University of Joensuu, PhD Dissertations in Biology

No: 54

Effects of temperature on the electrical excitability of fish cardiac myocytes

by
Jaakko Haverinen

Joensuu
2008
Haverinen, Jaakko. Effects of temperature on the electrical excitability of fish cardiac myocytes
University of Joensuu, PhD Dissertations in Biology, No. 54. ISSN 1795-7257 (printed), ISSN 1457-2486 (PDF)

Keywords: fish heart, atrium, ventricle, cardiac myocytes, temperature acclimation, resting membrane potential (RMP), action potential (AP), sodium current (I_{Na}), rapid component of the delayed rectifier potassium current (I_{Kr}), inward rectifier potassium current (I_{K1}), tetrodotoxin (TTX), rainbow trout, Oncorhynchus mykiss, crucian carp, Carassius carassius, burbot, Lota lota, pike, Esox lucius, roach, Rutilus rutilus, perch, Perca fluviatilis.

ABSTRACT
Introduction Fish can be subject to great and sometimes rapid changes in ambient temperature which directly affect the rate of body functions. As a counterreaction to chronic temperature changes, several fish show thermal compensation in metabolic rate and body functions including electrical activity of the heart. Electrical excitation of the fish heart originates from the sinoatrial pacemaker where a small number of pacemaker cells generate spontaneous action potential (APs) and determines the rate and rhythm of the heart. Cardiac AP is an outcome of the concerted action of several ion channels in the plasma membrane of cardiac myocytes. When AP arrives at the atrial and ventricular tissues, opening of the voltage-dependent Na\(^+\) channels causes an abrupt Na\(^+\) influx into the cell, depolarization of membrane potential and rapid
impulse spread over the heart. After a long plateau phase of the cardiac AP, K⁺ currents repolarize the membrane back to the resting membrane potential (RMP) (about -80 mV) and thereby adjust the duration of AP to comply with heart rate at each temperature. The aim of this study was to examine how temperature acclimation affects excitability of the fish heart through modification of sodium (I_{Na}), the delayed rectifier potassium (I_{Kr}) and inward rectifier potassium (I_{K1}) currents of cardiac myocytes in species that have different temperature preferences and tolerances. The exact anatomical site of the fish cardiac pacemaker and the effects of thermal acclimation on the activity of pacemaker cells were examined in rainbow trout. The electrophysiological properties of the cardiac I_{Na} in three teleost species (rainbow trout, crucian carp and burbot) acclimated at two different temperatures were examined, and the molecular composition of the trout cardiac Na⁺ channels was clarified. Temperature-dependent regulation of AP duration by K⁺ currents was studies in six different teleost species.

**Materials and methods** The fish were acclimated for four weeks at two different temperatures within their normal thermal tolerance range. Atrial and ventricular APs were recorded with intracellular microelectrodes from the spontaneously beating hearts, and sarcolemmal ion currents were measured from enzymatically isolated cardiac myocytes by the whole-cell patch-clamp method. Molecular cloning was used to examine the α-subunit composition of the cardiac Na⁺ channels and their tetrodotoxin (TTX) binding sites. Sinoatrial pacemaker tissue of the rainbow trout heart was localized by conventional histological methods and by microelectrode recordings.

**Results** The spontaneous rhythm of the trout heart originates from a small circular tissue in the junctional area between sinus venosus, atrium and sinoatrial valve. The
discharge rate of this pacemaker was enhanced by cold-acclimation (4°C), possibly due to the increased density of the $I_{Kr}$. Three different types of pacemaker cells (spindle, spider and elongated spindle cells) were recognized on morphological grounds and two categories (primary and secondary pacemaker cells) on the basis of AP characteristics. Temperature acclimation modified atrial and ventricular $I_{Na}$ in a species-specific manner: the density of $I_{Na}$ was increased in rainbow trout and burbot, but depressed in crucian carp. The main $Na^+$ channel isoform of the trout heart is the ortholog of the mammalian cardiac skeletal muscle, omNa_v1.4, which unlike the mammalian cardiac isoform, Na_v1.5, is highly sensitive to TTX. Acclimation to cold decreased the duration of cardiac AP in all six species examined and this response was associated with increased density of the $I_{K1}$ and/or the $I_{Kr}$.

Conclusion Chronic temperature changes have a profound effect on ion currents of fish cardiac myocytes and provide explanations for temperature induced changes in heart rate and AP duration. Compensatory shortening of AP duration seems to be an almost ubiquitous response to cold-acclimation in fish, as is the up-regulation of the $I_{Kr}$. By contrast, the $I_{K1}$ and $I_{Na}$ show more often species-specific responses. The depression of $I_{Na}$ in crucian carp may be associated with their winter dormancy in anoxic winter waters, while different responses of the $I_{K1}$ to temperature acclimation may partly be a phylogenetically determined trait.

Jaakko Haverinen, Faculty of Biosciences, University of Joensuu, P.O. Box 111, FI-80100 Joensuu, Finland
ACKNOWLEDGEMENTS

This study was supported by the Academy of Finland (project no. 210400). I would like to express my warmest gratitude to my supervisor and mentor Professor Matti Vornanen. His encouragement has been the key that unlocked the doors to the world of scientific thinking and writing. While working with Matti, I have learned a great deal about many aspects of science and life.

I am also grateful to the reviewers of this thesis, Dr. Holly A. Shiels and Docent Tomi Streng.

I wish to thank my colleagues in the electrophysiological research group and all who have assisted me in my research. Dr. Vesa Paajanen helped me to solve many technical problems and advised me on the analysis of electrophysiological data. I am grateful to Ms Minna Hassinen, MSc., for making all the molecular work of my thesis. Ms Hanna Korajoki, MSc., advised me on many scientific and computing questions, for which I wish to express my gratitude to her. Laboratory technician Anita Kervinen prepared countless numbers of saline solutions for me, always quickly and reliably. Research technician Harri Kirjavainen caught the fish for my experiments and with Harri I took part in an adventurous fishing trip in the fishery of Juuka. Animal attendant Leena Koponen took care of the welfare of the fish in the lab. All your help has been invaluable!

I wish to acknowledge the whole staff of the Faculty of Biosciences at the University of Joensuu and all of my numerous friends including Pentti, now sadly deceased, for their support and encouragement during this work. Riikka-Liisa, I first saw you on the first of May six years ago and since then your joy in life and success at school have been stimulants for me during this work.
I warmly thank my parents, Mirjami and Juhani. With the love and the wisdom of experience of life, you have always advised and motivated me to go forward in my studies, work and in life. Father, through your heritage and guidance I have received an inextinguishable spark of interest in fishing, hunting and nature. The inspiration I received from these activities has helped me to complete this work. My sisters and brothers, Jukka, Päivi, Jouni, Hanna, Kaisa, Janne and Sanna and their families, thanks that you are all present in my life. In common moments and remembrances have been the important resources for me. Special thanks to the men in my family (also the small “men”) for unforgettable moments in fishing waters and in the wilderness of Kainuu.

Finally, my dearest and greatest thanks belong to Tuija. I cannot hope to cover the past years in these few sentences, I have not words enough, but they are unnecessary - you understand. Thanks.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>APD</td>
<td>action potential duration</td>
</tr>
<tr>
<td>RMP</td>
<td>resting membrane potential</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>the maximum rate of AP upstroke</td>
</tr>
<tr>
<td>$I_{Na}$</td>
<td>sodium current</td>
</tr>
<tr>
<td>$I_{Kr}$</td>
<td>rapid component of the delayed rectifier potassium current</td>
</tr>
<tr>
<td>$I_{Ks}$</td>
<td>slow component of the delayed rectifier potassium current</td>
</tr>
<tr>
<td>$I_{K1}$</td>
<td>inward rectifier potassium current</td>
</tr>
<tr>
<td>$I_{o0}$</td>
<td>transient outward current</td>
</tr>
<tr>
<td>$I_f$ or $I_h$</td>
<td>hyperpolarization-activated pacemaker current</td>
</tr>
<tr>
<td>$I_{Ca, L}$</td>
<td>L-type calcium current</td>
</tr>
<tr>
<td>$I_{Ca, T}$</td>
<td>T-type calcium current</td>
</tr>
<tr>
<td>NCX</td>
<td>sodium-calcium exchanger protein</td>
</tr>
<tr>
<td>$Na_v$,1.1-1.9</td>
<td>sodium channel isoforms</td>
</tr>
<tr>
<td>om$Na_v$</td>
<td>sodium channel of rainbow trout</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>half maximal inhibition of current</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>Ry</td>
<td>ryanodine</td>
</tr>
</tbody>
</table>
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by chapter numbers:

**Chapter 4**  

**Chapter 5**  

**Chapter 6**  

**Chapter 7**  

**Chapter 8**  
Articles 4-7 were reprinted with permission from the publishers. The copyright for publication 4 is held by the American Physiological Society, for publications 5-7 by the Company of Biologists and for publication 5 by the Blackwell Synergy.

