

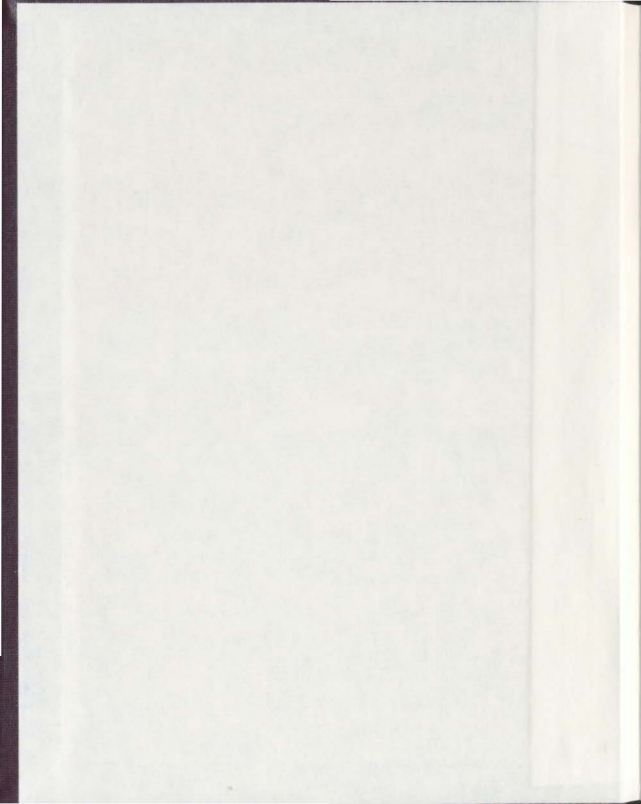
ATP-SENSITIVE POTASSIUM CHANNELS (K_{ATP})
IN FISH CARDIAC MUSCLE DURING
ANOXIA AND RECOVERY

CENTRE FOR NEWFOUNDLAND STUDIES

**TOTAL OF 10 PAGES ONLY
MAY BE XEROXED**

(Without Author's Permission)

TYSON MacCORMACK



INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI[®]



National Library
of Canada

Acquisitions and
Bibliographic Services

365 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale
du Canada

Acquisitions et
services bibliographiques

365, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file name reference

Our file name reference

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-73610-5

Canada

**ATP-sensitive Potassium Channels (K_{ATP}) in Fish Cardiac Muscle
During Anoxia and Recovery**

By

Tyson MacCormack

**A thesis submitted to the School of Graduate Studies
in partial fulfilment of the requirements
for the degree of Master of Science**

**Biology Department,
Ocean Sciences Centre
Memorial University of Newfoundland**

October 15, 2001

St. John's Newfoundland, Canada

Abstract

Cardiac muscle from anoxia tolerant and intolerant fishes was used to investigate the importance of ATP-sensitive potassium channels (K_{ATP}) in the control of anaerobic cardiac function. K_{ATP} channels contribute to anaerobic cardioprotection in mammals yet little is known of their action in more hypoxia tolerant animals. Isometrically contracting ventricular muscle preparations were used to study sarcolemmal and mitochondrial K_{ATP} channel activity in the myocardium of three species of teleost fishes (*Lipossarcus pardalis*, *Limanda ferruginea*, and *Gadus morhua*) with varying cardiac hypoxia tolerances. Channel activity was assessed pharmacologically using the non-specific K_{ATP} channel blocker glibenclamide, the mitochondrial specific blocker 5-hydroxydecanoic acid and the agonist, diazoxide to determine the involvement of K_{ATP} channels in anaerobic cardiac performance. Results suggest that cardiac K_{ATP} channels from hypoxia tolerant fishes are tonically active, resulting in a constant cardioprotection similar to the “preconditioned” state which can be induced by various methods in mammalian hearts. These studies clearly show that K_{ATP} channels are important for anaerobic cardiac function in anoxia tolerant fish.

Acknowledgements

I would like to thank my supervisor, Dr. William Driedziec for inviting me into his lab and providing me with guidance both academically and personally as well as with financial support. Thanks to Adalberto and Vera Val who kindly allowed us the use of their lab and equipment and provided us with animals for our work in Brazil. I also thank Jason Treberg for his technical assistance and valuable input on the work. I am also indebted to Connie Short and Nicola Legate for their excellent technical assistance, as well as Trevor Avery for statistical advice. I also thank my parents, Jim and Emma Lee MacCormack as well as Johanne Lewis for their support during the study. I also thank Tony Manning for his help with yellowtail flounder, and Angela Tate, Mike Riehl, Marcianna Ptak, Robert Evans, Nichole Richoux, Robert Stead, and Patty Saunders. Acknowledgement is also extended to my committee members Drs. David Heeley and Derek Burton for comments on the manuscript, as well as to Drs. Holly Shiels, Kurt Gamperl, Garrett Gross, Matti Vormanen, and Hans Gesser for helpful comments on the study. This work was partially funded by an NSERC PGS A fellowship and a Memorial University School of Graduate Studies fellowship.

Table of Contents

Abstract.....	ii
Acknowledgements.....	iii
Table of Contents.....	iv
List of Tables.....	vi
List of Figures.....	vii
List of Abbreviations.....	viii
1 Ion Flux and Anaerobic Contractility in the Fish Heart.....	1
1.1 Introduction.....	1
1.2 Cardiac Anatomy and Anoxic Exposure in Fish.....	1
1.3 Ca^{2+} Regulation.....	4
1.3.1 Ca^{2+} Influx.....	6
1.3.2 Cardiac Relaxation and Ca^{2+} Efflux.....	8
1.4 Na^+ and K^+ regulation.....	11
1.5 Ion Flux and Cardiac Performance During Anoxia.....	14
1.6 References.....	19
2 Mitochondrial ATP-sensitive K^+ Channels Contribute to Heart Anoxic Resistance in the Amazonian Armoured Catfish Acari-bodo (<i>Lipossarcus pardalis</i>).	27
2.1 Introduction.....	27
2.2 Materials and Methods.....	29
2.2.1 Animals.....	29
2.2.2 Ventricular strip preparations.....	30
2.2.3 Drugs.....	31

2.2.4	Statistics.....	32
2.3	Results.....	32
2.3.1	Direct impact of anoxia and reoxygenation.....	32
2.3.2	Ryanodine treatment.....	33
2.3.3	5HD treatment.....	34
Discussion	37
2.3.4	Down regulation is probably due to decreased Ca^{2+} activity.....	39
2.3.5	Mito K_{ATP} channels under anoxia.....	40
2.3.6	Blocking Mito K_{ATP} channels results in hypercontracture.....	41
2.3.7	Conclusions.....	42
2.4	References.....	44
3	Evidence for Myocardial ATP-sensitive K^+ Channels in Yellowtail Flounder but not Atlantic Cod.....	50
3.1	Introduction.....	50
3.2	Materials and Methods.....	52
3.2.1	Tissue preparation.....	52
3.2.2	Drugs.....	55
3.2.3	Data analysis and statistics.....	56
3.3	Results.....	57
3.3.1	Anoxia / reoxygenation.....	57
3.3.2	K_{ATP} contribution.....	59
3.4	Discussion.....	63
3.5	References.....	70
4	Summary.....	74

List of Tables

Table 1.1 Comparison of anoxia tolerance using time to 50 % force loss under simulated anoxia in ventricular strip preparations of various species of fish.

List of Figures

Figure 1.1 Cross sectional view of a pelagic elasmobranch (*Alpioides vulpinus*) ventricle showing the lumen as well as the spongy and compact myocardium.

Figure 1.2 Schematic of Ca^{2+} regulating mechanisms in cardiac muscle.

Figure 1.3 Schematic drawing of major ion regulating mechanisms in the fish cardiomyocyte.

Figure 2.1 Twitch force and resting tension (expressed as % of initial force development) for ventricular strip preparations from acari-bodo heart exposed to oxygenated conditions and anoxia followed by reoxygenation.

Figure 2.2 Twitch force and resting tension for ventricular strips from acari-bodo exposed to anoxia and reoxygenation. *A*: untreated *B*: ryanodine treated *C*: 5HD treated.

Figure 2.3 Twitch force and resting tension for acari-bodo ventricle strip preparations treated with 5HD throughout anoxia and reoxygenation and strips treated with 5HD 5 min before reoxygenation.

Figure 3.1 Twitch force and resting tension for yellowtail flounder and Atlantic cod ventricular preparations exposed to oxygenated conditions and to 35 min of anoxia followed by reoxygenation.

Figure 3.2 Twitch force and resting tension for yellowtail flounder ventricular preparations subjected to anoxia and reoxygenation and treated with agents to affect K_{ATP} channel activity. *A*: DMSO treated *B*: glibenclamide treated *C*: 5HD treated *D*: diazoxide treated.

Figure 3.3 Twitch force and resting tension for Atlantic cod ventricular preparations subjected to anoxia and reoxygenation and treated with agents to affect K_{ATP} channel activity. *A*: DMSO treated *B*: glibenclamide treated *C*: 5HD treated *D*: diazoxide treated.

List of Abbreviations

- AP: Transmembrane action potential
- APD: Transmembrane action potential duration
- ADP: Adenosine diphosphate
- ATP: Adenosine 5'-triphosphate
- Ca^{2+}_{ex} : Extracellular calcium
- Ca^{2+}_i : Intracellular calcium
- CN: Cyanide
- DMSO: Dimethyl-sulfoxide
- E-C coupling: Excitation-contraction coupling
- 5HD: 5-Hydroxydecanoic acid
- K_{ATP} : ATP-sensitive potassium channel
- K^{+}_{ex} : Extracellular potassium
- K^{+}_i : Intracellular potassium
- Mito K_{ATP} : Mitochondrial ATP-sensitive potassium channel
- Na^{+}_{ex} : Extracellular sodium
- Na^{+}_i : Intracellular sodium
- P_i : Inorganic phosphate
- ROS: Reactive oxygen species
- Sarc K_{ATP} : Sarcolemmal ATP-sensitive potassium channel
- SE: Standard error
- SR: Sarcoplasmic Reticulum

1 Ion Flux and Anaerobic Contractility in the Fish Heart

1.1 Introduction

The vertebrate heart has been extensively studied and much of its complex physiology has been uncovered. Fish hearts have been the focus of substantial research, in part due to their relative anatomical simplicity when compared to mammals, and also because fish show an exceptional ability to maintain cardiac function during temperature extremes, low oxygen levels and over a wide range of extracellular ion concentrations. This review will outline the effects of extreme oxygen deprivation (anoxia) on ion flux and contractility in the cardiac muscle of teleost fish. Where relevant, comparisons are made to information available for elasmobranch fishes, reptiles and mammalian species.

1.2 Cardiac Anatomy and Anoxic Exposure in Fish

The fish heart is a simple linear pump system composed of four chambers aligned in series and situated between the systemic and gill circulations of the animal. The sinus venosus, which collects venous blood from the systemic circulation, is a thin walled vessel made up mostly of connective tissue with a small complement of cardiac muscle. The atrium has a higher proportion of cardiac muscle than the sinus venosus and a luminal capacity almost equivalent to that of the ventricle. Atrial contraction moves blood into the lumen of the highly muscular ventricle which then contracts to pump blood

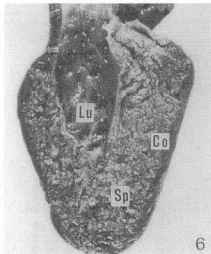
into the bulbus arteriosus. The bulbus arteriosus is an elastic chamber made up of connective tissue which serves as a compliance vessel in teleost fish (Olson 1998).

Fish hearts show considerable interspecific differences in myocardial anatomy. Compared to mammals however, they have a much higher proportion of spongiosum (Fig 1.1) or trabeculated myocardium in the ventricle. Compact myocardium (compactum), which is untrabeculated and more dense than spongiosum, makes up the outermost layer of the heart in those fish possessing it, and the fraction of compactum correlates well with the cardiac demands of teleost fishes. For instance, active pelagic species such as tuna have a high proportion of compactum (>30 %) while sedentary demersal species such as cod have no compactum (Satchell 1991). It is important to note that the proportion of spongiosum to compactum is determined developmentally in fish and is not influenced by exercise (Olson 1998). Interestingly, all elasmobranchs examined to date display some development of compactum regardless of perceived cardiac demand (i.e. activity levels) (Tota 1999).

Important anatomical differences between the hearts of fish and those of birds and mammals exist in the degree of coronary artery development. Most species of fish completely lack a system of coronary arteries and the heart must receive oxygen and nutrients from the venous blood it pumps. Only in highly active teleosts showing a significant fraction of compactum do coronary arteries supply the heart with oxygenated blood, and even then capillaries rarely extend beyond the compact layer of muscle (Olson 1998). All elasmobranchs show some degree of coronary artery development regardless of activity level. Spongiosum is highly trabeculated to increase the surface area of the lumen and enhance the diffusion of gasses and solutes into and out of the muscle.

Trabeculae also form “mini hearts”, partially sub-dividing the ventricle into smaller cavities or chambers in the ventricular wall. Cross-luminal bridges may also be formed which may increase the efficiency of contraction in fish hearts (Olson 1998). The degree of coronary artery development in fish may also be influenced by the size of the heart (Olson 1998). Oxygen diffusion would be restricted to only the luminal surface layers in very large hearts lacking coronary arteries. Though extensive comparisons have been made concerning cardiac anatomy in smaller fish, information is sparse on arterial development in very large fish.

