present in hagfish hearts. (Chapter 4)

This hypothesis was supported by Western blotting and immunofluorescence of sAC. I discovered a 112 KDa band corresponding to sAC in homogenized hagfish hearts, a finding then confirmed by the staining and imaging of sAC peptide throughout the myocardium of both hagfish cardiac chambers via immunofluorescence. This is the first time sAC peptide presence has been partially mapped in a heart. Since sAC is an adenylyl cyclase, it provides a new pathway for

cAMP production in hearts and further control of cAMP regulated processes, including heart rate.

Hypothesis: sAC produces cAMP in response to bicarbonate stimulation. (Chapter 4)

This hypothesis was supported by a 2-fold increase in cAMP production in homogenized hagfish hearts stimulated with 40 mM HCO_3^-. This increase in cAMP production was blocked in homogenized hearts in the presence of both HCO_3^- and the sAC antagonist KH7. This discovery shows the relationship between HCO_3^- stimulation and cAMP production by the sAC peptide that I have previously shown to be present in the myocardium.

Hypothesis: sAC stimulation is involved in hagfish cardiac control. (Chapter 4)

This hypothesis was supported by the fact that heart rates in isolated hagfish hearts increased by up to 146% of the normoxic control rates when HCO_3^- was added following an anoxia-induced bradycardia. As with the increase in cAMP above, this increase in heart rate was blocked by the addition of KH7 in the presence of HCO_3^-. This is the first time heart rate has been shown to increase in the presence of HCO_3^-. I propose that, in addition to tmAC mediated cAMP production, sAC can also produce cAMP in order to stimulate I_f flow through HCN by stimulation of the CNBD and phosphorylation. This is a novel pathway of cardiac control and, providing sAC

is present in all craniate hearts, a pathway that may provide an important role in cardiac control in all of these hearts.

5.4 An integrated model for cardiac control in the Pacific hagfish

Prior to my thesis, the only information regarding the initiation of the hagfish heartbeat was limited to action potentials recorded from atrial and ventricular tissue (Jensen 1965). Together with spontaneous contractions of both isolated chambers, this indicated that the heartbeat was initiated by a similar mechanism to vertebrate hearts. The pacemaker mechanisms involved in producing the pacemaker potential in hagfish hearts was unknown. In addition, control of heart rate was limited to a few studies that determined that that there was a tonic catecholamine stimulation of heart rate, but it was unknown how this varied, and how the heart rate could increase or decrease given the lack of vagal innervation. Thus, it was not possible to formulate an integrated model of cardiac control in the ancestral hagfish heart. The collective discoveries in my thesis have altered this understanding, and allow for the inclusion of a cardiac control mechanism not previously considered in any craniate hearts.

My proposed model (summarized in Fig. 4.6.) is detailed below, building from the experimental observations summarized above.

In the Pacific hagfish, the heartbeat is initiated by I_f flowing through HCN channels, with no contribution from SR-Ca^{2+} cycling as supported by pharmacological and mRNA expression studies. Therefore, it appears that the calcium clock mechanism of cardiac pacing may have evolved later in the vertebrate lineage. It may be that the calcium clock, working in tandem with the underlying membrane clock, allows for larger depolarization rates, and, in turn, faster heart rates, or for fine tuning of heart rate control. The two mechanisms working together would account for the bradycardic effects of ryanodine, an antagonist of SR Ca^{2+} cycling, and HCN antagonists such as zatebradine on mammalian hearts (Rubenstein and Lipsuis, 1989). In addition to I_f, there appears to be a role of I_{NaTTX} adding to the depolarization during the pacemaker potential, however, since zatebradine completely stops atrial contractions, I_{NaTTX} appears to be secondary to I_f.

It is well established that the hagfish heart contains catecholamines (Johnels and Palmgren, 1960; Östlund, 1960; Bloom et al., 1961; von Euler and Fänge, 1961; Jensen, 1961; Perry et al., 1993). Based upon my finding that nadolol reduces heart rate in isolated hearts, I can suggest that paracrine release of catecholamines provides tonic stimulation of cardiac tmAC, as opposed to circulating catecholamines. Therefore, the hagfish heart does appear to act as an endocrine gland, providing paracrine stimulation of pacemaker cells and likely the myocardium as a whole.

Control of hagfish heart rate is then achieved by two pathways that produce cAMP from ATP; the well known tmAC pathway stimulated by catecholamines, and a sAC mediated pathway stimulated by HCO_3^- (Fig. 4.5). Hagfish hearts receive tonic catecholamine stimulation of tmAC and contracts at approximately 13 bpm. At routine rates, tmAC is producing cAMP at its maximum rate, as was shown with forskolin. This catecholamine stimulation is removed in anoxic conditions, resulting in a bradycardia to about 8 bpm, and further effects of anoxia cause a further drop to approximately 5 bpm. During normoxic recovery from anoxia, resumed catecholamine stimulation of tmAC, together with HCO_3^- stimulation of sAC produces a tachycardia up to 23 bpm. Therefore, this model can explain how heart rate can change over 4-fold in the isolated, aneural hagfish heart.