I participated in the design of the studies and conducted most of the electrophysiological experiments. I analyzed all the electrophysiological data and carried out the statistical analyses. I prepared the first version of the papers and processed the articles in collaboration with the co-authors.
CONTENTS

CHAPTER 1
General introduction 15
1.1 Thermal tolerance of fish 15
1.2 Fish heart 17
1.3 Initiation of the heartbeat 17
1.4 Cardiac action potential of atrial and ventricular myocytes 19
1.5 Sodium channels 20
1.6 Cardiac potassium channels 25
1.7 Importance of cardiac function in thermal tolerance of fish 26
1.8 Aims of the study 28

CHAPTER 2
Synopsis of methods 31
2.1 Whole-cell patch-clamp of ion currents 31
2.2 Microelectrode recording of action potentials 32

CHAPTER 3
Synopsis of results 35
3.1 Cardiac pacemaker of the rainbow trout heart (Chapter 4) 35
3.2 Regulation of cardiac excitability by sodium channels (Chapters 5-7) 35
3.3 Potassium currents and thermal action potential duration (Chapter 8) 37

References 39

CHAPTER 4
Temperature acclimation modifies sinoatrial pacemaker of the rainbow trout heart 53

CHAPTER 5
Temperature acclimation modifies Na\(^+\) current in fish cardiac myocytes 65

CHAPTER 6
Significance of Na\(^+\) current in the excitability of atrial and ventricular myocardium of the fish heart 79

CHAPTER 7
Fish cardiac sodium channels are tetrodotoxin sensitive 91

CHAPTER 8
Responses of action potential and K\(^+\) currents to temperature acclimation in fish hearts: Phylogeny or thermal preferences? 101

CHAPTER 9
General discussion 141
9.1 Rate and rhythm of the heart 141
9.2 Significance of sodium current in excitation of fish heart 145
9.3 Potassium currents and action potential duration 148
9.4 Conclusion 151
References
CHAPTER 1

GENERAL INTRODUCTION
GENERAL INTRODUCTION

Fish evolved around 500 million years ago and currently they form the oldest and most diverse vertebrate group which includes more species than all other vertebrate groups combined. The evolutionary position of fish and their ability to adapt to wide variety of environments have made them frequently used models essentially in every biological discipline including neurobiology, physiology, toxicology, endocrinology, developmental biology and environmental research (Powers 1989).

1.1 Thermal tolerance of fish

Most fish are ectothermic and they have successfully occupied the most diverse thermal habitats of aquatic ecosystems, from the subzero temperatures of the Southern Ocean to the hot waters of desert caves (Brett 1956, Eastman 1997, Beitinger & Bennett 2000). Indeed, the thermal tolerance range of fish extends from -2 to +44°C (Beitinger & Bennett 2000). In north-temperate climates fish are exposed to great seasonal changes of temperature which often require physiological plasticity, i.e. the ability to change the physiological phenotype according to the seasonal temperature conditions. The process of phenotype change in an individual or in a tissue occurs within the framework of the same genome and is called temperature acclimation/acclimatization: it is used to adjust physiology and performance in response to an imposed chronic change of ambient temperature (Hazel & Prosser 1974, Bennet 1984). Thermal acclimation is often based on the ability to express different proteins in response to changing temperature or to produce proteins that are relatively insensitive to temperature (Somero 1975, Johnston & Temple 2002, Watabe 2002, Gracey et al. 2004, Vornanen et al. 2005).
Even in the changing temperature regimes of north-temperate latitudes fish show quite high variability in their temperature tolerance. Some species, e.g. burbot (*Lota lota*), prefer cold waters and probably tolerate only relative small temperature changes, and they are therefore called temperate stenotherms (Bernard *et al.* 1993). For temperate fish, rainbow trout have a relatively low upper thermal tolerance limit (25°C) and are therefore often regarded as stenothermic (Hokanson 1977). By contrast, eurythermic fish tolerate great temperature changes and are likely to show a high physiological plasticity. Cyprinid fish, crucian carp (*Carassius carassius* L.) among them, are well-known for their eurythermicity (Horoszevicz 1973). Fish with intermediate thermal tolerance are called mesotherms and include such species as pike (*Esox lucius*), perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) (Black 1953, Horoszevicz 1973, Currie *et al.* 1998).

Physiological responses to chronic temperature changes are often such that they compensate, partially or completely, for the effects of temperature on the rate of body functions i.e. they tend to maintain metabolism, locomotion and cardiac function relatively independent of temperature changes (Rome *et al.* 1985, Guderley 1990, Driedzic *et al.* 1996, Aho & Vornanen 1999, Tiitu & Vornanen 2001). Under some specific environmental conditions, e.g. in anoxic winter waters, it may be more meaningful to allow temperature to exert its total impact to reduce the metabolic rate and thereby diminish the use of limited energy reserves. Sometimes animals even actively exaggerate the effects of temperature by suppressing the rate of physiological processes more than would be caused by a simple temperature effect ($Q_{10}$). This process is called inverse acclimation and usually leads to metabolic depression under harsh environmental conditions and thereby extends the survival time of the individual (Crawshaw 1984, Driedzic & Gesser 1994, Vornanen 1994, Jackson 2000).
1.2 Fish heart
The heart of teleost fish consists of four compartments, two contracting muscular chambers, the atrium and the ventricle, which propel blood into the vasculature and two collecting cavities, the sinus venosus and the bulbus arteriosus which connect the heart to the vasculature. The sinus venosus receives oxygen-poor venous blood from the body and directs it to the atrium through an opening guarded by the sinoatrial valve, while the bulbus arteriosus provides a blood pressure stabilizing and compliant exit route for blood from the ventricle to the ventral aorta and further to the gills (Santer 1985, Olson & Farrell 2006).

1.3 Initiation of the heartbeat
The function of the heart is to maintain continuous circulation of blood to active tissues and provide them with oxygen and nutrients. The fish heart pumps blood via the ventral aorta to the gills and further into the dorsal aorta and all body tissues (Santer 1985). Each heartbeat is initiated by a pulse of electrical excitation or AP that begins in a group of spontaneously active pacemaker cells in an anatomically and functionally specialized cardiac tissue and subsequently spreads to the atrial and ventricular muscles, eliciting sequential contraction of the muscular chambers. In the mammalian heart, this center is located in the wall of the right atrium and is called the sinoatrial node (Keith & Flack 1907, Bleeker et al. 1980, Bouman & Jongsma 1986). In the fish heart, the pacemaker area is assumed to be located in the sinus venosus or sinoatrial junction, but this has been experimentally verified in only a few species (Mackenzie 1913, Saito 1969, Yamauchi et al. 1973).