Figure 1.1 Cross sectional view of a pelagic elasmobranch (*Alpoias vulpinus*) ventricle showing the lumen (Lu) as well as the spongy (SP) and compact myocardium (Co). Taken from Tota 1989.



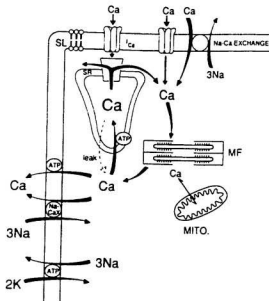
The deficiency of adequate oxygen delivery mechanisms renders the majority of fish highly prone to cardiac anoxia. Fish inhabiting cool waters with high dissolved oxygen levels may encounter cardiac hypoxia during vigorous exercise when the metabolism of skeletal muscle depletes blood oxygen content. Ventral aortic dissolved oxygen levels have been shown to fall from 4.43 to 2.13 kPa during maximal activity in the rainbow trout (Driedzic and Gesser 1994). Environmental hypoxia is commonly incurred by species inhabiting warm waters or areas of high organic activity such as estuaries or eutrophicated lakes. Many bottom dwelling species also bury themselves in sediment for camouflage, predation, or thermal regulation. Winter flounder (*Pseudopleuronectes americanus*) are routinely found buried in 12-15 cm of sediment during winter months when epibenthic temperatures are slightly warmer than water temperatures (Fletcher 1977). Even schooling fish which do not generally inhabit oxygen deprived waters may be exposed to environmental hypoxia. Theoretically, the respiration of a substantial number of fish could also drastically reduce dissolved oxygen levels in the centre of a large school. This is especially applicable to marine fish such as Atlantic cod, which can aggregate into schools that may be many kilometres in diameter.

1.3 Ca²⁺ Regulation

The Ca²⁺ necessary for contraction in fish cardiomyocytes is derived largely from extracellular stores and must cycle into and out of the cell during each contractile cycle (Fig 1.2) (Tibbits et al. 1991). The diffusion distance between Ca²⁺ and the contractile elements of the cardiomyocyte would therefore be expected to be larger in fish than in

mammals. This is at least partially overcome in fish by significantly smaller cardiomyocyte diameters. Mammalian cardiomyocytes range in size from 15-20 μm while those of teleosts are only 2-10 μm in diameter (Tibbits et al. 1991). This increases the surface area to volume ratio of the cell which reduces diffusion distances across the sarcolemmal membrane.

Figure 1.2 Schematic of Ca^{2+} regulating mechanisms in cardiac muscle. ATP, adenosine triphosphate; MITO, mitochondrion; MF, myofilaments; Na-CaX, $\text{Na}^+/\text{Ca}^{2+}$ exchanger; SL, sarcolemma; SR, sarcoplasmic reticulum; I_{Ca} , L-type Ca^{2+} channel. Taken from Tibbits et al. 1991.



The transmembrane action potential duration (APD) of fish cardiomyocytes is in the upper range of that seen in mammals (Moller-Nielsen and Gesser 1992; Vornanen

1996) and this may also help to compensate for the poor development of sarcoplasmic reticulum (SR) in most fish. Prolonged action potentials should allow more time for Ca^{2+} to reach and bind with the contractile proteins of the cell, as well as facilitating increased Ca^{2+} influx across the sarcolemma (Driedzic and Gesser 1994). In addition, the contractile elements of trout cardiomyocytes are inherently more sensitive to Ca^{2+} than those of mammals and amphibians (Churcott et al. 1994), meaning less Ca^{2+} is required to initiate a contraction in fish than in other vertebrates. Increased Ca^{2+} sensitivity in fish may also facilitate the maintenance of cardiac activity at low temperatures.

1.3.1 Ca^{2+} Influx

Ca^{2+} enters the fish cardiomyocyte through several channels on the sarcolemma (Fig. 1.2). The L-type Ca^{2+} channel is generally thought to contribute the majority of Ca^{2+} required for contraction (activator Ca^{2+}). L-type Ca^{2+} channel activity is temperature sensitive in fish (Shiels et al. 2000) and can be affected by circulating hormones, catecholamines, ATP supply, voltage and intracellular free Ca^{2+} (Vornanen 1998). Vornanen (1998; 1999) has shown that approximately 2/3 of activator Ca^{2+} enters through the L-type channel in the crucian carp ventricle with the remaining Ca^{2+} contributed through $\text{Na}^+/\text{Ca}^{2+}$ exchange. In its forward orientation the $\text{Na}^+/\text{Ca}^{2+}$ exchanger takes in three Na^+ ions and extrudes one Ca^{2+} ion from the myocyte (Reeves et al. 1994). This exchanger is voltage gated and during the majority of the cardiac cycle it is pumping Ca^{2+} out of the cell. However, during the initial portion of the action potential the direction of $\text{Na}^+/\text{Ca}^{2+}$ exchange can be reversed, allowing Ca^{2+} to flow into the cell (Vornanen 1999). Ca^{2+} influx through the exchanger is driven by transient elevations in Na^+ near the

sarcolemmal membrane (Reeves et al. 1994), resulting presumably from influx through voltage gated Na^+ channels. In agreement with this assumption, small changes in $[\text{Na}^+]$ have been shown to cause significant alterations in Ca^{2+} flux in the mammalian cardiomyocyte (Reeves et al. 1994). Exchanger activity and molecular makeup of the protein have been characterised by Tibbits et al. (1990) in isolated sarcolemmal membranes of rainbow trout.

Physiological data on $\text{Na}^+/\text{Ca}^{2+}$ exchange at the tissue level is not available for fish; however, Gesser and Mangor-Jensen (1984) have shown that lowering Na^+_{ex} with no concomitant change in osmolarity results in an increase in $^{45}\text{Ca}^{2+}$ uptake and potentiates twitch force in the flounder *Platichthys (Pleuronectes) flesus*. This characteristic may be of physiological importance during hypoxia in the intact cardiomyocyte since increases in intracellular Na^+ (Na^+_i) are well documented during periods of metabolic stress in mammals (Carmeliet 1999). Plasma Na^+ concentrations have been shown to fluctuate seasonally by as much as 40 mM in cold water species such as Atlantic cod (Fletcher et al. 1982) and winter flounder (Fletcher 1977) to enhance freezing point depression for survival in sub-zero waters. Exhaustive exercise has been shown to cause small (1-3 mM) increases in Na^+_i and Ca^{2+}_i in rainbow trout (*Oncorhynchus mykiss*) white muscle as well (Wang et al. 1994); however, it is not known whether parallel changes occur in cardiac muscle. There is also evidence that sub-sarcolemmal Na^+ concentrations may be higher than those in the rest of the cytoplasm (Carmeliet 1992) but the physiological importance of this is difficult to assess experimentally.

1.3.2 Cardiac Relaxation and Ca^{2+} Efflux

In order for the fish cardiomyocyte to relax, $[\text{Ca}^{2+}]_i$ must be lowered to approximately 100 nM to remove Ca^{2+} from troponin C and reset the contractile mechanisms of the cell (Tibbits et al. 1991). In fish, $[\text{Ca}^{2+}]_i$ is lowered mainly through passive and active efflux across the sarcolemma. The contribution of the SR to lowering $[\text{Ca}^{2+}]_i$ varies widely amongst species and is influenced by the acclimation temperature of the fish. The Ca^{2+} -ATPase pump on the SR is inefficient in sequestering Ca^{2+} at low temperatures. Aho and Vornanen (1997) have shown that the SR is able to retain Ca^{2+} at low temperatures in the heart of rainbow trout, negating previous notions that SR Ca^{2+} release channels may remain open at low temperatures (Aho and Vornanen 1998; Keen et al. 1992). SR Ca^{2+} uptake is also strongly influenced by interspecies differences in whole animal activity levels. The SR of extremely active fish such as yellowfin tuna (*Thunnus albacores*) plays a major role in cardiac relaxation (Shiels et al. 1999), while in a moderately active teleost, the rainbow trout, SR function is not as important to contractile performance (Aho and Vornanen 1999). SR contribution was found to be negligible in flounder (*Pleuronectes flesus*) (El-Sayed and Gesser 1989) and crucian carp (*Carassius auratus*) (Aho and Vornanen 1998) but unimpaired SR function was required for relaxation in the American eel (*Anguilla rostrata*) heart at high $[\text{Ca}^{2+}]_o$ (Bailey et al. 2000). Most data suggest a long term Ca^{2+} buffering role for the SR in fish cardiomyocytes rather than a direct participation in beat to beat Ca^{2+} cycling.

Mitochondria may also have the potential to be involved in beat to beat Ca^{2+} regulation in fish myocardium. Mitochondrial Ca^{2+} contribution is often dismissed in routine cardiac ion cycling; however, recent mammalian studies have shown that

mitochondria are intimately involved in long term Ca^{2+}_i homeostasis and that Ca^{2+}_i cycling across cardiac mitochondrial membranes may be rapid enough to participate on a beat to beat basis (reviewed by Bernardi 1999). Evidence is sparse concerning mitochondrial Ca^{2+}_i cycling in ectothermic vertebrates, although mitochondrial Ca^{2+} contribution has been implicated in contractile force recovery of ventricular muscle from the snake *Vipera berus* during acidosis (Gesser and Poupa 1979). A similar recovery is seen under acidosis in ventricular muscle from *Platichthys flesus* (Gesser and Poupa 1979) but not in muscle from other teleosts such as cod (*Gadus morhua*) (Gesser and Poupa 1979) and carp (*Cyprinus carpio*) (Gesser and Poupa 1978). Without further investigation it is difficult to quantify the significance of mitochondrial Ca^{2+}_i cycling in fish cardiomyocytes, although it generally does not seem critical to the beat to beat functioning of cardiac muscle in the fishes examined to date.

In most fish the majority of Ca^{2+} in the cardiomyocyte must be extruded passively by $\text{Na}^+/\text{Ca}^{2+}$ exchange or pumped out actively by the sarcolemmal Ca^{2+} -ATPase. Cardiac Ca^{2+} pump activity has not been investigated at all in fish and only minimally in other ectothermic vertebrates. Brommundt and Kavalier (1985) suggested that ATP-dependent Ca^{2+} efflux was more important than Na^+ -dependent Ca^{2+} efflux in the bullfrog heart. This has been questioned however, since the author's interpretations were based on an extremely slow Ca^{2+} efflux which would be irrelevant at physiological frequencies of Ca^{2+} cycling during normal contractions (Tibbits et al. 1991). Despite the paucity of direct information, it seems generally accepted that the sarcolemmal Ca^{2+} -ATPase pump in fish cardiac muscle is not critical for the majority of Ca^{2+} efflux and that it serves only to maintain low $[\text{Ca}^{2+}_i]$ during diastole (Tibbits et al. 1991).

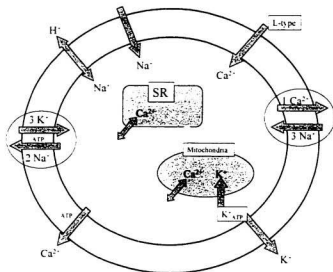
The hypothesis that Ca^{2+} -ATPase contribution is insignificant in fish cardiac relaxation is supported by a high density of $\text{Na}^+/\text{Ca}^{2+}$ exchange current in teleost sarcolemmal membranes. Vornanen (1999) noted that in crucian carp, exchange current densities were higher than those observed in adult mammals but lower than those seen in embryonic mammals. This suggests a correlation between the density of $\text{Na}^+/\text{Ca}^{2+}$ exchange current and the development of SR in the hearts of vertebrates. In heart muscle with highly developed SR, such as that of adult mammals, $\text{Na}^+/\text{Ca}^{2+}$ exchange does not play a dominant role in relaxation and high current densities are not necessary. In hearts with poorly developed SR, such as those of embryonic mammals and many ectothermic vertebrates, the SR does not dominate contractile Ca^{2+} flux and $\text{Na}^+/\text{Ca}^{2+}$ exchange becomes more important.

Kinetic data from mammals suggests that at physiological levels of Na^+ and Ca^{2+} , the exchanger is only working at a portion of its maximal capacity. This may not apply in fish however, since in mammals the exchanger competes with the Ca^{2+} -ATPase pump on the SR for Ca^{2+} which would not be the case in cardiomyocytes lacking a well developed SR. Intracellular compartmentalisation of ions may also significantly affect Ca^{2+} efflux through $\text{Na}^+/\text{Ca}^{2+}$ exchange, but this phenomenon can not be readily studied using current technology (Reeves et al. 1994). Tibbits et al. (1992) have shown that $\text{Na}^+/\text{Ca}^{2+}$ exchange in rainbow trout sarcolemmal membranes is relatively insensitive to temperature changes ($Q_{10} \sim 1.2$) when compared with that of mammals ($Q_{10} > 2$). This is consistent with the adequate cardiac performance observed in fish at low temperatures which would be cardioplegic to homeothermic mammals.