While the implications of sAC supporting hagfish heart rate in addition to tmAC appear very conclusive, one caveat to this hypothesis is that calculated plasma HCO_3^- concentrations were much lower in recovery than the 40 mM presumably required by the hypothetical model. One explanation for this is that because the HCO_3^- concentration was calculated from sinus blood, the recovery value may be lower than that seen by the heart in the venous circulation. The sinus system is a low turnover blood chamber, with turnover rates being suggested at between 8 and 18 h (Forster et al., 1989). Therefore the recovery period may be too short for thorough mixing of venous and sinus blood. Additionally, as discussed in Chapter 4, intracellular HCO_3^- may be higher in the cell compared to the blood, and all of the HCO_3^- added to the saline is unlikely to have made it into the cell due to a lack of

CO_2 in the saline. However, if HCO_3^- concentrations fail to reach high enough levels in order to fully stimulate sAC during recovery from anoxia, sAC may have a minor role in controllong heart rate, only adding slightly to that provided by tmAC. If this is the case, what other hypothetical mechanisms might increase heart rate during recovery? In the dog, vasoactive intestinal peptide (VIP) and peptide histidine isoleucine both bind to the VIP receptor on pacemaker cells increasing heart rate, however, in fishes VIP has no effect on heart rate (Rigel, 1988; Jensen et al., 1991; Holmgren et al., 1992). Additionally, in the guinea pig, endothelins 1 and 2 have been demonstrated to increase heart rate, possibly by binding to the endothelin receptors that have been found in the right atrium (Beaulieu and Lambert, 1998). Histamine has also been shown to have tachycardiac effects in snakes (Levi, 1972; Skovgaard et al., 2009). These hormones and peptides could play a role in increasing heart rate, and should be examined as possibilities in isolated hearts. Futhermore, my thesis only considers modulation of the HCN channels. However, I also discovered a role of I_{NaTTX} in setting heart rate. It is possible that I_{NaTTX} also responds to reoxygenation following prolonged anoxia, however, this is likely to have to be studied using electrophysiological techniques in order to separate and distinguish between I_{NaTTX} and I_f.

5.5 Limitations, Further Work and Implications

The above model has been generated from the novel findings of my thesis; however, further testing beyond the scope of the current work is required to answer some of

the new questions that have arisen and to address the limitations of the thesis. Pharmacology was used as a tool to probe the pacemaking mechanism of the hagfish heart. From this, I was able to deduce the channels involved in setting the heart rate in this ancestral heart. However, the inferences made were based upon the literature from teleost and mammalian hearts. As a result, electrophysiological measurements will be necessary to confirm the presented findings. These measurements may be hard to gather due to hagfish cardiac myocytes being surrounded by a thick glycocalyx compared to other craniates, and having highly concentrated extracellular fluid due to hagfish being osmoconformers.

The phylogeny is limited because it was created using only 37 amino acid long sequences due to the short partially cloned gene segments discovered. The shortness of the sequences reduces the accuracy of the phylogenetic tree produced, hence the low bootstrap values. Ideally, this analysis will be repeated following the sequencing of the hagfish genome or proteome. Knowing the full genome of the hagfish would also divulge whether or not HCN1 exists in the hagfish, or if it evolved in the vertebrate taxon. Finally, mRNA expression data was used to discuss possible cardiac regulation by assuming that changes in mRNA expression would be transferred to protein expression. This may not be the case, and in order to assess the effects of anoxia on HCN expression, protein expression data will have to be recorded to complete the picture.

The elasmobranchs and other primitive fishes represent taxa that are under-represented in the cardiovascular literature. In this regard, it would be if interest to study HCN mRNA and sAC in these animal groups. It would make the most sense to start with the dogfish, *Squalus acanthias*, due to its use a model eslasmobranch, and sAC has been well characterized in other tissues such as gills this fish. Indeed, the antibodies used to detect sAC in the hagfish were originally developed for use in dogfish, however, no work has been done on the shark heart.

A major discovery of my work is a novel pathway of cardiac control in a craniate heart. Indeed, the implications of sAC mediated, HCO_3^- stimulated-cAMP production may be very far reaching. The roles of sAC in controlling heart rate in vertebrate hearts are presently unknown and need to be explored. If sAC control is present in vertebrate hearts, control of sAC produced cAMP, or blood-HCO_3^- concentrations may represent new forms of cardiac therapy in the medical field. With the aneural state of the hagfish heart mirroring the human condition *in utero* and following a transplant, therapeutics revolving around sAC may be of particular importance under these conditions where neural control is absent. Additionally, manipulating sAC activity may be useful for restarting a heart following cardiac arrest in place of adrenaline while minimizing the risk of fibrillation. Furthermore, as mentioned in chapter 4, the roles of cAMP as a second messenger are many, and therefore, sAC produced cAMP is unlikely to affect solely heart rate. Indeed, via cAMP-dependant protein kinase A (PKA), cAMP affects include, but are not limited to, SR Ca^{2+} cycling, cardiac muscle contractility and cardiac muscle relaxation. Therefore, the findings of

my thesis have the potential to open up a new field of cardiac physiological research.

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