Rhythmic activity of the cardiac pacemaker is due to the presence of a spontaneous diastolic depolarization or pacemaker AP that is driven by a net inward
current through the sarcolemma of the pacemaker cells. The pacemaker AP is an outcome of a complicated interaction between several ion channels and ion transporters. The net inward current results from deactivation of an outward current, i.e. the delayed rectifier $K^+$ currents ($I_{K,a}$ and $I_{K,r}$), and the activation of inwards currents, including a $Na^+$-dependent background current ($I_{b,Na}$), a hyperpolarization-activated pacemaker current ($I_f$ or $I_h$) and T- and L-type $Ca^{2+}$ currents ($I_{Ca,T}$, $I_{Ca,L}$) (Brown 1982, Irisawa et al. 1993, Boyett et al. 2000). Of the other cell membrane mechanisms the sodium pump, sodium-calcium exchanger (NCX) and intracellular $Ca^{2+}$ stores of the sarcoplasmic reticulum (SR) seem to be involved in cardiac pacemaking, too (Zhou & Lipsius 1993, Ju & Allen 1999). Although large number of ion channels and transporters are known to be involved in pacemaker function, there is no consensus about the pacemaker mechanism of the vertebrate heart. In some models deactivation of the delayed rectifier current(s) drives the diastolic depolarization (Irisawa et al. 1993, Ono & Ito 1995, Verhecjk et al. 1995), in another the hyperpolarization-activated “pacemaker” current is in the central position (DiFransesco et al. 1995), while in still others spontaneous $Ca^{2+}$ release from the SR and subsequent activation of the sarcolemmal NCX play the dominant roles (Maltsev et al. 2006).

Thus far, the pacemaker mechanism of the fish heart has not been studied at cellular and molecular level. The heart rate in fish is strongly modified by thermal acclimation and this effect seems to be largely mediated by temperature-dependent changes in the activity of the autonomic nervous system (von Skramlik 1935, Farrell & Jones 1992). In particular, inhibitory cholinergic innervation seems to be involved and is up-regulated in warm environments (Seibert 1979, Axelsson et al. 1987). Thus, the current paradigm assumes that the pacemaker mechanism itself does not
contribute to temperature-induced changes in heart rate. However, this has not been directly tested.

1.4 Cardiac action potential of atrial and ventricular myocytes

Although molecular composition and functional characteristics of the fish heart are quite different from those of the mammalian heart (Santer 1985), its electrical properties obey the same biophysical principles and are in many respects similar to those of humans and other mammals (Sedmera et al. 2003, Kopp et al. 2005, Milan et al. 2006, Arnaout et al. 2007). The normal electrical excitability of cardiac myocyte results from an elaborate balance between depolarizing (inward) and repolarizing (outward) currents. Depolarization is mediated by channels or transporters that enable positively charged Na\(^+\) or Ca\(^{2+}\) ions to enter the cell (or negatively charged Cl\(^-\) ions to exit) and repolarization results when the K\(^+\) efflux exceeds the sum of the Na\(^+\) and Ca\(^{2+}\) influx (Hodgkin & Huxley 1952a). When the ion channels open, they drive the membrane potential of the cell towards the equilibrium potential of the ion in question. The opening of the K\(^+\) channels drives the cell towards -90 mV and the opening of the Na\(^+\) and Ca\(^{2+}\) channels forces the membrane potential towards positive levels, +60 mV or more. The resulting electrical signal or cardiac AP consists of five phases, numbered 0-4 (Figure 1), which are produced by the opening and closing of the Na\(^+\), K\(^+\), Ca\(^{2+}\) and Cl\(^-\) specific ion channels. Six major ion currents govern the vertebrate cardiac AP. I\(_{\text{Na}}\) generates the rapid upstroke (phase 0). Transient outward current (I\(_{\text{to}}\), carried by K\(^+\) and Cl\(^-\) ions) causes the small and rapid repolarization following the AP upstroke (phase 1). Potassium currents (I\(_{\text{Kr}}\), I\(_{\text{Ks}}\), I\(_{\text{K1}}\)) regulate APD (phases 2 and 3) together with I\(_{\text{Ca}}\) and maintain the negative resting membrane potential (I\(_{\text{K1}}\) (phase 4) (Opie 1998, Hille 2001).
Figure 1. Action potential (AP) of the rainbow trout atrium and schematic presentation of different phases of cardiac AP and the underlying ion currents. The rapid phase 0 depolarization is mediated by Na\(^+\) entry into the cells due to a marked increase in the number of open Na\(^+\) channels in the cell membrane. In phase 1 the transient outward current (I\(_{to}\)) is due to the movement of K\(^+\) and Cl\(^-\) ions in opposite directions. Note that this phase is poorly developed in the AP of the fish heart. Phase 2 of the cardiac action potential is sustained by a balance between inward movement of Ca\(^{2+}\) through L-type calcium channels (I\(_{Ca, L}\)) and outward movement of K\(^+\) through potassium channels (I\(_{Ks}\), I\(_{Kr}\), I\(_{K1}\)). During phase 3 of the action potential, the L-type Ca\(^{2+}\) channels close and net outward, positive current causes the cell to repolarize. I\(_{Ks}\) and I\(_{Kr}\) disappear when the membrane potential is restored to about -80 to -85 mV, while I\(_{K1}\) remains conducting throughout the phase 4. The cell remains at the resting membrane potential (RMP) until it is stimulated by an AP spreading from the cardiac pacemaker over the entire heart. Modified from Opie (1998).

1.5 Sodium channels

The Amazonian electric eel (Electrophorus electricus) and electric ray (Torpedo californica) have electroplax tissue which they use to generate powerful electrical shocks to repel predators and to stun prey animals (Powers 1989). Because the electric organ is a rich source of Na\(^+\) channels, the first vertebrate Na\(^+\) channels were cloned and purified from this fish tissue (Agnew et al. 1978, Noda et al. 1984).
Subsequently, several Na\(^+\) channel isoforms have been found in various tissues of mammals and fish, including the heart.

Voltage-gated Na\(^+\) channels are composed of a pore-forming \(\alpha\) subunit and 1 or more auxiliary \(\beta\) subunits (Catterall 1992, 1996). During the past two decades, altogether ten genes encoding Na\(^+\) channel \(\alpha\) subunits have been identified and nine orthologs of these channels have been found in different mammalian tissues (Goldin 2001) and eight orthologs in fish tissues (Lopreato \textit{et al.} 2001). The \(\alpha\) subunit consists of four repeat domains (I-IV), each of them having six transmembrane segments (S1-S6) (Figure 2). The S4 segments form positively charged voltage sensors, which coordinate the opening of the Na\(^+\) channel in a voltage-dependent manner. The short intracellular loop connecting domains III and IV in channel forms “the plug”, that closes the channel upon inactivation (Catterall 1992).
Figure 2. Structures of three ion channel types of the vertebrate cardiac muscle examined in the present study. Kir channels (top) are tetramers of proteins with two-transmembrane segments (S1 and S2) and they generate the cardiac I_{K1} current. Voltage-gated potassium channels (middle) are tetramers of four identical proteins each having six transmembrane segments (S1-S6). This type of channel produces e.g. the I_{Kr} current. The voltage-sensitive sodium channels (bottom) consist of one large protein which has four similar membrane-spanning domains (I-IV), each containing six transmembrane segments (S1-S6). Segments and/or domains are connected by intracellular and extracellular loops in each channel type. In the sodium channel, TTX binding sites in the pore loop region between S5 and S6 of the domains I-IV are marked with gray circles. Modified from Goldin (2001).

The concentration of Na\(^+\) ions in extracellular and intracellular compartments is about 140 and 10 mM, respectively, creating an electrochemical gradient of +60 mV across the plasma membrane, towards which membrane potential is driven by opening of the Na\(^+\) channels (Hille 2001, Bers et al. 2003). The functional cycle of the Na\(^+\) channel (and other voltage-gated channels) involves three states (resting, open and
inactivated) and four gating transitions (activation, deactivation, inactivation and recovery from inactivation) (Figure 3). In the resting state, the inactivation gate (h) is in the open position and the activation gate (m) in the closed position, and consequently the channel is non-conducting. Depolarization of atrial and ventricular myocytes by a pacemaker potential or an external stimulus over the threshold value (positive to -60 mV) changes channels from the resting state to the open state (activation), i.e. both gates are open and Na$^+$ ions flow into the cell. Depolarization of the cell membrane causes movement of the inactivation gate to the closed position (inactivation) and switches off the Na$^+$ conductance. The activation gate has to recover its open position (deactivation) and the inactivation gate its closed position (recovery from inactivation) before a new cycle can be elicited, i.e. the channel cannot be opened from the inactivated state (Hodgkin & Huxley 1952 b, Hille 1978, 2001, Grant 2001).
Figure 3. Scheme of sodium channel gating. A depolarizing voltage step (top), an associated $I_{Na}$ of a rainbow trout atrial myocyte (centre) and a physical model to account for the transient $I_{Na}$ are shown. Sodium channel proteins form a water-filled pore with two voltage-sensitive gates in the lipid membrane of the cardiac myocyte. In the resting state, the activation (m) gate is in the closed position and the inactivation (h) gate is in the open position. When the threshold voltage for sodium channel opening is exceeded, the m gate assumes an open position (activation) and with both gates open, sodium ions diffuse into the cell. The h gate then moves into the closed position (inactivation), blocking the channel. When membrane potential returns to the resting level (-80 mV), the m gate moves into the closed position (deactivation). In a time- and voltage-dependent process termed recovery from inactivation, the h gate moves into the open position (not shown). A similar scheme applies to other voltage-gated ion channels. Modified from Grant (2001).