1.4 Na⁺ and K⁺ regulation

Figure 1.3 illustrates the basic elements involved in ion regulation in the fish cardiomyocyte. Na⁺ and K⁺ ions are intimately associated in cardiomyocytes of all vertebrates and the maintenance of their gradients is crucial to the function of excitable cells in general. K⁺ is the major cytoplasmic and mitochondrial cation and its concentration is elevated in the cytoplasm relative to the extracellular fluid. Na⁺ is the dominant cation in the extracellular fluid and cytoplasmic concentrations are sustained at low levels. The Na⁺/K⁺-ATPase is the central mechanism responsible for the maintenance of Na⁺ and K⁺ gradients, actively cotransporting two Na⁺ ions out of the cell in exchange for the influx of three K⁺ ions. The pump is sensitive to ATP concentrations, or more specifically to the free energy derived through ATP hydrolysis (phosphate potential). This is a significant factor to account for when studying hypoxic ion regulation in fish cardiac muscle since oxygen deprivation is often associated with depolarisation of the cell membrane and increases in intracellular inorganic phosphates (P_i), both of which reduce the phosphate potential (Carmeliet 1999).

Figure 1.3 Schematic drawing of major ion fluxes and regulating mechanisms in cardiomyocytes.



Although the Na⁺/K⁺-ATPase is an active cotransporter, it also possesses some qualities common to ion channels which allows pump rate to be modulated somewhat by changes in [Na⁺] and [K⁺]. [Na⁺]_o generally exerts a more powerful influence on pump activity than [K⁺]_i because [Na⁺]_o is at less than saturating levels and is subject to greater change than [K⁺]_i which is usually near saturation (Carmeliet 1999).

The Na⁺/K⁺-ATPase pump is either directly or indirectly responsible for regulating almost all ionic activity in cardiac muscle. As discussed above, changes in [Na⁺]_o in either the intracellular or extracellular fluid, can cause profound alterations in Ca²⁺ flux via Na⁺/Ca²⁺ exchange, which will effect cardiac performance through changes in contractile strength. K⁺ concentrations control the degree of polarisation across the sarcolemma which is central to the function of the cell since most membrane proteins are

sensitive to the electrical state of their environment. Small changes in the polarisation state of the sarcolemma caused by K^+ flux can therefore control contractile performance via modulation of membrane bound Ca^{2+} transporters. The transmembrane action potential (AP) is also intimately tied to the electrical characteristics of the sarcolemma and it directly controls contractile function in the fish cardiomyocyte. Resting membrane potential, which is set by K^+ gradients, affects the amplitude of the AP, which in turn influences the duration that the Na^+/Ca^{2+} exchange remains in its reverse orientation. For example, Vornanen (1999) found that little Ca^{2+} influx occurred through Na^+/Ca^{2+} exchange at membrane potentials below -20 mV, but that Ca^{2+} influx increased significantly according to membrane potential at more positive voltages in cardiomyocytes from both warm and cold-acclimated crucian carp.

K^+ flux is also regulated by adenosine 5'-triphosphate sensitive potassium channels (K_{ATP}) located on both the sarcolemmal and inner mitochondrial membranes. Sarcolemmal K_{ATP} channels (sarc K_{ATP}) facilitate K^+ efflux passively across the sarcolemmal membrane while mitochondrial K_{ATP} channels (mito K_{ATP}) facilitate K^+ flux into the mitochondria. Both sarcolemmal and mito K_{ATP} channels are controlled by the ratio of ATP to adenosine diphosphate (ADP) as well as a host of other modulators. Channel activity is inhibited by high ratios of ATP/ADP and enhanced when the ratio of ATP/ADP declines. Sarc K_{ATP} channels have been associated with membrane electrical activity, including modulation of APD and loss of K^+ gradients during metabolic inhibition (Gross 1998). Mito K_{ATP} channels influence inner mitochondrial membrane polarisation and control electrical activities as well, but they seem to play a more active role in regulating mitochondrial matrix volume through osmotic mechanisms. Mito K_{ATP}

channels have also been implicated in mitochondrial Ca^{2+} homeostasis through membrane potential effects on Ca^{2+} release channels (Bernardi 1999). K_{ATP} channels have been intimately linked with the beneficial effects of cardiac “preconditioning” in the mammalian heart (Grover and Garlid 2000). Preconditioning is the phenomenon by which one or more short periods of hypoxia protect the heart from a subsequent, prolonged period of hypoxia. The functional aspects of K_{ATP} channels in fish cardiac muscle will be discussed more in later sections and investigated and discussed fully in Chapters 2 and 3.

1.5 Ion Flux and Cardiac Performance During Anoxia

Anoxia affects cardiac muscle most profoundly through creation of an energy debt. The hydrolysis of ATP to ADP provides the free energy necessary to fuel active cellular activities and the majority of ATP production occurs oxidatively. Anoxia directly inhibits oxidative production of ATP and the cell must rely on anaerobic ATP generation to maintain function. Anaerobic ATP production is inherently less efficient than oxidative mechanisms and this eventually leads to a decrease in intracellular [ATP] if metabolic demand remains constant.

The actual free energy derived from the hydrolysis of ATP can also fall during anoxia as explained earlier. Anoxia is routinely associated with an intracellular build up of ADP and P_i , which reduces the phosphate potential and limits energy availability. Phosphate potential has been found to drop from a normoxic value of ~ 61 kJ/mol to less than 50 kJ/mol during hypoxia in rat heart (Carmeliet 1999). The drop in phosphate potential tends to occur more quickly than any significant decrease in [ATP] and it seems

more important than actual [ATP] in determining the initial response of a tissue to anoxia (Hartmund and Gesser 1996). Degradation of contractile performance in anoxic fish cardiac muscle is not accompanied by a gross decrease in intracellular [ATP] (Purup Hansen and Gesser 1987; Nielsen and Gesser 1984) suggesting some type of down-regulation of ATP consumption.

Sarc K_{ATP} channels are particularly sensitive to intracellular [ATP] and activated with decreases in the ratio of ATP/ADP. During hypoxia in mammals, modest decreases in [ATP] have been shown to cause activation of Sarc K_{ATP} channels (Deutsch et al. 1991). Sarc K_{ATP} channels are very abundant in rat heart (Terzic et al. 1995) and the activation of only a small portion of these channels can cause a significant increase in K^+ efflux across the sarcolemma. Though large changes in cytoplasmic ATP concentrations are not common under hypoxia in fish, [ATP] at the sarcolemmal margin may be more variable due to activity of active ion pumps.

Hypoxia also affects K^+ flux through the Na^+/K^+ -ATPase pump. The activity of the pump is sensitive to membrane polarisation, extracellular K^+ and intracellular Na^+ , and the availability of energy derived from ATP hydrolysis. The latter is potentially responsible for initial inhibition of the pump during hypoxia. The pump has a huge capacity and relatively little inhibition is required to bring about significant changes in the concentration of ions in the cytoplasm and extracellular fluid (Carmeliet 1999). The accumulation of K^+_{ex} observed during hypoxia may also be mediated through Na^+/K^+ -ATPase inhibition. A decrease in K^+ influx through the pump along with an increase in K^+ efflux through sarc K_{ATP} channels is likely responsible for the loss of K^+_{i} in mammals

during hypoxia, though it is not clear at this time how each of these processes influences the other (Kabakov 1998; Haruna et al. 1998).

Significant increases in Na^+ have been directly observed in mammalian cardiomyocytes during hypoxia though the cause of this phenomenon is still under debate. Studies have implicated decreased Na^+ efflux through inhibition of the Na^+/K^+ -ATPase pump by mechanisms discussed above (Dizon et al. 1998), while others feel that activation of a Na^+/H^+ exchanger is responsible for the observed changes (Park et al. 1999; Tani and Neely 1989; Ho et al. 2000; reviewed by Murphy et al. 1999). Direct observations on $[\text{Na}^+]_i$ and the Na^+/H^+ exchanger during hypoxia are not available for fish cardiac muscle, though there is no evidence suggesting that the dynamics of Na^+ flux will be different than in mammals. Aho and Vornanen (1997) have reported a decrease in the activity of the cardiac Na^+/K^+ -ATPase during a 100 hour laboratory anoxia in crucian carp. Alterations in Na^+ may lead to significant alterations in contractile performance in fish due to their reliance on trans-sarcolemmal Ca^{2+} flux for contractility and the relative lack of intracellular Ca^{2+} buffers.

When confronted with either environmental or physiological hypoxia, some fish release catecholamines into the circulatory system. High levels of catecholamines can significantly stimulate heart rate and ventilation in fish through influence on nervous control of the heart (Perry and Gilmour 1996). Adrenaline also has a stimulating effect on the function of the Na^+/K^+ -ATPase as well as on Ca^{2+} release from the SR in rainbow trout (Hove-Madsen and Gesser 1989) though it is not clear how this may affect hypoxic cardiomyocyte function in the presence of other changes in the cell. During normoxia the addition of high levels of adrenaline (10 μM) to isolated ventricular muscle strips of

rainbow trout potentiated force production by up to 50 % (Shiels and Farrell 1997; Hove-Madsen and Gesser 1989). At the whole animal level, contractile force potentiation by adrenaline would be beneficial in helping a fish to escape a hypoxic environment, but it may be deleterious to the survival of fish forced to endure extended periods of hypoxia. An increase in heart rate and force production would elevate ATP demand at a time when the conservation of energy could be paramount to the animal's survival.

Table 1.1 Comparison of anoxia tolerance using time to 50 % force loss under simulated anoxia in ventricular strip preparations of various species of fish.

	<i>Species</i>	<i>Beats per min</i>	<i>Time to 50 % force loss (min)</i>	<i>Reference:</i>
1	<i>Squalus acanthias</i>	12	46	Gesser and Poupa (1974)
2	<i>Gadus morhua</i>	12	4	Gesser and Poupa (1974)
3	<i>Sprattus sprattus</i>	12	22	Gesser and Poupa (1974)
4	<i>Scomber scomber</i>	12	30	Gesser and Poupa (1974)
5	<i>Pleuronectes platessa</i>	12	70	Gesser and Poupa (1974)
6	<i>Labrus ossifragus</i>	12	75	Gesser and Poupa (1974)
7	<i>Hippoglossoides platessoides</i>	12	47	Gesser and Poupa (1974)
8	<i>Oncorhynchus mykiss</i>	30	11	Bailey et al. (1999)
9	<i>Perca fluviatilis</i>	30	11	Bailey et al. (1999)
10	<i>Astronotus ocellatus</i>	30	10	Bailey et al. (1999)
11	<i>Colossoma macropomum</i>	30	9	Bailey et al. (1999)
12	<i>Hoplosternum littorale</i>	30	11	Bailey et al. (1999)
13	<i>Lipossarcus pardalis</i>	30	11	Bailey et al. (1999)
14	<i>Anguilla anguilla</i>	12	>60 *	Hartmund and Gesser (1992)
15	<i>Anguilla rostrata</i>	30	11 **	Bailey et al. (1999)
16	<i>Ictalurus punctatus</i>	30	21 **	Bailey et al. (1999)

* Force development remained above 60% of initial for the duration of the experiment.

** These species showed rapid loss of force followed by stabilisation below 50 % initial for the duration of experiment.

Variability in cardiac anoxia tolerance is illustrated in Table 1.1, which gives representative values for time to 50 % force loss under simulated anoxia in isolated, contracting ventricular strips from various species of fish. Twitch force generation under anoxia is considered a good indicator of cardiac anoxia tolerance and it generally responds in one of several characteristic manners in fish. Force generation in anoxia intolerant species such as rainbow trout (*Oncorhynchus mykiss*) decays in a linear and continuous fashion with a concomitant increase in resting tension, presumably due to a failure of the Ca^{2+} extruding mechanisms of the cell (Driedziec and Gesser 1994). Twitch force development under anoxia in highly tolerant species, such as American eel (*Anguilla rostrata*), falls rapidly during the onset of anoxia, but then stabilises at a lower level where it remains for the duration of the insult with little change in resting tension (Bailey et al. 1999). These responses will be studied in the following chapters with respect to the activity of K_{ATP} channels.

In conclusion, fish cardiac muscle exhibits substantial differences from mammalian cardiac muscle with the most notable being the general lack of SR function. The anoxic contractile performance of teleost cardiac muscle is variable amongst species and anoxia tolerance seems to be related to physiological adaptations to both metabolic and ion regulating mechanisms in the cardiomyocyte. Altering the dynamics of anaerobic energy production and consumption in the heart may help to avoid depletion of intracellular ATP and allow for the maintenance of contractile function during anoxia. The adaptation of ion regulating mechanisms may aid in the preservation of E-C coupling in the cardiomyocyte during metabolic inhibition and aids in avoiding permanent cellular injury through the accumulation of ions.

1.6 References

- Aho E. and Vornanen M. Seasonality of ATPase activities in crucian carp (*Carassius auratus* L.) heart. *Fish Physiol Biochem* 16: 355-364, 1997.
- Aho E. and Vornanen M. Ca^{2+} -ATPase activity and Ca^{2+} uptake by sarcoplasmic reticulum in fish heart: effects of thermal acclimation. *J Exp Biol* 201: 525-532, 1998.
- Aho E. and Vornanen M. Contractile properties of atrial and ventricular myocardium of the heart of rainbow trout *Oncorhynchus mykiss*: effects of thermal acclimation. *J Exp Biol* 201: 2663-2677, 1999.
- Bailey JR, Val AL, Almeida-Val VMF, and Driedziec WR. Anoxic cardiac performance in Amazonian and north-temperate-zone teleosts. *Can J Zool* 77: 683-689, 1999.
- Bailey JR, Barter T, and Driedziec WR. Maintenance of resting tension in the American eel (*Anguilla rostrata* L.) heart is dependant upon exogenous fuel and the sarcoplasmic reticulum. *J Exp Zool* 286: 707-717, 2000.
- Bernardi P. Mitochondrial transport of cations: channels, exchangers, and permeability transition. *Physiol Rev* 79: 1127-1155, 1999.