$Na^+$ channels are effectively blocked by guanidium compounds, most notably saxitoxin and TTX, produced by the glands of some poisonous animals. TTX is a very potent toxin with a lethal dose ($LD_{50}$) value of 0.1 mg/kg in mouse, and it is abundantly present in the ovaries and liver and in lesser amounts in the intestine and skin of the puffer fish (fugu, *Tetradon viridis*) and its relatives from the family *Tetraodontidae* as well as in some newts and marine invertebrates (Denac et al. 2000). TTX blocks the ion permeation of $Na^+$ channels from an extracellular site, but has no
effect on channel gating (Lipkind & Fozzard 1994). Based on their sensitivity to TTX, Na\(^+\) channels are categorized either as TTX-sensitive (K\(_{d}\)=1-1000 nM) or TTX-resistant (K\(_{d}\)=1000-30000 nM). Among mammalian Na\(^+\) channels, Na\(_v\)1.5, Na\(_v\)1.8 and Na\(_v\)1.9 are TTX-resistant, whereas Na\(_v\)1.1, Na\(_v\)1.2, Na\(_v\)1.3, Na\(_v\)1.4 and Na\(_v\)1.6 are inhibited by nanomolar concentrations of TTX, as is the isoform expressed in skeletal muscle (Na\(_v\)1.4) (Fozzard & Hanck 1996, Goldin 2001, Lei et al. 2004). TTX-resistant Na\(_v\)1.5 is the main cardiac isoform in mammalian hearts, even though TTX-sensitive isoforms Na\(_v\)1.4, Na\(_v\)1.1, Na\(_v\)1.2 and Na\(_v\)1.6 are expressed in small amounts in different parts of the heart (Maier et al. 2002). Thus, the mammalian cardiac I\(_{Na}\) is quite generally termed TTX-resistant.

### 1.6 Cardiac potassium channels

Voltage-gated K\(^+\) channels (K\(_v\)) (Figure 2) are tetramers of proteins which have six membrane spanning sequences, segments 1-4 being associated with voltage-sensing and -gating, while the loops between segments 5 and 6 form the K\(^+\) selective pore of the channel (Warmke & Ganetsky 1994, Jan & Jan 1997). The inward rectifier K\(^+\) channels (K\(_i\)) are much simpler in structure than voltage-gated K\(^+\) channels since they lack the voltage-sensing and -gating structures. They are hetero- or homotetramers of proteins having only two membrane spanning segments. In the absence of the voltage-sensitive sequences, the characteristic voltage-dependence of the inward rectifier current is produced by a current-dependent blocking of the channel by intracellular Mg\(^{2+}\) and polyamines (spermine and spermidine) (Nichols & Lopatin 1997).

Until recently, one voltage-gated K\(^+\) current (I\(_{Kr}\)) (Vornanen et al. 2002 a) and three inward rectifier K\(^+\) currents (ATP-sensitive K current, I\(_{K,ATP}\); background inward rectifier current, I\(_{K1}\); and acetylcholine-activated inward rectifier, I\(_{K,Ach}\)) (Aho &
Vornanen 2002, Paajanen & Vornanen 2002, 2004, Vornanen et al. 2002 a) have been found in fish hearts. The inward rectifier $K^+$ channels of the vertebrate heart are tetramers of $K_{ir}2.1$, $K_{ir}2.2$ and $K_{ir}2.3$ proteins, the $K_{ir}2.1$ being the main isoform in mammals (Wang et al. 1998, Dhamoon et al. 2004). The inward rectifier of the rainbow trout heart is formed by $K_{ir}2.1$ and $K_{ir}2.2$ (Hassinen et al. 2007), while crucian carp heart expresses three $K_{ir}2$ channels ($K_{ir}2.1$, $K_{ir}2.2$ and $K_{ir}2.5$) (Hassinen et al. 2008).

1.7 Importance of cardiac function in thermal tolerance of fish

The function of the heart is intimately associated with the thermal preferences of the fish. In both stenothermic (narrow thermal tolerance) and eurythermic (wide thermal tolerance) species the optimum temperature for heart function is close to the preferred body temperature of the fish, and the activity of the heart is depressed towards both low and high extremes of thermal tolerance (Brett 1971, Matikainen & Vornanen 1992). Due to thermal limitation of the heart at both low and high extreme of temperatures, the animal is likely to suffer from systemic hypoxia much below its acute thermal tolerance limits (Pörtner et al. 2004), and may eventually succumb to hypoxia.

The hypothesis that a mismatch between the demand for oxygen and the capacity of oxygen supply to tissues restricts whole-animal tolerance to thermal extremes has recently been presented (Pörtner et al. 2006). It is proposed that when the temperature approaches the upper or lower limits of thermal tolerance, oxygen delivery systems may fail to provide enough oxygen to the tissues and the animal will die to hypoxia or anoxia, mainly due to temperature-dependent limitation of the circulation. Central to this hypothesis is the assumption that heart function is
compromised at extremes of low and high temperatures and therefore the heart fails to nourish the tissues with oxygen. This hypothesis is made particularly fascinating by a recent finding that changes in the distribution of fish populations due to global warming are caused by limitation of cardiac function (Pörtner & Knust 2007). Understanding thermal tolerance of the heart function in ectothermic vertebrates may thus improve our knowledge of the impact of global warming on fish populations.

Cardiac muscle is an electrically excitable tissue, where ion channel function plays a vital role in setting the rate and rhythm of the heart, initiating contraction and regulating the force production of cardiac myocytes. Electrical excitation or cardiac AP is produced by delicate and complex interaction between several ion channels in the plasma membrane of the cardiac myocyte (Opie 1998, Schram et al. 2002). As a complex phenomenon, cardiac AP is sensitive to disturbances in ion channel function, which may generate arrhythmias, cause conduction failures and compromise the force of cardiac contraction (Wang & Rudy 2000). Relatively small changes in body temperature can elicit severe malfunction of the ion channels, as exemplified by fatal cardiac arrhythmias in hypothermic humans (Walpoth et al. 1997, Marban 2002). Yet, many ectothermic vertebrates, including several fish species, routinely experience seasonal temperature changes of 20-25°C. In these animals, the cardiac ion channels must have high resistance to temperature changes. On the other hand, such an extension of thermal tolerance may have brought ion channel function close to the limits of temperature-dependent failure. As electrical excitation of myocyte plasma membrane governs all vital aspects of heart function (rate, rhythm, impulse conduction and force), ion channels could be critical for the adaptation and acclimation of ectothermic hearts to temperature. However, relatively little is known about the effects of temperature on cardiac ion currents and the molecular
composition of the cardiac ion channels. Most studies have dealt with L-type Ca\(^{2+}\) current (Hove-Madsen & Tort 1998, Hove-Madsen et al. 2000, Vornanen 1997, 1998, Shiels et al. 2000, 2002), and some data also exists for ATP-sensitive K\(^+\) current and the delayed rectifier K\(^+\) current (Paajanen & Vornanen 2002, 2004). However, practically nothing (Warren et al. 2001) is known about the fish cardiac Na\(^+\) current, especially regarding its molecular background.

1.8 Aims of the study
In this thesis my aim was to examine how temperature acclimation affects the electrical excitability of the fish heart in species living in different aquatic habitats and having different thermal preferences and tolerances. The location of the cardiac pacemaker tissue and the effects of thermal acclimation on initiation of heart beat was investigated in rainbow trout sinoatrial tissue and isolated pacemaker cells (Chapter 4). The electrophysiological characteristics and molecular composition of cardiac Na\(^+\) channels in the atrial and ventricular myocytes of three teleost species were studied with regard to acute and chronic temperature changes (Chapters 5, 6 and 7). Finally, temperature-dependent regulation of APD by repolarising K\(^+\) currents was investigated in six different teleost species in order to observe species-specific differences in electrical excitability (Chapter 8).
CHAPTER 2

SYNOPSIS OF METHODS
SYNOPSIS OF METHODS

Established methods of electrophysiology were used to measure the electrical activity of the fish heart in multicellular cardiac preparations and single cardiac myocytes (Chapters 4-8). The sodium channel composition of the trout heart and the amino acid sequence of the tetrodotoxin (TTX) binding site of the Na$^+$ channels were obtained by molecular cloning and sequencing (Chapter 7).

2.1 Whole-cell patch-clamp of ion currents

The whole-cell patch-clamp method (Hamill et all. 1981) was used to measure the density and kinetics of Na$^+$ and K$^+$ currents from enzymatically isolated cardiac myocytes of the fish heart. Atrial and ventricular myocytes were isolated by enzymatic digestion of the intercellular connective tissue of the heart. To this end, the heart was cannulated and perfused with a saline solution containing trypsin (10 mg/ml) and collagenase (15 mg/ml) for 10-15 minutes (Vornanen 1997). Small pieces of softened atrial and ventricular muscle were agitated through the opening of a Pasteur pipette to release single cardiac myocytes. Single cells were “patched” in a small flow-through chamber with continuous temperature control of the saline solution. Unwanted ion currents were prevented from using specific blockers (TTX, nifedipine and E-4031 for the Na$^+$ current, L-type Ca$^{2+}$ current and rapid component of the delayed rectifier K$^+$ current, respectively) in the external saline solution and/or from replacing external and internal K$^+$ with Cs$^+$ to block K$^+$ currents. Capacitive cell surface area was routinely determined and amplitudes of ion currents were always given as current densities (pA/pF).
2.2 Microelectrode recording of action potentials

The action potentials (APs) of ventricle, atrium and pacemaker tissue were measured from multicellular cardiac preparations using sharp microelectrodes (Ling & Gerard 1949). A temperature controlled tissue chamber (10 ml) was filled with a physiological saline solution and a sinoatrial preparation, or a small whole-heart was mounted on the bottom of the chamber with small needles. Myocytes of the selected heart area were impaled on electrodes filled with 3M KCl solution (resistance 10-20 MΩ) and APs were recorded through a voltage amplifier and a digitizer on the computer. Resting membrane potential and characteristics of the APs (amplitude, overshoot, duration and rate of the upstroke) were measured off-line using Clampfit software.
CHAPTER 3

SYNOPSIS OF RESULTS
SYNOPSIS OF RESULTS

3.1 Cardiac pacemaker of the rainbow trout heart (Chapter 4)
Using electrophysiological and histological methods, the pacemaker of the trout heart was located in a small ring-form tissue in the border area between the sinus venosus and the atrium. Two categories of pacemaker cells (primary and secondary) were characterized from this tissue on the basis of action potential (AP), while three types of pacemaker cells (spindle, spider and elongated spindle cells) were recognized on the basis of myocyte shape. Cold acclimation (+4°C) increased AP rate (heart rate) and decreased the duration of pacemaker AP. These differences were attributed to the increased density of the delayed rectifier potassium current ($I_{Kr}$) in the cold-acclimated trout hearts. By contrast, inhibition of sarcoplasmic reticulum (SR) function with ryanodine (Ry) and thapsigargin did not contribute to acclimation-dependent changes in AP rate.

3.2 Regulation of cardiac excitability by sodium channels (Chapters 5-7)
Sodium current ($I_{Na}$) is considered to determine the rate of the AP upstroke and conduction velocity of AP over the heart. Temperature acclimation modified $I_{Na}$ of the fish cardiac myocytes differently, depending on the animal’s lifestyle and/or thermal preferences (Table 1). In cold-active species (rainbow trout and burbot) cold-acclimation increased the density of $I_{Na}$, whereas in the cold-dormant crucian carp, the density of $I_{Na}$ was depressed in the cold-acclimated winter fish, suggesting that increased $I_{Na}$ is needed to maintain high electrical excitability and conductivity in the cold.
The $I_{\text{Na}}$ of atrial and ventricular myocytes was compared in the cold-acclimated rainbow trout. Although the maximum rate of action potential upstroke was 21% greater in atrial than in ventricular tissue, the density of $I_{\text{Na}}$ did not differ between atrial and ventricular myocytes. This contradiction might be due to the more extensive resistive coupling with myocytes and fibroblasts in the ventricle than in the atrium, since fibroblasts can function as current sinks for $I_{\text{Na}}$ and thereby reduce the effect of $I_{\text{Na}}$ on the AP upstroke. Thus, in atrial muscle the high velocity of impulse conduction would be attributable to weak electric coupling of myocytes to fibroblasts, and not to $I_{\text{Na}}$. The voltage-dependence of $I_{\text{Na}}$ activation was more negative in atrial than in ventricular myocytes. This together with much higher input resistance of atrial myocytes makes atrial muscle readily excitable by weak depolarizing voltages from pacemaker cells.

The $I_{\text{Na}}$ of the trout heart is about 1000 times more sensitive to tetrodotoxin (TTX) ($IC_{50} = 1.8–2 \text{ nM}$) than the mammalian cardiac $I_{\text{Na}}$ and it is produced by three sodium channels ($Na_v$) $\alpha$-subunits which are orthologs to mammalian skeletal muscle $Na_v1.4$, cardiac $Na_v1.5$ and peripheral nervous system $Na_v1.6$ isoforms respectively. $OmNa_v1.4a$ is the predominant isoform of the trout heart accounting for over 80% of the $Na_v$ transcripts, while $omNa_v1.5a$ forms about 18% and $omNav1.6a$ only 0.1% of the transcripts. Thus, the ortholog of the mammalian skeletal muscle isoform, $omNa_v1.4a$, is the main cardiac isoform of fish hearts. Unlike the mammalian cardiac $Na_v1.5a$, all trout cardiac $Na^+$ channels, including the $omNav1.5a$ have aromatic amino acids (phenylalanine or tyrosine) instead of non-aromatic cysteine or serine in the critical TTX binding position of the domain I and this confers their high TTX sensitivity. It is likely that the residues involved in TTX binding close to the selectivity filter of the channel are functionally important, and thus phylogenetic
sequence differences in TTX binding site reflect the adaptation to variable internal body conditions and functional requirements of cardiac $I_{Na}$ in different vertebrate groups.

Table 1. Effects of cold-acclimation (+4°C) on the properties of ventricular $I_{Na}$ of the fish heart at 11°C.

<table>
<thead>
<tr>
<th></th>
<th>$I_{Na}$ (pA/pF)</th>
<th>SSAct. (mV)</th>
<th>SSInact. (mV)</th>
<th>Recovery from inactivation</th>
<th>Rested-state inactivation</th>
<th>Kinetics of inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burbot</td>
<td>↑</td>
<td>-</td>
<td>-</td>
<td>↓</td>
<td>-</td>
<td>↓</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>↑</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>Crucian carp</td>
<td>↓</td>
<td>-</td>
<td>-</td>
<td>↓</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

SSAct. voltage-dependence ($V_{0.5}$) of steady-state activation; SSInact. voltage-dependence ($V_{0.5}$) of steady-state inactivation. Upward (↑) and downward (↓) arrows indicate increase and decreases in the value, respectively. A hyphen (-) signifies the absence of thermal effects.