Brommundt G. and Kavalier F. Greater ATP dependence than sodium dependence of radioactive efflux in bullfrog ventricle. *Am J Physiol Cell Physiol* 249: C129-C139, 1985.

Carmeliet E. A fuzzy subsarcolemmal space for intracellular Na^+ in cardiac cells? *Cardiovasc Res* 26: 433-442, 1992.

Carmeliet E. Cardiac ionic currents and acute ischemia: from channels to arrhythmias. *Physiol Rev* 79: 917-1017, 1999.

Churcott CS, Moyes CD, Bressler BH, Baldwin KM, and Tibbits GF. Temperature and pH effects on Ca^{2+} sensitivity of cardiac myofibrils: a comparison of trout with mammals. *Am J Physiol Regulatory Integrative Comp Physiol* 267: R62-R70, 1994.

Deutsch N, Klitzner TS, Lamp ST, and Wiess JN. Activation of cardiac ATP-sensitive K^+ current during hypoxia: correlation with tissue ATP levels. *Am J Physiol Heart Circ Physiol* 261: H671-H676, 1991.

Dizon J, Burkhoff D, Tauskela J, Whang J, Cannon P, and Katz J. Metabolic inhibition in the perfused rat heart: evidence for glycolytic requirement for normal sodium homeostasis. *Am J Physiol Heart Circ Physiol* 274: H1082-H1089, 1998.

Driedzic WR. and Gesser H. Energy metabolism and contractility in ectothermic vertebrate hearts: hypoxia, acidosis, and low temperature. *Physiol Rev* 74: 221-257, 1994.

El-Sayed MF. and Gesser H. Sarcoplasmic reticulum, potassium, and cardiac force in rainbow trout and plaice. *Am J Physiol Regulatory Integrative Comp Physiol* 257: R599-R604, 1989.

Fletcher GL. Circannual cycles of blood plasma freezing point and Na⁺ and Cl⁻ concentrations in Newfoundland winter flounder (*Pseudopleuronectes americanus*): correlation with water temperature and photoperiod. *Can J Zool* 55: 789-795, 1977.

Fletcher GL, Slaughter D, and Hew CL. Seasonal changes in plasma levels of glycoprotein antifreeze, Na⁺, Cl⁻, and glucose in Newfoundland Atlantic cod (*Gadus morhua*). *Can J Zool* 60: 1851-1854, 1982.

Gesser H. and Mangor-Jensen A. Contractility and ⁴⁵Ca fluxes in heart muscle of flounder at a lowered extracellular NaCl concentration. *J Exp Biol* 109: 201-207, 1984.

Gesser H. and Poupa O. Relations between heart muscle enzyme pattern and directly measured tolerance to acute anoxia. *Comp Biochem Physiol* 48A: 97-103, 1974.

Gesser H. and Poupa O. The role of intracellular Ca^{2+} under hypercapnic acidosis of cardiac muscle: comparative aspects. *J Comp Physiol* 127: 307-313, 1978.

Grover GJ. and Garlid KD. ATP-sensitive potassium channels: a review of their cardioprotective pharmacology. *J Mol Cell Cardiol* 32: 677-695, 2000.

Gross GJ. Recombinant cardiac ATP-sensitive potassium channels and cardioprotection. *Circulation* 98: 1479-1480, 1998.

Hartmund T. and Gesser H. Temperature, contractility and high energy phosphates in anoxic fish heart muscle. *J Comp Physiol B* 162: 714-721, 1992.

Hartmund T. and Gesser H. Cardiac force and high-energy phosphates under metabolic inhibition in four ectothermic vertebrates. *Am J Physiol Regulatory Integrative Comp Physiol* 271: R946-R954, 1996.

Haruna T, Horie M, Kouchi I, Nawada R, Tsuchiya K, Akao M, Otani H, Murakami T, and Sasayama S. Coordinate interaction between ATP-sensitive K^+ channel and Na^+ , K^+ -ATPase modulates ischemic preconditioning. *Circulation* 98: 2905-2910, 1998.

Ho HS, Liu H, Cala PM, and Anderson SE. Hypertonic perfusion inhibits intracellular Na and Ca accumulation in hypoxic myocardium. *Am J Physiol Cell Physiol* 278: C953-C964, 2000.

Hove-Madsen L. and Gesser H. Force frequency relation in the myocardium of rainbow trout: effects of K⁺ and adrenaline. *J Comp Physiol B* 159: 61-69, 1989.

Kabakov AY. Activation of K_{ATP} channels by Na/K pump in isolated cardiac myocytes and giant membrane patches. *Biophys J* 75: 2858-2867, 1998.

Keen JE, Farrell AP, Tibbits GF, and Brill RW. Cardiac physiology in tunas: II. Effect of ryanodine, calcium and adrenaline on force-frequency relationships in atrial strips from skipjack tuna, *Katsuwonus pelamis*. *Can J Zool* 70: 1211-1217, 1992.

Moller-Nielsen T. and Gesser H. Sarcoplasmic reticulum and excitation-contraction coupling at 20 and 10 °C in rainbow trout myocardium. *J Comp Physiol B* 162: 526-534, 1992.

Murphy E, Cross H. and Steenbergen C. Sodium regulation during ischemia versus reperfusion and its role in injury. *Circ Res* 84: 1469-1470, 1999.

Nielsen KE, and Gesser H. Eel and rainbow trout myocardium under anoxia and / or hypercapnic acidosis, with changes in (Ca²⁺)_o and (Na⁺)_o. *Mol Physiol* 5: 189-198, 1984.

Olson K. The cardiovascular system. In: *Fish physiology*, second edition, edited by Evans D. New York, CRC press. 1998, 129-154.

Park C, Xiao X, and Allen D. Changes in intracellular Na^+ and pH in rat heart during ischemia: role of Na^+/H^+ exchanger. *Am J Physiol Heart Circ Physiol* 276: H1518-H1590, 1999.

Perry SF, and Gilmour KM. Consequences of catecholamine release on ventilation and blood oxygen transport during hypoxia and hypercapnia in an elasmobranch (*Squalus acanthias*) and a teleost (*Onchorhynchus mykiss*). *J Exp Biol* 199: 2105-2118, 1996.

Purup Hansen S, and Gesser H. Extracellular Ca^{2+} , force, and energy state in cardiac tissue of rainbow trout. *Am J Physiol Regulatory Integrative Comp Physiol* 253: R838-R847, 1987.

Reeves JP, Condrescu M, Chernaya G, and Gardener JP. $\text{Na}^+/\text{Ca}^{2+}$ antiport in the mammalian heart. *J Exp Biol* 196: 375-388, 1994.

Satchell GH. The Heart. In: *Physiology and form of fish circulation*, edited by Satchell GH. Cambridge: Cambridge University Press, 1991, p.11-40.

Shiels HA, and Farrell AP. The effect of temperature and adrenaline on the relative importance of the sarcoplasmic reticulum in contributing Ca^{2+} to force development in isolated ventricular trabeculae from rainbow trout. *J Exp Biol* 200: 1607-1621, 1997.

Shiels HA, Freund E, Farrell AP, and Block B. The sarcoplasmic reticulum plays a major role in isometric contraction in atrial muscle of yellowfin tuna. *J Exp Biol* 202: 881-890, 1999.

Shiels HA, Vornanen M, and Farrell AP. Temperature-dependence of L-type Ca^{2+} channel current in atrial myocytes from rainbow trout. *J Exp Biol* 203: 2771-2780, 2000.

Tani M, and Neely JR. Role of intracellular Na^+ in Ca^{2+} overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. Possible involvement of H^+ - Na^+ and Na^+ - Ca^{2+} exchange. *Circ Res* 65: 1045-1056, 1989.

Terzic A, Jahangir A, and Kurachi Y. Cardiac ATP-sensitive K^+ channels: regulation by intracellular nucleotides and K^+ channel-opening drugs. *Am J Physiol Cell Physiol* 269: C525-C545, 1995.

Tibbits GF, Kashihara H, Thomas MJ, Keen JE, and Farrell AP. Ca^{2+} transport in myocardial sarcolemma from rainbow trout. *Am J Physiol Regulatory Integrative Comp Physiol* 259: R453-R460, 1990.

Tibbits GF, Hove-Madsen L, and Bers DM. Calcium transport and the regulation of cardiac contractility in teleosts: a comparison with higher vertebrates. *Can J Zool* 69: 2014-2019, 1991.

Tibbits GF, Philipson KD, and Kashihara H. Characterisation of myocardial Na^+ - Ca^{2+} exchange in rainbow trout. *Am J Physiol Cell Physiol* 262: C411-C417, 1992.

Tota B. Myoarchitecture and vascularization of the elasmobranch heart ventricle. *J Exp Zool Supplement* 2: 122-135, 1989.

Tota B. Heart: In: Sharks, skates, and rays. The biology of elasmobranch fishes, edited by Hamlett WC. Baltimore, Johns Hopkins Univ. Press, 1999, p. 238-272.

Vornanen M. Effect of extracellular calcium on the contractility of warm and cold-acclimated crucian carp heart. *J Comp Physiol B* 165: 507-517, 1996.

Vornanen M. L-type Ca^{2+} current in fish cardiac myocytes: effects of thermal acclimation and B-adrenergic stimulation. *J Exp Biol* 201: 533-547, 1998.

Vornanen M. Na^+ / Ca^{2+} exchange current in ventricular myocytes of fish heart: contribution to sarcolemmal Ca^{2+} influx. *J Exp Biol* 202: 1763-1775, 1999.

Wang Y, Heigenhauser GJF, and Wood CM. Integrated responses to exhaustive exercise and recovery in rainbow trout white muscle: acid-base, phosphogen, carbohydrate, lipid, ammonia, fluid volume and electrolyte metabolism. *J Exp Biol* 195: 227-258, 1994.

2 Mitochondrial ATP-sensitive K⁺ Channels Contribute to Heart Anoxic Resistance in the Amazonian Armoured Catfish Acari-bodo (*Lipossarcus pardalis*).

2.1 Introduction

Many fishes inhabiting the waters of the Amazon drainage basin exhibit extreme whole animal and tissue hypoxia resistance (Almeida-Val and Farias 1996). Several behavioural and biochemical strategies are employed by these fishes to deal with extended hypoxia, including aerial respiration, surface skimming, elevated anaerobic metabolism and metabolic depression (Almeida-Val et al. 1999; Muusze et al. 1998; Val 1996). Ventricular muscle preparations from the air-breathing armoured catfish, acari-bodo (*Lipossarcus pardalis*), showed exceptional anaerobic capacity, maintaining roughly 50 % of isometric force development after 75 min of cyanide (CN) poisoning at 25 °C (Bailey et al. 1999). In addition to the maintenance of twitch force development, ventricle preparations from Amazonian fishes were better able to preserve resting tension under CN poisoning than north-temperate species. However, when comparing the two groups of fishes, West et al. (1999) could find no obvious correlation between maximal *in vitro* activities of glycolytic enzymes and the ability of heart tissue to function under impaired oxidative phosphorylation. The objective of this study was to identify mechanisms responsible for the extreme anoxic resistance observed in the heart of acari-bodo with reference to Ca²⁺ and K⁺ trafficking.

In mammals, cardiac ATP-sensitive potassium channels (K_{ATP}) are thought to play an important role in reducing infarct size and improving recovery of performance following a hypoxic challenge (Gross and Fryer 1999). K_{ATP} channels, which are activated when the ATP/ADP ratio declines, have been described on both the sarcolemmal membrane (sarc K_{ATP}) (Noma 1983) and on the inner mitochondrial membrane (mito K_{ATP}) (Inoue et al. 1991) of mammalian cardiomyocytes. As sarc K_{ATP} channels facilitate K^+ efflux, their activity has been associated with effects on transmembrane action potential duration (Ganim et al. 1998) and the concentration of extracellular K^+ (Kantor et al. 1990; Venkatesh et al. 1991; Wilde et al. 1990). Mito K_{ATP} channels promote K^+ influx across the inner mitochondrial membrane, leading to a loss of membrane potential and osmotic swelling of the matrix. The depolarisation of the inner membrane can affect mitochondrial Ca^{2+} handling and decrease the rate of ATP synthesis. Mito K_{ATP} channels are also activated during chronic hypoxia in the immature rabbit heart (Eells et al. 2000). This investigation examines the role of K_{ATP} channels in anoxic cardiac function and recovery of the acari-bodo heart. The problem was approached by assessing the impact of 5-hydroxydecanoic acid (5HD), a highly specific mito K_{ATP} channel antagonist, on heart performance. Based on the available literature I would expect mito K_{ATP} channels to open when acari-bodo heart muscle is exposed to anoxia. The effects of opening mito K_{ATP} channels on contractility in the fish heart is difficult to predict. These channels have not previously been investigated in fish and their contribution to contractility in mammalian cardiac muscle is still unclear.