3.3 Potassium currents and thermal action potential duration (Chapter 8)

The responses of AP and $K^+$ currents to temperature acclimation were examined in six different teleost fish species. Cold-acclimation shortened action potential duration (APD) in all species regardless of the thermal tolerances, lifestyles or phylogenies of the fish, suggesting that this response is a common characteristic of the teleost heart (Table 2). However, the strength of the response differed among the phylogenetic groups *Salmoniformes* fish showing the greatest and the perch (*Perciformes*) the weakest acclimation capacity of APD. The underlying ionic mechanisms were also partly phylogeny-dependent. Modification of the delayed rectifier potassium current ($I_{Kr}$) current was almost ubiquitously involved in the acclimation response of fish cardiac myocytes to temperature, while the ability to change the inward rectifier potassium current ($I_{K1}$) under chronic thermal stress was absent or showed inverse compensation in *Salmoniformes* species. Thus, in *Salmoniformes* fish the thermal
plasticity of APD is strongly based towards $I_{Kr}$, while other fish groups rely on both $I_{Kr}$ and $I_{K1}$.

Table 2. Effect of cold acclimation on the delayed rectifier potassium current ($I_{Kr}$), inward rectifier potassium current ($I_{K1}$) and duration of action potential at 90% of repolarization (APD90) in six different teleost fish atrial and ventricular myocytes.

<table>
<thead>
<tr>
<th></th>
<th>$I_{Kr}$ (pA/pF)</th>
<th>$I_{K1}$ (pA/pF)</th>
<th>APD90 (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>V</td>
<td>A</td>
</tr>
<tr>
<td>Burbot</td>
<td>↑</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>↑</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td>Crucian carp</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Roach</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Perch</td>
<td>↑</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pike</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Upward (↑) and downward (↓) arrows indicate increases and decreases in value, respectively. The hyphen (-) signifies the absence of thermal effects. The letter A refers to the atrial and V to the ventricular cell.
References


Horoszewicz L (1973) Lethal and ‘disturbing’ temperatures in some fish species from lakes with normal and artificially elevated temperature. *J. Fish Biol.* 5: 165-181.


TEMPERATURE ACCLIMATION MODIFIES SINOATRIAL PACEMAKER MECHANISM OF THE RAINBOW TROUT HEART

Haverinen J. and Vornanen, M (2007)

*Am. J. Physiol.* 292: R1023-R1032
CHAPTER 5

TEMPERATURE ACCLIMATION MODIFIES NA⁺ CURRENT IN FISH CARDIAC MYOCYTES


_J. Exp. Biol._ 207: 2823-2833
SIGNIFICANCE OF NA$^+$ CURRENT IN THE EXCITABILITY OF ATRIAL AND VENTRICULAR MYOCARDIUM OF THE FISH HEART

Haverinen J. and Vornanen, M (2006)

*J. Exp. Biol.* 209: 549-55
CHAPTER 7

FISH CARDIAC SODIUM CHANNELS ARE TETRODOTOXIN SENSITIVE


Acta Physiol. 191: 197-204
CHAPTER 8

RESPONSES OF ACTION POTENTIAL AND K⁺ CURRENTS TO TEMPERATURE ACCLIMATION IN FISH HEARTS.

PHYLOGENY OR THERMAL PREFERENCES?

Haverinen J. and Vornanen, M (2008)

*Physiol. Biochem. Zool.* 000: 000-000 (under revision)
CHAPTER 9

GENERAL DISCUSSION
GENERAL DISCUSSION

The vertebrate heart is a muscle pump which propels blood through the closed circulatory system. The volume of blood circulation is determined by cardiac output which is the product of heart rate and stroke volume. Temperature has a strong effect on the rate of all biological processes including the rate functions of the heart. The heart rate, rate of impulse conduction through the heart and shortening velocity of the cardiac muscle are strongly retarded by low temperatures and accelerated by high temperatures (Farrell & Jones 1992, Opie 1998). As a consequence, the cardiac function of ectothermic fish is strongly modulated by chronic temperature changes in different seasons and by acute temperature changes e.g. when fish make transitions through the thermocline. Since the whole body of the fish (with exception of some partially endothermic species) is ectothermic, ambient temperature will similarly affect the metabolic rate of the fish and function of the heart, and therefore a relative close match will prevail between the metabolic need for oxygen and the circulatory supply of oxygen (Johnston & Temple 2002, Pörtner et al. 2004, Pörtner et al. 2006). Correspondingly, when physiology of the animal responds to chronic temperature changes by acclimation/acclimatization, similar changes are expected to happen in the metabolism and pump function of the heart in order to satisfy the metabolic needs of different tissues, i.e. the cardiac function should reflect temperature-dependent changes in the metabolism of the fish.

9.1 Rate and rhythm of the heart

The excised heart of vertebrate animals beats in physiological saline solution with a rate determined by its own pacemaker center (intrinsic rate). In the intact animal body, heart rate is under the constant control of the autonomic nervous system through
acceleratory adrenergic nerves and inhibitory cholinergic nerves, using adrenaline and acetylcholine, respectively, as neurotransmitters. Furthermore, circulating catecholamines may also affect the heart rate in fish (Laurent et al. 1983, Farrell and Jones 1992). In particular, the inhibitory cholinergic tone has a significant modulating effect on the heart rate in fish, while the acceleratory influences of the adrenergic nerves are often much weaker (von Skramlik 1932, Cameron 1979, Lillywhite et al. 1999). Several studies have shown that acclimation-induced changes in the heart rate of fish are due to changes in the cholinergic control of the heart; in cold conditions, inhibitory cholinergic tone is decreased, resulting in a compensatory increase in heart rate (Seibert 1979, Sureau et al. 1989). Thus, the prevailing paradigm on temperature-induced changes in the heart rate of fish maintains that they are solely produced by the autonomic control of the heart. Contrary to this hypothesis, the effect of temperature acclimation on heart rate in rainbow trout is still evident in excised hearts, indicating that autonomic nerves cannot be totally responsible for the temperature-induced changes in heart rate (Aho & Vornanen 2001). Consistent with the latter finding, it was shown that the effect of temperature acclimation is present in individual pacemaker cells of the trout heart (Chapter 4). Thus the hypothesis of temperature-induced changes in heart rate must be reformulated to include temperature-dependent modulation of the pacemaker mechanism, i.e. ion regulation of the diastolic depolarization of the primary pacemaker cells. Since temperature-induced changes in heart rate are common among teleost fish (Chapter 8), it remains to be shown what the relative significance of autonomic nervous control and cellular pacemaker mechanism is in thermal acclimation of the heart rate.

Several ion currents contribute to the diastolic depolarization of the cardiac pacemaker cells (Irisawa 1978, Hagiwara et al. 1988, Campbell et al. 1992). There are
several ionic models which try to explain the initiation of pacemaker depolarization and the steady rhythm of the heart beat. Three major hypotheses on the pacemaker mechanism of the vertebrate heart have been worked out using mammalian and frog pacemaker tissues and cells, which emphasize different ionic mechanisms as the primary cause of diastolic depolarization. In the earliest model of the cardiac pacemaker mechanism, deactivation of the delayed rectifier $K^+$ current (the fast and slow components of the delayed rectifier were not yet known) was considered to be crucial for the initiation of the pacemaker action potential (AP) (Irisawa et al. 1993).

The pacemaker current ($I_f$ or $I_h$) of the mammalian heart was described for the first time in 1980 and a hypothesis proposing a master role for to this current has subsequently elaborated, mainly by Difrancesco and his colleagues (Brown & Difrancesco 1980, Difrancesco 1981, Difransesco et al. 1986). In this hypothesis $Na^+$ and $K^+$ influx through the pacemaker channels initiates diastolic depolarization and determines the heart rate. The activity of the pacemaker channel is regulated by adrenergic and cholinergic agonists, thereby providing an explanation for autonomic control of the heart rate. An alternative hypothesis to that offered by the pacemaker current is based on the interaction of sarcoplasmic reticulum (SR) $Ca^{2+}$ release and sodium-calcium exchanger (NCX) in the sarcolemma (Li et al. 1997, Ju & Allen 1999, Bogdanov et al. 2001, Mangoni et al. 2003, Lakatta et al. 2006). In this model, a small spontaneous release of $Ca^{2+}$ from the subsarcolemmal SR activates an extrusion of $Ca^{2+}$ out of the cell through the NCX and as an electrogenic system the NCX generates a small inward current that primes the rate of the pacemaking.