In order to address the observed preservation of resting tension in the acari-bodo heart, we investigated the contribution of the sarcoplasmic reticulum (SR) to intracellular

Ca^{2+} cycling. Increases in resting tension are considered to result from elevations in cytoplasmic Ca^{2+} (Ca^{2+}_i) activity (Driedzic and Gesser 1994). A reduction of Ca^{2+}_i activity is generally achieved via active Ca^{2+} efflux across the sarcolemma or by the SR actively sequestering Ca^{2+} from the cytoplasm. Although this function of the SR is thought to be minor under most conditions in north-temperate fish (Driedzic and Gesser 1994) it is considered important to Ca^{2+} cycling in ventricular muscle from rainbow trout (Shiels et al. 1997) and tide pool fish (Rantin et al. 1998) at high temperatures and in atrial muscle of yellowfin tuna (Shiels et al. 1999). The hypothesis that SR function is important under anoxia and reoxygenation was tested by treating ventricle strips with ryanodine, an agent that locks the SR Ca^{2+} release channel in the open position.

The key findings of this study are that mito K_{ATP} channels play an important role in the anoxia defence strategies of the acari-bodo heart. The opening of mito K_{ATP} channels down regulates force development, possibly to preserve energy and minimise tissue damage during anoxia and recovery. Blocking SR function did not effect the response of acari-bodo ventricle preparations to anoxia or reoxygenation.

2.2 Materials and Methods

2.2.1 Animals

Armoured catfish commonly referred to as acari-bodo (*Lipossarcus pardalis*) (body mass 144.6 ± 8.3 g) were purchased from an aquaculture facility (Amazon Fish, Itacoatiara Road, AM-10, km 251, Manaus) and held at the Instituto Nacional Pesquisa da Amaz3nia (INPA), Manaus, Brazil, in indoor tanks supplied with recirculating, aerated tap water.

2.2.2 Ventricular strip preparations

Fish were killed by a sharp blow to the head, the heart was immediately excised and placed in oxygenated medium. The bathing medium for isolated muscle preparations included 125 mM NaCl, 3.0 mM KCl, 1.0 mM MgSO_4 , 1.5 mM CaCl_2 , 0.18 mM NaH_2PO_4 , 3.12 mM Na_2HPO_4 , and 5.0 mM glucose was added as a metabolic fuel (Driedzic and Bailey 1994). Medium was gassed with 100 % O_2 and pH set to 7.8 at 25 °C. Normally for ventricular preparations, medium is gassed with a mixture containing 0.5 % CO_2 to mimick the physiological environment of the fish heart. Mixed gases were not available for the experiments however. For the initial isolation, bathing medium was maintained at room temperature (~22 °C) to avoid rapid cooling effects on the preparation. The ventricle was dissected free of the bulbus arteriosus and atrium and then splayed open via a longitudinal cut through the dorsal wall. Two strips approximately 5-7 mm long and 1 mm wide were cut. Each strip was mounted in a tissue bath via a Plexiglas clamp between two platinum electrodes and tied to a Harvard Apparatus isometric force transducer (model 60-2994) using surgical silk. Chambers contained 30 ml of medium held at 25 °C and were continuously gassed with 100 % O_2 . Strips were stimulated to contract by field stimulation using a Grass model S9 square wave generator set at 150 % threshold voltage and 5 msec duration. Preparations were stretched to the optimum length for force production and equilibrated for 20 min at a pacing rate at 0.5 Hz. Stimulation rate was maintained at 0.5 Hz and temperature at 25 °C for all experiments.

Anoxia was quickly and reversibly induced in some experiments by flushing the tissue bath with 150 ml of medium equilibrated with 100 % N₂ and gassing with 100 % N₂. Tissue disturbance during the switch was minimal with the procedure requiring approximately 1 min to complete and resulting in dissolved oxygen levels of 0.1 mg/l or less. Preliminary experiments on other species using oxygenated medium during the switch have shown the procedure to have no effect on tissue contractility. Following the anoxic period, medium was regassed with 100 % O₂ and reached saturated levels in less than 1 min.

Force transducers were interfaced with a MacLab/2E (ADInstruments) and data were collected using Chart software for the Macintosh. Twitch force and resting tension were calculated using Chart software and are expressed as percent of initial force development.

2.2.3 Drugs

The contribution of the SR during normoxia, anoxia and reoxygenation was assessed using ryanodine (Sigma, St. Louis, MO, USA), a specific agent which, at the concentration used, locks the SR Ca²⁺ release channel in the open position (Coronado et al. 1994). A 1 mM stock solution was prepared in ethanol and stored at -20 °C until just before use. This stock solution resulted in a final ethanol concentration of 0.1 % in the tissue chamber. Preliminary trials found that this concentration of ethanol did not affect twitch force development. Mitochondrial K_{ATP} channel activity was addressed using sodium 5-hydroxydecanoic acid (5HD) (ICN Biomedicals, Aurora, OH, USA), which

selectively inhibits mito K_{ATP} channel activity without affecting sarc K_{ATP} channels at the concentration used (Hu et al. 1999). A 100 mM stock solution was prepared in bathing medium and frozen at -20°C until just before use.

2.2.4 Statistics

For ventricular performance studies statistical significance between treatments was tested using a parametric repeated measures analysis with a Bonferonni adjustment for multiple comparisons. Due to a limitation in the availability of experimental animals, most ventricle strip experiments were not run in concert with paired controls with the exception of trials involving 5HD. A P value < 0.05 was considered to be significant.

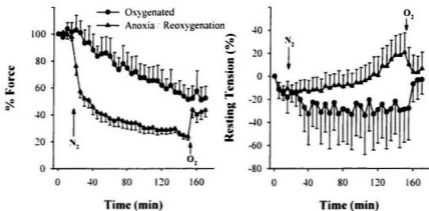
2.3 Results

2.3.1 Direct impact of anoxia and reoxygenation

Figure 2.1 shows twitch force development and resting tension for ventricular strips from acari-bodo subjected to oxygenation and anoxia-reoxygenation. After one hour under oxygenated conditions, force decreased by approximately 15 %. Twitch force decay was accompanied by a highly variable decrease in resting tension (i.e. strips lengthened). When exposed to anoxia, force development fell rapidly during the first 15 min but then stabilised at approximately 40 % of initial oxygenated levels. Rate of force decay following stabilisation was less than oxygenated controls for the duration of the insult. Twitch force was significantly lower under anoxia than oxygenation for all time points. Strips maintained resting tension for the majority of anoxia, showing a trend to increase only after 2 hours, which recovered following reoxygenation. Twitch force also

recovered quickly upon reoxygenation to a level not significantly different from normoxic controls. Further experiments showed that neither anoxic force development nor recovery were affected by reducing the duration of the anoxia from 135 to 30 min (see below).

Figure 2.1 Twitch force and resting tension (expressed as % of initial force development) (\pm SE) for ventricular strip preparations from acari-bodo heart exposed to oxygenated conditions (\bullet)(n = 4) and anoxia followed by reoxygenation (\blacktriangle)(n = 4). All points under anoxia are significantly different than oxygenated treatment.



2.3.2 Ryanodine treatment

A separate series of experiments was conducted over a shorter time course to assess the effects of ryanodine and 5HD on anoxic heart performance. Figure 2.2A presents the appropriate untreated, anoxia-reoxygenated control for these studies. During the initial oxygenated period force development decreased to 60 % after 40 min, under

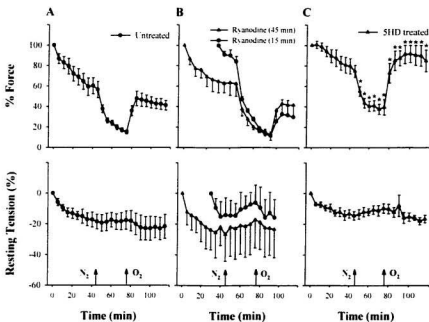
anoxia to 15 %, and upon reoxygenation force recovered to 45 %. Recovered force was similar to preanoxic levels. Figure 2.2B shows force and resting tension data for ryanodine (10 μ M) treated ventricular strips from acari-bodo. After 15 min and 45 min of ryanodine treatment under oxygenated conditions, force development was about 80 % and 65 % of the initial level respectively. Anoxia resulted in a rapid decrease in force development to approximately 15 % of initial force after 30 min. Preparations declined to this level of performance regardless of length of pretreatment with ryanodine. The degree of recovery following reoxygenation was not affected by changes in the duration of ryanodine treatment and was similar to untreated controls. Untreated preparations recovered to approximately 48 % of initial force development while strips treated with ryanodine for 15 and 45 min showed force recovery to 33 % and 43 % of initial levels, respectively. Ryanodine had no noticeable effect on resting tension during either oxygenated conditions or anoxia.

2.3.3 5HD treatment

Figure 2.2C illustrates force and resting tension for 5HD (100 μ M) treated preparations. Under oxygenation, blocking mito K_{ATP} channels with 5HD tended to attenuate twitch force decay in ventricle strips. After 40 min, force development in 5HD treated preparations was 79.8 ± 7.5 % of initial compared to 60.2 ± 7.9 % for untreated controls. Following the onset of anoxia, force fell significantly less in 5HD treated than in untreated strips so that after 30 min 5HD treated preparations maintained more than twice the % initial force of untreated preparations (39.5 ± 7.5 % vs. 15.0 ± 1.7 %). Upon reoxygenation, 5HD treated tissue recovered significantly more contractile force than

untreated tissue, to a maximum of over 91 % at 20 min reoxygenation, compared to approximately 45 % of initial for untreated strips. Treating strips with 5HD did not noticeably affect resting tension.

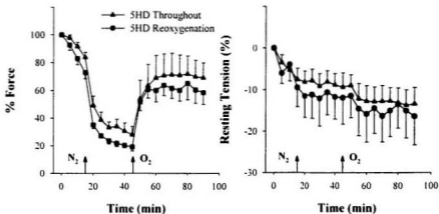
Figure 2.2 Twitch force and resting tension (\pm SE) for ventricular strips from acari-bodo exposed to anoxia (at 45 min for all panels) and reoxygenation (at 75 min for all panels). *A*: untreated ($n = 5$) *B*: ryanodine ($10 \mu\text{M}$) treated ($n = 4$ for 45 min incubation, $n = 2$ for 15 min incubation) *C*: 5HD ($100 \mu\text{M}$) treated ($n = 5$). * $P < 0.05$ vs. untreated preparations exposed to anoxia and reoxygenation (*A*).



A further experiment was performed to discern the time frame when 5HD was exerting its effects. Paired ventricular preparations were exposed to 30 min anoxia followed by reoxygenation with each strip receiving 5HD ($100 \mu\text{M}$) at a different time

(Fig 2.3). One preparation was treated throughout the experiment while the other received 5HD in the final 5 min of the anoxic period, just before reoxygenation. Preparations pretreated with 5HD tended to be stronger during anoxia. Recovery was similar in both treatments, suggesting that 5HD was affecting recovery during reoxygenation and not during anoxia. Interestingly, the force recovery observed in both treatments of this protocol was less than that seen in the previous set of experiments in which strips were incubated with 5HD for 45 min before being subjected to anoxia / reoxygenation. Changes in the length of 5HD incubation prior to ischemia can alter the agent's ability to inhibit preconditioning in rat heart (Fryer et al. 2000). The mechanism underlying this observation is not known.

Figure 2.3 Twitch force and resting tension (\pm SE) for acari-bodo ventricle strip preparations treated with 5HD (100 μ M) throughout anoxia and reoxygenation (\blacktriangle) and strips treated with 5HD 5 min before reoxygenation (\blacksquare)(n = 5).



Discussion

Hearts from acari-bodo exhibited a remarkable ability to recover contractility following anoxia, especially considering the relatively high temperature (25 °C) and pacing rate (0.5 Hz) used in the present study. Anoxia consistently resulted in a rapid decrease in force in preparations without pharmacological agents (Fig 2.1, 2.2). Preparations then seemed to reach a lower, controlled steady state which they maintained for at least 2 hours, substantiating previous observations on the exceptional anoxia tolerance of this tissue (Bailey et al. 1999). Ventricular strips maintained a level of force development approximately half that of oxygenated controls after 2 hours of anoxia. A previous observation of recovery of force during simulated anoxia (1 mM sodium CN) (Bailey et al. 1999) was not seen with the experimental approach used in this study. One explanation offered by Bailey et al. (1999) was that ATP production may have been impaired so quickly by CN that energy demand initially outstripped supply but ATP production was then activated, leading to recovery. The lack of a recovery phase during anoxia in this study is consistent with this viewpoint, since oxidative phosphorylation would be impaired more progressively using N₂. The novel finding of the current study is that following reoxygenation twitch force development recovers to levels equivalent to oxygenated controls.