Until the present, the pacemaker mechanism has not been examined in fish hearts. Two of the prevailing hypotheses on pacemaker mechanisms of the heart were tested with regard to temperature induced changes in the heart rate of the rainbow
trout. Although the $I_h$ current has been demonstrated in embryonic cardiac cells of zebrafish (Baker et al. 1997, Warren et al. 2001), it could not be found in trout atrial or pacemaker cells (unpublished results). The first hypothesis was therefore abandoned as a relevant pacemaker mechanism of the trout heart. The two other hypotheses were tested by using ryanodine (Ry) and E-4031, specific blockers of the SR Ca$^{2+}$ release channel and the delayed rectifier potassium current ($I_{Kr}$) to inhibit the prime molecules of the two hypotheses. It could be demonstrated that the effect of E-4031 differed between warm-acclimated and cold-acclimated trout sinoatrial preparations from warm-acclimated and cold-acclimated trout, in that the beating rate of warm-acclimated hearts is more strongly depressed by E-401 in comparison to that of cold-acclimated hearts (Chapter 4). This result together with the finding that $I_{Kr}$ current is greater in cold-acclimated than in warm-acclimated myocytes (Vornanen et al. 2002 a), suggests that the $I_{Kr}$ current contributes at least partly to the higher heart rate of the cold-acclimated fish. In situ hybridization indicates the presence of the delayed rectifier potassium channels (ERG) in the pacemaker tissue of the rainbow trout (Hassinen et al. 2008 b). On the other hand, Ry had only a minor effect on the beating rate of the trout heart, suggesting that SR Ca$^{2+}$ release and NCX are not central operators in trout pacemaker tissue (Chapter 4). It should be noted, however, that the heart rate of warm-acclimated trout was slightly inhibited by Ry, suggesting that the third hypothesis might have some bearing at high temperatures.

The ion currents of the fish cardiac pacemaker have not yet been examined, and this is an interesting open area of research. Careful examination of ion currents, ion exchangers and the function of the SR are needed in order to clarify the pacemaker mechanism of the fish heart and its modification by temperature and other environmental factors.
9.2 Significance of sodium current in excitation of fish heart

In every heart beat, voltage-sensitive Na$^+$ channels initiate atrial and ventricular excitation by generating the rapid upstroke (phase 0) of the cardiac AP, and therefore are responsible for fast impulse conduction through the heart (Grant 1991, Catterall 1992). The generation of APs and the coordinated spread of excitation are essential for the orderly pumping action of the heart and depend largely on the density and kinetics of the sodium current (I_{Na}) (Furue et al. 1998). Without any temperature-induced compensation in the Na$^+$ channel function, excitation and impulse conduction of the atrial and ventricular muscle would vary according to ambient temperature changes which might compromise heart function or make the heart vulnerable to arrhythmias. In many fish species the frequency of cardiac beating increases when the animals are exposed to chronic cold (Seibert 1979, Sureau et al. 1989, Bowler & Tirri 1990, Aho & Vornanen 2001), which is expected to require compensatory changes in the density of I_{Na} and/or its kinetics. Consistent with this, increases in the density of cardiac I_{Na} were found in cold-acclimated individuals of rainbow trout and burbot, i.e. species which show positive thermal compensation in the heart rate. Cold acclimation depressed I_{Na} density in the heart of crucian carp, a species which, it is suggested to become inactive in the cold and anoxic winter waters (Table 1, Chapters 5).

There were prominent species-specific differences in the density of I_{Na}, burbot having clearly the highest current and crucian carp the lowest current, while the I_{Na} of the rainbow trout heart was intermediate between the two (Table 1, Chapter 5). This suggests that excitability and rate of impulse conduction varies in the order burbot>rainbow trout>crucian carp. In particular, high I_{Na} density may be needed in burbot and rainbow trout at low temperatures to support cardiac function at relatively high heart rates (at 4°C 19 and 30 beats/min for burbot and rainbow trout,
respectively). Such demands are much smaller in crucian carp, in which the heart beats only about 10 times per min at 4°C (Chapter 8).

In both mammals and fish, the contractile properties and AP characteristics of the atrial and ventricular muscle differ in that the shortening velocity of contraction is higher and the duration of AP less in the atrium than in the ventricle (Hume & Uehara 1985). It might therefore be hypothesized that the properties of the I_{Na} would also differ between atrial and ventricular myocytes. This hypothesis was tested in the rainbow trout heart. The maximum rate of AP upstroke (V_{max}) was indeed significantly higher in the atrial than in the ventricular muscle, suggesting that I_{Na} density might be greater in the atrial than in the ventricular myocytes. Surprisingly, no such difference was found in the density of I_{Na}. However, slight differences were evident in the voltage-dependence of steady-state activation, steady-state inactivation and inactivation kinetics between the atrial and the ventricle in that the values of atrial myocytes shifted towards more negative voltages in comparison to those of ventricular myocytes (Chapter 6). These findings agree well with similar experiments in the zebrafish heart (Warren et al. 2001) and values obtained from the rabbit heart (Li et al. 2002).

Tetrodotoxin (TTX) is a specific blocker of the voltage-gated Na^{+} channels and on the basis of TTX sensitivity Na^{+} channels can be categorized as either TTX-sensitive or TTX-resistant. The TTX binding affinity is mainly determined by the amino acid in position 401 of domain I, where an aromatic residue (fenylalanine or tyrosine) confers high TTX binding sensitivity and a nonaromatic serine or cysteine a gives a low TTX binding sensitivity (Satin et al. 1992, Plummer & Meisler 1999). The mammalian cardiac I_{Na} is well-known for its low sensitivity to TTX, which is due to the major cardiac Na^{+} channel isoform, Na_{v}1.5, having a non-aromatic cysteine in
the critical position 401 (Gellens et al. 1992, Heinemann et al. 1992). On the basis of
the generally accepted paradigm about the TTX resistance of the cardiac I_{Na} in
mammals, this concept seems to be expected also to apply the fish heart, but without
adequate evidence (Venkatesh et al. 2005, Novak et al. 2006 a, b). In the some
previous studies transcripts of the Na_{v}1.4 and Na_{v}1.5 isoforms have been found in the
cardiac muscle of Takifugu rupripes (fugu) and zebrafish, but little attention has been
paid to on their relative amounts and the TTX sensitivity of the I_{Na} (Venkatesh et al.
2005, Novak et al. 2006 a, Soong & Venkatesh 2006). The TTX sensitivity of the
fish cardiac I_{Na} and Na^{+} channel composition of the fish heart are thus still unresolved.

Interestingly, the present findings indicate that the I_{Na} of the rainbow trout heart
is about 1000 times as sensitive (K_{d} = 1.8-2 nM) to TTX as the mammalian I_{Na} (K_{d} =
1-10 µM) (Chapter 7), which raised a question of its molecular basis (the main
isoform of the mammalian heart, Na_{v}1.5, is TTX-resistant). Three different isoforms
of the Na^{+} channels (omNa_{v}1.5, omNa_{v}1.4 and omNa_{v}1.6) were found in the trout
heart and surprisingly, the ortholog of the mammalian skeletal muscle isoform,
omNa_{v}1.4, was clearly the main isoform (over 84 % of all Na^{+} channel transcripts) in
the trout heart. This isoform is TTX-sensitive in mammals, and therefore consistent
with high TTX sensitivity of the trout cardiac I_{Na}. Quite surprisingly, it was also found
that the omNa_{v}1.5 (forming 16% of Na^{+} channel transcripts), which is orthologous to
the mammalian cardiac isoform, has the sequence structure of a TTX-sensitive Na^{+}
channel: an aromatic phenylalanine instead of a non-aromatic cysteine or serine in the
TTX binding site of Na^{+} channel (Chapter 7). Thus, in the rainbow trout heart both
isoforms, omNa_{v}1.5, omNa_{v}1.4, are TTX-sensitive. This seems to apply to other
teleost fish species as well (unpublished results).
The present findings show that the composition of the cardiac Na\(^+\) channels has radically diverged in evolution so that Na\(_v\)1.4 has taken on a dominating role in fish hearts in contrast to the Na\(_v\)1.5 of mammalian hearts (Chapter 7). What physiological advantages could the dominance of Na\(_v\)1.4 provide for the trout heart? Previous studies have shown that Na\(_v\)1.4 has faster activation and inactivation kinetics than Na\(_v\)1.5 (Zimmer et al. 2002). The fast kinetics of the Na\(_v\)1.4 might therefore have provided a selective advantage in ectothermic animals, which have relatively low body temperature, i.e. the Na\(_v\)1.4 could better support the cardiac function in the cold than the kinetically slower Na\(_v\)1.5. In addition, it is known that the critical amino acid residue for TTX binding (position 401) also contributes to the pH dependence of Na\(_v\)1.4 and Na\(_v\)1.5 generated currents (Khan et al. 2006). Specifically, cysteine in position 401 of mammalian Na\(_v\)1.5 makes this isoform more sensitive to protons than channels having tyrosine, phenylalanine or serine in this position. Because fish hearts are mainly supplied by venous blood, which can have a relatively low pH due to the lactic acid released by the muscle tissue, the less pH-sensitive Na\(_v\)1.4 might provide more stable Na\(^+\) currents than the pH-sensitive Na\(_v\)1.5 isoform.