The loss of force observed during oxygenation has been observed for other species in previous experiments of this type (Bailey et al. 1999), but the mechanism of force loss is unknown. Gesser and Poupa (1981) found that in resting flounder (*Platichthys flesus*) myocardium, ⁴⁵Ca²⁺ efflux decreased over 60 min, suggesting an overall loss of Ca²⁺_i. Though similar evidence is not available for acari-bodo myocardium, the observed force

loss may have occurred because of a similar loss in Ca^{2+}_i activity over the experimental period. The composition of the bathing medium used may not have been optimal for this species, resulting in a loss of Ca^{2+}_i to the medium and a concomitant decline in force. It is also possible that preparations were over oxygenated and that the formation of reactive oxygen species (ROS) damaged the myocardium. It is well established that ROS are a major contributor to myocardial damage in mammalian hearts (Sawyer and Colucci 2000). In preliminary experiments on yellowtail flounder (*Limanda ferruginea*), the ROS scavenger N-(2-mercaptopropionyl)-glycine reduced force loss over a similar time period (data not shown), suggesting ROS may impact on performance in the fish heart during oxygenation.

Preparations also maintained resting tension during anoxia, suggesting no significant increase in Ca^{2+}_i activity. Only following 2 hours of anoxia was there any evidence of an increase in resting tension. This is in contrast to studies with north temperate fish that show large increases in resting tension following an impairment of oxidative phosphorylation, usually attributed to an increase in Ca^{2+}_i activity (Driedzic and Gesser 1994). The heart of acari-bodo clearly has mechanisms to defend against such. Many preparations actually lengthened during the experimental period. The mechanism responsible for loss of resting tension during oxygenation and anoxia is not clear. It is possible that preparations were unable to maintain Ca^{2+}_i or that the composition of the bathing medium used in this study resulted in osmotic swelling of cardiomyocytes, which lead to an increase in the overall length of the preparation (Carmeliet 1999). Low-flow ischemia has also been shown to increase the dimensions of mammalian cardiomyocytes, possibly as a result of collagen damage (Lu et al. 1999). Regardless, the important point

in the context of this study is that resting tension is maintained under anoxia in acari-bodo heart for a considerable length of time.

2.3.4 Down regulation is probably due to decreased Ca^{2+} activity

Ryanodine was used as an agent to assess the role of SR in the down regulation of twitch force and maintenance of resting tension. Ryanodine had no effect on twitch force or resting tension during anoxia, indicating that the SR does not contribute to Ca^{2+} trafficking on a beat to beat basis in acari-bodo heart. If the SR was important, ryanodine treatment should result in a decrease in force as seen in yellowfin tuna (Shiels et al. 1999) or an increase in resting tension as occurs in eel heart preparations (Bailey et al. 2000). The Ca^{2+} required to activate contraction in acari-bodo heart is probably derived from influx across the sarcolemmal membrane via L-type Ca^{2+} channels and the Na^+/Ca^{2+} exchanger as is the case in other fish species (Vornanen 1998; 1999). Mitochondrial Ca^{2+} trafficking may also impact contractility in the acari-bodo heart, and this will be discussed below.

The rapid down regulation of force development and maintenance of resting tension may in part be a result of action potential shortening under anoxia leading to decreased Ca^{2+} entry across the sarcolemmal membrane mediated by sarc K_{ATP} channels as has been hypothesised for mammalian heart (Noma 1983). Studies of this nature are limited for fish heart, but in rainbow trout the rapid phase of force decrease under anoxia is associated with a transient decrease in action potential duration followed by a lengthening to preanoxic values in association with an increase in resting tension (Gesser and Høglund 1988). In addition, Ganim et al. (1998) have shown that glibenclamide, a

general K_{ATP} antagonist, can affect action potential duration under some circumstances in goldfish. A component of force down regulation may be attributable to sarc K_{ATP} opening.

2.3.5 Mito K_{ATP} channels under anoxia

5HD is considered to be a highly selective antagonist of mito K_{ATP} activity in mammalian heart (Hu et al. 1999). The concentration of 5HD used in this study (100 μ M) is at the lower end of those typically used in investigations on isolated perfused mammalian hearts (for example Baker et al. 1998). Although nothing is known concerning effective concentrations of this agent in fish, the nature of the current model made it impossible to establish an optimal dose without carrying out an inordinate number of additional trials.

Hearts treated with 5HD developed greater force under anoxia than control preparations. This is the first evidence, albeit indirect, of mito K_{ATP} channels in the fish heart. I am not aware of any studies that relate mito K_{ATP} channel activity to performance during oxygen limitation in hearts from any other species. A mitochondrial contribution to Ca^{2+}_i may be involved in this response. Mito K_{ATP} channels have been linked to changes in mitochondrial Ca^{2+} flux during ischemia in mammalian heart. Activation of mito K_{ATP} channels results in a depolarisation of the mitochondrial membrane and leads to reduced Ca^{2+} uptake and a release of stored mitochondrial Ca^{2+} (Holmuhamedov et al. 1998; Holmuhamedov et al. 1999). Since force development is intimately associated with Ca^{2+}_i activity, alterations in mitochondrial Ca^{2+} cycling resulting from 5HD treatment could conceivably affect twitch force development. Irrespective of their mechanism of

action, the physiological implications of mito K_{ATP} channel activation during anoxia appear to be important in the heart of acari-bodo. Resetting contractility to a lower steady state during anoxia should conserve glycolytic energy stores and protect the heart from injury at reoxygenation. Cardiac muscle is susceptible to the accumulation of Na^+ and Ca^{2+} ions during anoxia, which, along with several other factors, can damage the tissue during reoxygenation.

2.3.6 Blocking Mito K_{ATP} channels results in hypercontracture

The extent of recovery in acari-bodo heart following an anoxic challenge is masked by the fact that preparations maintained under oxygenated conditions also showed a decrease in twitch force. Reoxygenating untreated ventricular strips following a period of anoxia resulted in a recovery of twitch force development to levels similar to oxygenated controls. Treatment with 5HD either before anoxia, or immediately prior to reoxygenation resulted in significant hypercontracture during reoxygenation. The question arises as to why the blockade of opened mito K_{ATP} channels leads to hypercontracture in a tissue that has the capacity to recover to control levels after an anoxic episode. Studies with rabbit heart may lend insight into this paradox. Heart mitochondria isolated from chronically hypoxic rabbits have higher rates of ATP synthesis than hearts from control animals in association with increased mito K_{ATP} channel activity (Eells et al. 2000). 5HD treatment results in a decrease in mitochondrial ATP synthesis in hearts from hypoxic but not normoxic rabbits.

I suggest that application of 5HD to the post-anoxic acari-bodo heart leads to the closure of open mito K_{ATP} channels and subsequently impairs ATP synthesis. Given that

SR function is apparently lacking under these conditions, the dependence of cardiomyocytes on extracellular Ca^{2+} may cause decreases in ATP production to transiently potentiate force development via changes in Na^+_i . Inhibition of the Na^+/K^+ -ATPase, owing to decreases in [ATP], would result in increased $[\text{Na}^+_i]$ and a concomitant rise in sarcolemmal Ca^{2+} influx via reverse $\text{Na}^+/\text{Ca}^{2+}$ exchange. Vornanen (1999) has shown that reverse $\text{Na}^+/\text{Ca}^{2+}$ exchange contributes at least 1/3 of the Ca^{2+} required to activate contraction in the fish heart. In addition, decreasing extracellular Na^+ results in a large increase in twitch force in eel and rainbow trout heart (Nielsen and Gessel 1984). Since the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is sensitive to the electrochemical gradients for Na^+ and Ca^{2+} (Vornanen 1999), increasing Na^+_i via inhibition of the Na^+/K^+ -ATPase should mimic the effects of decreasing extracellular Na^+ , resulting in increased sarcolemmal Ca^{2+} influx and the observed potentiation of twitch force. Once more, although this interpretation must be viewed with caution the importance of mito K_{ATP} channels in acari-bodo heart seems clear.

2.3.7 Conclusions

This study clearly shows the presence of mito K_{ATP} channels in the fish heart and indicates their involvement in a controlled down-regulation of twitch force during impaired oxidative phosphorylation. Resetting contractility to a lower steady state during anoxia should conserve glycolytic energy stores and protect the heart from injury at reoxygenation. Further study is necessary to characterise the activity of both mito and sarc K_{ATP} channels in the anoxia tolerant fish heart and define the mechanism by which these channels control contractility, and to assess the functional differences between fish

and mammals in the role of K_{ATP} channels. The extreme anaerobic capacity along with the relative cellular and whole organ simplicity of the acari-bodo heart may prove to be a powerful tool in clarifying the role of K_{ATP} channels in clinical models of hypoxic and ischemic heart disease.

2.4 References

Almeida-Val VMF, and Farias IP. Respiration in fishes of the Amazon: metabolic adjustments to chronic hypoxia. In: Physiology and biochemistry of the fishes of the Amazon, edited by Val AL, Almeida-Val VMF, and Randall DJ. Manaus: INPA, 1996, p. 257-271.

Almeida-Val VMF, Val AL, and Walker I. Long- and short-term adaptation of Amazon fishes to varying O₂-levels: intraspecific phenotypic plasticity and interspecific variation. In: Biology of tropical fishes, edited by Val AL, and Almeida-Val VMF. Manaus: INPA, 1999, p.185-206.

Bailey JR, Barter T, and Driedzic WR. Maintenance of resting tension in the American eel (*Anguilla rostrata* L.) heart is dependant upon exogenous fuel and the sarcoplasmic reticulum. *J Exp Zool* 286: 707-717, 2000.

Bailey JR, Val AL, Almeida-Val VMF, and Driedzic WR. Anoxic cardiac performance in Amazonian and north-temperate-zone teleosts. *Can J Zool* 77: 683-689, 1999.

Baker JE, Holman P, and Gross GJ. Preconditioning in immature rabbit hearts: role of K_{ATP} channels. *Circulation* 99: 1249-1254, 1999.

Carmeliet E. Cardiac ionic currents and acute ischemia: from channels to arrhythmias. *Physiol Rev* 79: 917-1017, 1999.

Coronado R, Morrissette J, Sukhareva M, and Vaughan DM. Structure and function of ryanodine receptors. *Am J Physiol Cell Physiol* 246: C1485-C1504, 1994.

Driedzic WR, and Bailey JR. Isolated perfused fish hearts. In: *Biochemistry and molecular biology of fishes*, vol 3, edited by Hochachka PW, and Mommsen TP. Amsterdam: Elsevier Science B.V., 1994, p. 119-125.

Driedzic WR, and Gesser H. Energy metabolism and contractility in ectothermic vertebrate hearts: hypoxia, acidosis, and low temperature. *Physiol Rev* 74: 221-257, 1994.

Eells JT, Henry MM, Gross GJ, and Baker JE. Increased mitochondrial K_{ATP} channel activity during chronic myocardial hypoxia. Is cardioprotection mediated by improved bioenergetics? *Circ Res* 87: 915-921, 2000.

Fryer RM, Eells JT, Hsu AK, Henry MM, and Gross GJ. Ischemic preconditioning in rats: role of mitochondrial K_{ATP} channel in preservation of mitochondrial function. *Am J Physiol Heart Circ Physiol* 278: H305-H312, 2000.

Ganim R, Peckol E, Larkin J, Ruchhoeft M, and Cameron J. ATP-sensitive K⁺ channels in cardiac muscle from cold-acclimated goldfish: characterisation and altered response to ATP. *Comp Biochem Physiol* 119A: 395-401, 1998.

Gesser H, and Hoglund L. Action potential, force and function of the sarcoplasmic reticulum in the anaerobic trout heart. *Exp Biol* 47: 171-176, 1988.

Gesser H, and Poupa O. Acidosis and Ca²⁺ distribution in myocardial tissue of flounder and rat. *J Comp Physiol* 143: 245-251, 1981.

Gross GJ, and Fryer RM. Sarcolemmal versus mitochondrial ATP-sensitive K⁺ channels and myocardial preconditioning. *Circ Res* 84: 973-979, 1999.

Holmuhamedov EL, Jovanovic S, Dzeja PP, Jovanovic A, and Terzic A. Mitochondrial ATP-sensitive K⁺ channels modulate cardiac mitochondrial function. *Am J Physiol Heart Circ Physiol* 275: H1567-H1576, 1998.

Holmuhamedov EL, Wang L, and Terzic A. ATP-sensitive K⁺ channel openers prevent Ca²⁺ overload in rat cardiac mitochondria. *J Physiol* 519.2: 347-360, 1999.

Hu H, Sato T, Seharaseyon J, Liu Y, Johns DC, O'Rourke B, and Marbán E. Pharmacological and histochemical distinctions between molecularly defined

sarcolemmal K_{ATP} channels and native cardiac mitochondrial K_{ATP} channels. *Mol Pharmacol* 55: 1000-1005, 1999.

Inoue I, Nagase K, Kishi K, and Higuti T. ATP-sensitive K^+ channel in the mitochondrial inner membrane. *Nature* 352: 244-247, 1991.

Kantor PF, Coetzee WA, Carmeliet EE, Dennis SC, and Opie LH. Reduction of ischemic K^+ loss and arrhythmias in rat hearts. Effect of glibenclamide, a sulfonylurea. *Circ Res* 66: 478-485, 1990.

Lu L, Xu Y, Greyson CR, Ursell PC, and Schwartz GG. Non-elastic deformation of myocardium in low-flow ischemia and reperfusion: ultrastructure-function relations. *J Mol Cell Cardiol* 31: 1157-1169, 1999.

Muusze B, Marcon J, van den Thillart G, and Almeida-Val VMF. Hypoxia tolerance in Amazon fish: respirometry and energy metabolism of the cichlid *Astronotus ocellatus*. *Comp Biochem Physiol A* 120: 151-156, 1998.

Nielsen K, and Gesser H. Eel and rainbow trout myocardium under anoxia and/or hypercapnic acidosis, with changes in $(Ca^{2+})_o$ and $(Na^+)_o$. *Mol Physiol* 5: 189-198, 1984.

Noma A. ATP-regulated K^+ channels in cardiac muscle. *Nature* 305: 147-148, 1983.

Purup Hansen S. and Gesser H. Extracellular Ca^{2+} , force, and energy state in cardiac tissue of rainbow trout. *Am J Physiol Regulatory Integrative Comp Physiol* 22: R838-R847, 1987.

Rantin FT, Gesser H, Kalinin AL, Guerra CDR, De Freitas JC, and Driedziec WR. Heart performance, Ca^{2+} regulation and energy metabolism at high temperatures in *Bathygobius soporator*, a tropical marine teleost. *J Therm Biol* 23: 31-39, 1998.

Sawyer DB, and Colucci WS. Mitochondrial oxidative stress in heart failure: "oxygen wastage" revisited. *Circ Res* 86: 119-120, 2000.

Shiels HA, and Farrell AP. The effect of temperature and adrenaline on the relative importance of the sarcoplasmic reticulum in contributing Ca^{2+} to force development in isolated ventricular trabeculae from rainbow trout. *J Exp Biol* 200: 1607-1621, 1997.

Shiels HA, Freund EV, Farrell AP, and Block BA. The sarcoplasmic reticulum plays a major role in isometric contraction in atrial muscle of yellowfin tuna. *J Exp Biol* 202: 881-890, 1999.

Val AL. Surviving low oxygen levels: lessons from fishes of the Amazon. In: *Physiology and biochemistry of the fishes of the Amazon*, edited by Val AL, Almeida-Val VMF, and Randall DJ. Manaus: INPA, 1996, p. 59-73.

Venkatesh N, Lamp ST, and Weiss JN. Sulfonylureas, ATP-sensitive K^+ channels, and cellular K^+ loss during hypoxia, ischemia, and metabolic inhibition in mammalian ventricle. *Circ Res* 69: 623-637, 1991.

Vornanen M. L-type Ca^{2+} current in fish cardiac myocytes: effects of thermal acclimation and β -adrenergic stimulation. *J Exp Biol* 201: 533-547, 1998.

Vornanen M. Na^+/Ca^{2+} exchange current in ventricular myocytes of fish heart: contribution to sarcolemmal Ca^{2+} influx. *J Exp Biol* 202:1763-1775, 1999.

Wang Y, and Ashraf M. Role of protein kinase C in mitochondrial K_{ATP} channel-mediated protection against Ca^{2+} overload injury in rat myocardium. *Circ Res* 84: 1156-1165, 1999.

West JL, Bailey JR, Almeida-Val VMF, Val AL, Sidell BD, and Driedzic WR. Activity levels of enzymes of energy metabolism in heart and red muscle are higher in north-temperate-zone than in Amazonian teleosts. *Can J Zool* 77: 690-696, 1999.

Wilde AA, Escande D, Schumacher CA, Thuringer D, Mestre M, Fiolet JW, and Janse MJ. Potassium accumulation in the globally ischemic mammalian heart. A role for the ATP-sensitive potassium channel. *Circ Res* 67: 835-843, 1990.

3 Evidence for Myocardial ATP-sensitive K⁺ Channels in Yellowtail Flounder but not Atlantic Cod

3.1 Introduction

Bottom dwelling flatfishes are known to exhibit substantial tolerance to cardiac acidosis and impairment of oxidative metabolism. When subjected to acidosis, cardiac force is initially potentiated in the flounder before slowly declining over time (Gesser and Poupa 1979). Gesser and Poupa (1978; 1979) have suggested that intracellular acidosis may trigger a release of stored mitochondrial Ca²⁺ resulting in the observed potentiation of force production, although this hypothesis remains untested in the flounder heart.

A recent study by Ganim et al. (1998) on goldfish, as well as the investigations presented in Chapter 2 on the Amazonian armoured catfish acari-bodo (*Lipossarcus pardalis*), have implicated adenosine 5'-triphosphate sensitive potassium (K_{ATP}) channels in the control of contractile function in anoxia tolerant fish hearts. K_{ATP} channels are activated by a decline in the ratio of ATP/ADP and are therefore most likely to contribute to cardiac function throughout periods of impaired ATP production, such as during hypoxia. The objective of this study was to evaluate whether K_{ATP} channels are involved in the phenomenon of hypoxic force potentiation in the yellowtail flounder (*Limanda ferruginea*) heart, and to investigate their role in cardiac performance during anoxia and reoxygenation in fish species with differing tolerances to cardiac anoxia. Atlantic cod (*Gadus morhua*) was chosen for comparison as it is generally believed to exhibit poor cardiac anoxia tolerance (Gesser and Poupa 1974; Hartmund and Gesser 1996).

In mammalian heart K_{ATP} channels have been described on both the sarcolemmal membrane (sarc K_{ATP}) (Noma 1983) and on the inner mitochondrial membrane (mito K_{ATP}) (Inoue et al. 1991) and their activity has been linked with the cardioprotection afforded by various means of preconditioning. Sarc K_{ATP} channels facilitate cellular K^+ efflux in mammalian cardiomyocytes and can therefore influence membrane electrical properties (Ganim et al. 1998) and extracellular K^+ concentrations (Kantor et al. 1990; Venkatesh et al. 1991; Wilde et al. 1990). Mito K_{ATP} channels allow mitochondrial K^+ influx, leading to declines in membrane potential and swelling of the matrix in the rat heart. Depolarisation can also effect mitochondrial Ca^{2+} handling (Holmuhamedov et al. 1999), increase respiration, and alter the rate of mitochondrial ATP synthesis (Holmuhamedov et al. 1998). Despite extensive study in mammals, it is still not clear whether the hypoxic cardioprotection associated with the opening of K_{ATP} channels is mediated by sarcolemmal or mitochondrial channels, or if both play an important role (Sato et al. 1999; reviewed by Gross and Fryer 1999).

Differences in excitation-contraction (E-C) coupling between fish and mammalian cardiac muscle may contribute to significant differences in the functionality of sarcolemmal and mito K_{ATP} channels in fish cardiomyocytes. Unlike mammalian cardiomyocytes which derive the Ca^{2+} needed for contraction from intracellular stores such as the sarcoplasmic reticulum (SR), fish cardiomyocytes rely heavily on trans-sarcolemmal Ca^{2+} influx to achieve contraction (Vornanen 1998; 1999). The dependence of fish cardiomyocytes on sarcolemmal Ca^{2+} flux enhances the importance of membrane bound ion channels and transporters in the modulation of contractility. The role of K_{ATP}

channels in fish cardiomyocytes may, therefore, be quite different than that of mammals and could be important in the beat to beat control of cardiac function in fish.

The contribution of K_{ATP} channels to heart performance during anoxia and reoxygenation was studied using isolated ventricular muscle strip preparations and pharmacological agents targeting both sarcolemmal and mito K_{ATP} channel activity. This study presents evidence that agents altering K_{ATP} channel activity can impact on anoxic contractility in ventricular muscle from yellowtail flounder, but not Atlantic cod. Species specific differences shown in fish cardiac K_{ATP} channels may therefore have implications in anaerobic heart performance.

3.2 Materials and Methods

Cultured yellowtail flounder (body mass 108.5 ± 5.1 g) and Atlantic cod (body mass 391.0 ± 99.9 g) were maintained in aerated, flow-through seawater tanks at between 6.5 and 7 °C and neutral photoperiod. Yellowtail flounder were held in either 215 or 1200 L tanks and fed commercial feed while cod were held in 8100 L tanks and fed either commercial feed or frozen herring.

3.2.1 Tissue preparation

Animals were killed by a sharp blow to the head and doubly pithed. The heart was quickly excised and placed in cold, oxygenated bathing solution. The bathing medium was a standard solution for marine teleosts and included 150 mM NaCl, 5.0 mM KCl, 0.17 mM $MgSO_4$, 1.5 mM $CaCl_2$, 0.17 mM NaH_2PO_4 , 2.33 mM Na_2HPO_4 , 11.0 mM $NaHCO_3$, with pH set to 7.8 at 6 °C. 5.0 mM glucose was added as a metabolic fuel

(Driedzic and Bailey 1994). The ventricle was dissected free of the atrium and bulbus arteriosus, bisected, and a strip approximately 1.5 mm wide and < 10 mm in length was cut longitudinally from each section.

Strips were mounted vertically in a tissue bath via a Plexiglas clamp and affixed to a Harvard Apparatus isometric force transducer (Model 60-2994) using 3-0 surgical silk. Each bath contained 30 ml of bathing medium held at 6 °C and gassed with either 0.5 % CO₂, balance O₂ (oxygenated) or 0.5 % CO₂, balance N₂ (anoxia). Gassing with a mixture rich in O₂ ensures adequate oxygen delivery, while the addition of a small amount of CO₂ provides a more physiological environment for the muscle preparation (Driedzic and Bailey 1994). Mixed gasses were not available for previous experiments (Chapter 2). Ganim et al. (1998) have shown K_{ATP} channels to be sensitive to acclimation temperature in fish, so in an attempt to reflect physiological conditions, experiments were run as close as possible to the acclimation temperature of the animals.

Strips were positioned between two platinum electrodes on the Plexiglas clamp and stimulated to contract via field stimulation using a Grass model S9 stimulator with voltage set at 150 % threshold and 5 msec duration. Strips were stretched to optimum length for force production and allowed 30 min to acclimate at a pacing rate of 0.2 Hz. Pacing frequency was 0.2 Hz for all experiments and spontaneously contracting strips were eliminated from statistical analysis. A pacing rate of 0.2 Hz was chosen for comparison with existing data on cardiac performance in other flatfish and on Atlantic cod (Gesser and Poupa 1974).

Anoxic conditions were induced rapidly and reversibly by replacing the oxygenated medium in the tissue bath with nitrogen gassed medium. A reservoir of

medium was maintained at 6 °C in a water-jacketed condenser and equilibrated with 0.5 % CO₂, balance N₂. During the switch to anoxia the tissue bath was gassed with 0.5 % CO₂, balance N₂ and flushed with 150 ml of anoxic medium. Mechanical disturbance was minimal during the switch and preliminary experiments using a reservoir of oxygenated medium found the process did not affect force development or the contractile characteristics of the preparation. The switch from oxygenated to anoxic medium required < 1 min and dissolved oxygen in the bath was routinely < 0.1 mg/l. To achieve reoxygenation the bath was gassed with 0.5 % CO₂, balance O₂, resulting in saturation within approximately 1 min.

The response of ventricular muscle to anoxia and reoxygenation was first assessed in the absence of pharmacological agents. Control strips were gassed with 0.5 % CO₂, balance O₂ for 85 min while treatment preparations were subjected to a 35 min period of anoxia followed by 30 min of reoxygenation.

The contribution of K_{ATP} channels to the response of yellowtail flounder and cod ventricular muscle to anoxia was next assessed using glibenclamide, an inhibitor of both sarcolemmal and mito K_{ATP} channels (Hu et al. 1999). Both control and treatment strips were subjected to a 35 min period of anoxia followed by 30 min of reoxygenation. Glibenclamide (5 µM) was initially applied to the treatment bath during the first minute following acclimation with the control bath receiving vehicle dimethyl-sulfoxide (DMSO). Chemicals were reapplied immediately following the switch to anoxia to maintain a constant concentration of agent in the bath. As discussed in Chapter 2, the nature of the current experimental model makes it difficult to establish optimal dosages for pharmacological agents. Dosages for all agents were chosen from the lower end of

the range of concentrations commonly employed in mammalian literature in an attempt to minimise the risk of non-specific or toxic side effects.

The functional contribution of mito K_{ATP} channels in the yellowtail flounder heart was next investigated using sodium 5-hydroxydecanoic acid (5HD), a specific inhibitor of mito K_{ATP} channel function. Trials were run as above with glibenclamide replaced by 5HD (100 μ M) in each instance. The concentration of 5HD used here is at the low end of those normally used in investigations on isolated perfused mammalian hearts (for example Baker et al. 1998).

In addition to 5HD, diazoxide, a specific mito K_{ATP} channel opener, was also used to assess the effects of mito K_{ATP} channels in anaerobic performance and recovery in yellowtail flounder and cod ventricular strips. Trials were run as above with 5HD replaced with diazoxide (50 μ M) (Hu et al. 1999).

3.2.2 Drugs

All chemicals were purchased from Sigma (St. Louis, MO, USA) with the exception of 5HD which was purchased from ICN Biomedicals Inc. (Aurora, OH, USA). Because of their low solubility in water, stock solutions of glibenclamide (5 mM) and diazoxide (18 mM) were prepared in DMSO and stored at -20°C in aliquots until just before use. A 100 mM stock solution of 5HD was prepared in bathing medium and was also frozen in aliquots until just before use. All chemicals were pipetted directly into the tissue bath.