9.3 Potassium currents and action potential duration

Under physiological conditions potassium channels mediate K\(^+\) efflux from the cardiac myocyte and thereby tend to bring the membrane potential to the K\(^+\) equilibrium potential of -80 -90 mV. In other words, K\(^+\) currents are repolarizing and regulate the duration of cardiac AP which is important for cardiac excitability and contractility (Giles & Imaizumi 1988, Sanguinetti & Jurkiewicz 1990, Barry & Nerbonne 1996). Action potential duration (APD) modulates the electrical and mechanical refractory periods of the atrial and ventricular muscle, i.e. the ability of
the heart to generate APs and to produce force at short diastolic intervals. APD also affects the duration of cardiac contraction (Opie 1998). Similar to mammalian hearts, in each species of fish examined, atrial AP was much shorter than ventricular AP, which probably contributes to the shorter duration of atrial contraction in comparison to ventricular twitch: AP characteristics are tuned to the contractile properties in each cardiac compartment (Chapter 8).

As heart rate increases, the total time available for each cardiac cycle decreases, i.e. the diastolic and systolic phases of the cycle must take place faster. However, proper cardiac function requires a definite time for diastolic filling which should be approximately 2/3 of the cardiac cycle (Opie 1998). Therefore, at high heart rates, the duration of AP must shorten in order to prevent excessive shortening of the diastolic period. In many teleost species there is a compensatory increase in the heart rate following cold-acclimation, and the inevitable shortening of the cardiac cycle is expected to require compensatory shortening of APD (Aho & Vornanen 2001). In agreement with this hypothesis, shortening of APD in both cardiac chambers was noticed in all six species of fish (Table 2, Chapter 8). Taken together, the present findings show that, regardless of the different thermal tolerances, lifestyles and phylogenies of the fish, the electrical excitability of the fish heart is increased following cold-acclimation, taking the form of shortened durations of atrial and ventricular APs (Chapter 8).

The AP plateau results from zero net current flow through the sarcolemma when inward (mainly Na\(^+\) and Ca\(^{2+}\)) and outward (mainly K\(^+\)) currents balance each other (Hille 2001). As shown above, in cold-acclimation cardiac I\(_{Na}\) was increased in rainbow trout and burbot and depressed in crucian carp (Table 1, Chapter 5). Since Na\(^+\) channels inactivate very rapidly, the contribution of I\(_{Na}\) to APD is probably
minor. On the other hand, temperature acclimation has little effect on $I_{\text{Ca,L}}$ of rainbow trout and crucian carp ventricular myocytes (Vornanen 1998). It should be noted, however, that acclimatization to winter has been shown to depress the $I_{\text{Ca,L}}$ in the heart of crucian carp. This study does not take into account the effects of thermal acclimation on inward currents which could also contribute to APD in addition to the $K^+$ currents that were objects of this study. In particular, this applies to the pike heart, where no changes in $K^+$ currents were noticed (Chapter 8).

Studies on mammalian cardiac myocytes indicate that $K^+$ currents are important regulators of APD and flexible entities in many pathophysiological situations (Nerbonne 2000). Several $K^+$ channels are known to exist in the heart (see chapter 1) including the delayed rectifier $K^+$ currents, $I_{Ks}$ and $I_{Kr}$, and the inward rectifier $K^+$ current, $I_{K1}$. Previous studies suggest that $I_{Kr}$ and $I_{K1}$ are the major $K^+$ currents of fish cardiac myocytes (Vornanen et al. 2002 a), and therefore they were also chosen as the targets of this study. $I_{Kr}$ activates earlier in the plateau phase than $I_{K1}$ and is a stronger repolarizing current than $I_{K1}$, which is mainly responsible for the rapid repolarization of AP phase 3 (Zeng et al. 1995; Sakmann & Trube 1984).

The $I_{Kr}$ of fish cardiac myocytes seems to be mainly responsible for shortening of APD in the cold conditions, since this current increased strongly in all fish species except the pike (Table 2, Chapter 8). The outward charge transfer by $I_{K1}$ was not changed by temperature acclimation in the pike heart and the current was depressed by chronic cold in the trout heart. This suggests that unlike the other teleost species the *Salmoniformes* fish are unable to upregulate the cardiac $I_{K1}$ in the cold. An explanation for the absence of positive thermal compensation in the density of $I_{K1}$ in *Salmoniformes* fish may lie in the molecular composition of the Kir2 channels. Three different isoforms of the Kir2 channels have been found in the fish heart, Kir2.1,
Kir2.2 and Kir2.5, which are variably present in different fish species and differently affected by temperature acclimation. It is evident that Kir2.2 channels are upregulated at high temperatures, while expression of the Kir2.5 is enhanced in cold conditions. Kir2.1 seems to be only weakly temperature-responsive. Crucian carp express Kir2.2 and Kir2.5, while rainbow trout express Kir2.1 and Kir2.2, but not Kir2.5 in the heart. Thus the absence of positive thermal compensation of the $I_{K1}$ in the rainbow trout heart may due to the absence of the cold isoform, Kir2.5, in this species (Hassinen et al. 2007, Hassinen et al. 2008 a). Examination of Kir2 gene expression in other teleost species would provide useful information on the molecular basis of temperature responses of cardiac $I_{K1}$, its constraints and phylogenetic origin.

9.4 Conclusions

Thermal acclimation modifies the cardiac pacemaker mechanism of the fish heart by increasing the intrinsic AP discharge rate of the primary pacemaker cells in cold conditions, which indicates that part of the temperature-induced response in heart rate is independent of autonomic nervous control and intrinsic to the pacemaker process proper. This is associated with shorter duration of the pacemaker AP and is likely to involve increased density of $I_{Kr}$, which allows a higher rate of diastolic depolarization.

Thermal acclimation induced compensatory and non-compensatory changes in the properties of fish cardiac $I_{Na}$. In cold-active species, rainbow trout and burbot, the density of $I_{Na}$ increased and in cold-dormant crucian carp $I_{Na}$ decreased in the cold. These responses suggest that the rate of impulse conduction is changed differently, depending on lifestyle and environment conditions in the habitat of the fish. Furthermore, it seems that the properties of $I_{Na}$ support higher excitability of the atrial muscle in comparison to the ventricular myocardium. $Na^+$ channel composition and
TTX sensitivity of the fish heart are notably different from those of the mammalian heart, which may be associated with the different functional demands of $I_{Na}$ in ectotherms and endotherms, e.g. as regards temperature and pH.

Positive thermal compensation is evident in electrical activity (APD) of the heart in all fish species with the exception of the perch atrium. The shortening of APD is due to cold-induced increase in the density of $I_{Kr}$ and/or $I_{K1}$ with the exception of the pike heart, where AP shortening may be mediated by inward currents. In contrast to other fish species, temperature compensation of $I_{K1}$ was absent or inverse in \textit{Salmoniformes} fish, suggesting phylogenetic differences in the underlying molecular mechanisms. $I_{Kr}$ seems to be temperature-responsive in most fish species, the pike being an exception among the studied species.

The results of the present study indicate that the ion channels of the fish heart are plastic entities which respond to chronic temperature changes and enable different cardiac electrical phenotypes depending on the environmental conditions and lifestyles of the fish.
References