3.2.3 Data analysis and statistics

Force transducers were interfaced to a MacLab /2E computerised unit and data were collected online using the accompanying Chart software for Macintosh. Data were recorded for a duration of 30 sec at 5 min intervals for all experiments and statistical analysis is based on the average of 6 contractions at each recording interval. Peak tension (% force) and resting tension were calculated using Microsoft Excel and are expressed as a percent of initial tension development. Due to the high variability observed, data from untreated, anoxia / reoxygenation trials were pooled for more accurate comparisons with untreated oxygenated preparations and 5HD treated preparations. Anoxia / reoxygenation trials in which DMSO was applied to preparations were also pooled to provide more accurate comparisons with glibenclamide and diazoxide treated strips. Statistical analysis of data was performed using the statistical package SPSS version 10.1 for Windows. Significance was tested between treatments using a parametric repeated measures analysis with a Bonferroni adjustment for multiple comparisons. Within treatment differences were tested using a one-way ANOVA. P values less than 0.05 were considered statistically significant.

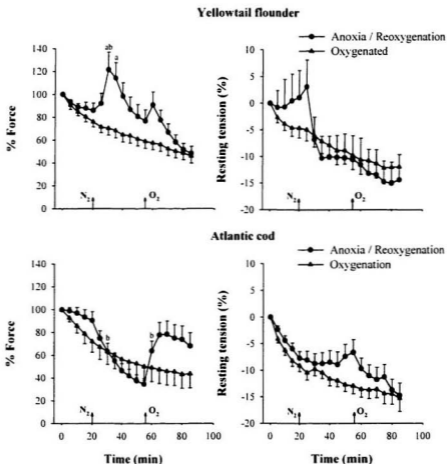
3.3 Results

3.3.1 Anoxia / reoxygenation

Yellowtail flounder. Figure 3.1 shows peak tension and resting tension for untreated yellowtail flounder ventricular preparations exposed to anoxia and reoxygenation. Ventricular strips from yellowtail flounder showed a consistent decay in force development under oxygenated conditions to approximately 57.5 % of initial after 60 min. Resting tension also fell by about 10 % during oxygenation. The decline in resting tension accounted for approximately 10 % of observed force loss after 85 min as the muscle lengthened and stretch dropped to less than optimum for peak tension development.

Force production increased significantly above oxygenated levels in yellowtail flounder following exposure to anoxia, peaking at 122 ± 13 % after 10 min. Force then declined over the balance of the anoxic period but remained above levels observed for preparations under oxygenated conditions. Force recovered significantly above anoxic levels at reoxygenation before continuing to decline at a rate approximately equal to that observed before reoxygenation. Resting tension consistently fell rapidly by approximately 10 % during the initial 10 min of anoxia, before stabilising and falling at a rate similar to oxygenated controls. Reoxygenation did not affect resting tension.

Figure 3.1 Twitch force and resting tension (\pm SE) for yellowtail flounder and Atlantic cod ventricular preparations exposed to oxygenated conditions (flounder $n = 6$, cod $n = 5$) and to 35 min of anoxia followed by reoxygenation ($n = 10$, both species). Arrows indicate points at which anoxia was induced (at time 20 min) and when preparations were reoxygenated (at time 55 min). *a* indicates significant difference between treatment. *b* indicates a significant decrease or increase from measurements within the treatment taken immediately before hand.



Atlantic Cod. Ventricle strips from cod exhibited a decay in force production and resting tension under oxygenated conditions similar to that observed for yellowtail flounder. Exposing preparations to anoxia resulted in a significant decline in force production relative to pre-anoxic levels, falling to 34.6 ± 6.3 % of initial after 30 min; however, anoxic force development was not significantly different than that observed for oxygenated controls. Following reoxygenation, force recovered to levels much higher than those observed for oxygenated controls (78.3 ± 10.9 % compared to 46.0 ± 15.4 %). Although preparations showed significant force recovery relative to anoxic levels, recovery was non-significant when compared to oxygenated preparations, owing to high variation in the recovering strips. Resting tension in cod preparations did not exhibit the same rapid decay at the onset of anoxia as exhibited by yellowtail flounder preparations, and was generally not affected by anoxia or reoxygenation.

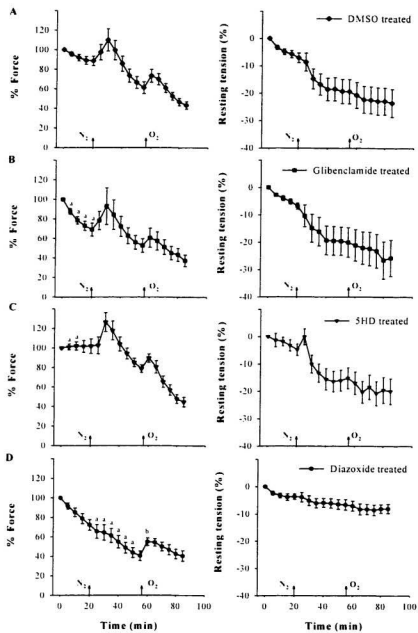
3.3.2 K_{ATP} contribution

Yellowtail flounder. Figure 3.2 shows peak tension and resting tension data for yellowtail flounder ventricular muscle preparations exposed to anoxia and reoxygenation and treated with agents to alter K_{ATP} channel activity. Blocking K_{ATP} channels with glibenclamide decreased force production significantly under oxygenated conditions in preparations from yellowtail flounder. Although, despite an overall decrease in force development, preparations continued to respond similarly to anoxia and reoxygenation. Glibenclamide had no effect on resting tension in yellowtail flounder ventricle preparations.

Inhibiting mito K_{ATP} channels with 5HD initially preserved force development under oxygenation in yellowtail flounder heart; however, it did not significantly affect peak tension during anoxia and reoxygenation. The mito K_{ATP} channel agonist diazoxide, on the other hand, significantly eliminated the potentiation of force production observed in untreated preparations exposed to anoxia. Diazoxide treated preparations did show significant force recovery over anoxic levels when reoxygenated, but still tended to be weaker than untreated strips. Resting tension also tended to be more stable during anoxia in diazoxide treated strips, with no rapid decline observed at the onset of anoxia. Differences were not statistically significant however.

Atlantic Cod. Figure 3.3 gives force and resting tension for Atlantic cod ventricular muscle preparations exposed to anoxia and reoxygenation and treated with agents to alter K_{ATP} channel activity. Neither glibenclamide, 5HD, nor diazoxide had any noticeable affect on force development or resting tension in cod ventricle preparations under the conditions tested. All preparations show a decrease in force development under anoxia to approximately 33 % of initial. Upon reoxygenation, strips immediately recover to approximately 88 % of initial force development.

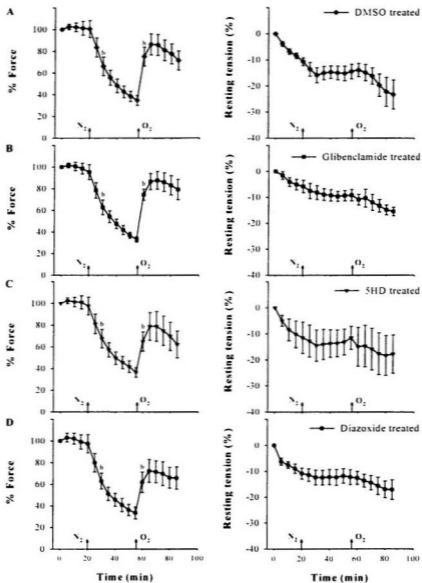
Figure 3.2 Twitch force and resting tension (\pm SE) for yellowtail flounder ventricular preparations subjected to anoxia and reoxygenation and treated with agents to affect K_{ATP} channel activity. Arrows indicate points at which anoxia was induced (at time 20 min) and when preparations were reoxygenated (at time 55 min). *A*: DMSO treated (●)(*n* = 13) *B*: glibenclamide treated (■)(*n* = 8) *C*: 5HD treated (▼)(*n* = 6) *D*: diazoxide treated (●)(*n* = 5). *a* indicates significant difference between treatment. *b* indicates a significant decrease or increase from measurements within the treatment taken immediately before hand.



3.4 Discussion

The potentiation of force exhibited by yellowtail flounder ventricle preparations exposed to nitrogen-induced anoxia is similar to that shown by other species of flatfish subjected to acidosis (Gesser and Poupa 1979; Hoglund and Gesser 1987; Poupa and Johansen 1975). Increases in cardiac twitch force production in fish are generally agreed to result from increased intracellular Ca^{2+} (Ca^{2+}_i) activity (Tibbitts et al. 1991). It has been hypothesised that acidotic force potentiation in ectothermic vertebrates may be due to a release of stored mitochondrial Ca^{2+} (Gesser and Poupa 1978). Gesser and Poupa (1978) found that ventricular strips from rainbow trout and *Vipera herus* exhibited increased resting tension when perfused with a medium free of Na^+ and Ca^{2+} . Altering sarcoplasmic reticulum activity with caffeine did not affect these changes, therefore leaving mitochondrial Ca^{2+} stores as the most likely explanation.

Figure 3.3 Twitch force and resting tension (\pm SE) for Atlantic cod ventricular preparations subjected to anoxia and reoxygenation and treated with agents to affect K_{ATP} channel activity. Arrows indicate points at which anoxia was induced (at time 20 min) and when preparations were reoxygenated (at time 55 min). *A*: DMSO treated (\blacklozenge)($n = 13$) *B*: glibenclamide treated (\blacksquare)($n = 8$) *C*: 5HD treated (\blacktriangledown)($n = 5$) *D*: diazoxide treated (\bullet)($n = 5$). *h* indicates a significant decrease or increase from measurements within the treatment taken immediately before hand.



In the current study diazoxide clearly eliminated anoxic force potentiation in the yellowtail flounder heart and it also tended to stabilise resting tension. Acute activation of mito K_{ATP} channels with diazoxide has been shown to depolarise the inner mitochondrial membrane in the rat heart at 30 °C, leading to a rapid reduction in mitochondrial Ca^{2+} content and inhibited mitochondrial Ca^{2+} uptake (Holmuhamedov et al. 1999). If the potentiation of force during anoxia in flounder is due to a bolus release of mitochondrial Ca^{2+} , as suggested by previous investigations, then our results seem to contrast those observed in mammals, in that the activation of mito K_{ATP} channels in the flounder heart seems to stabilise Ca^{2+} , during anoxia. Its possible that in the flounder heart, diazoxide releases mitochondrial Ca^{2+} more slowly than in the rat heart, likely due to the relatively extreme low temperature (6 °C) used in this experiment. Following a period of diazoxide treatment, mitochondrial Ca^{2+} content should be negligible, so that when subjected to anoxia, any large force potentiation resulting from a bolus release of mitochondrial Ca^{2+} would be eliminated.

The observed preservation of resting tension in diazoxide treated preparations supports the above hypothesis. Changes in resting tension are thought to reflect alterations in resting Ca^{2+}_i (Driedzic and Gesser 1994), therefore the observed decrease in resting tension at the onset of anoxia in untreated strips would presumably be due to a decrease in Ca^{2+}_i activity. If diazoxide treatment triggers a more gradual release of mitochondrial Ca^{2+} to the cytoplasm, it could help to maintain $[Ca^{2+}_i]$ during anoxia and overcome a net loss in Ca^{2+}_i activity, leading to a preservation of resting tension. Although the above result seems unexpected based on mammalian data, it is in line with

previous observations on the anoxia tolerant armoured catfish heart in which blocking active mito K_{ATP} channels at 25 °C caused an increase in anaerobic force development (chapter 2). 5HD had little effect on the flounder heart at 6 °C; however, it did significantly attenuate force loss during oxygenated conditions, suggesting that mito K_{ATP} channels may be active to some extent under these conditions. Since most studies involving 5HD have been carried out on mammalian tissues at relatively high temperatures, the efficacy of 5HD inhibition at low temperatures is unclear. It is possible that at 6 °C, 5HD is unable to effectively block mito K_{ATP} channels to an extent where they can influence contractility in the fish heart.

Glibenclamide, which inhibits both sarcolemmal and mito K_{ATP} channels, significantly reduced force development in flounder ventricle preparations during oxygenation, but did not affect the characteristics of force development during anoxia or recovery. This observation, along with fact that 5HD seemed to have the opposite effect on twitch force development in flounder ventricle strips, suggests that the force loss incurred with glibenclamide may be a result of a sarcolemma! rather than mito K_{ATP} channel contribution.

Sarc K_{ATP} channel activity increases in isolated goldfish cardiomyocytes acclimated to low temperatures (7 °C) (Ganim et al. 1998). Channel activity does not seem to influence the characteristics of the action potential however, as glibenclamide had no effect on action potential duration when tested at the acclimation temperature (Ganim et al. 1998). Taking into account the obvious species specific differences revealed by our study, however, we cannot rule out the possibility that glibenclamide could affect the characteristics of the action potential in yellowtail flounder cardiomyocytes. Our results

suggest that sarc K_{ATP} channels are normally active on a beat to beat basis in the yellowtail flounder heart at this temperature, and may therefore be important in the regulation of contractility.

The observation of impaired force development in glibenclamide treated preparations can not be explained based upon available literature dealing with E-C coupling in either mammalian or fish cardiomyocytes. Theoretically, blocking sarc K_{ATP} channel activity with glibenclamide should lengthen the duration of the action potential and enhance Ca^{2+} influx through L-type channels. Inhibiting sarc K_{ATP} activity would decrease net cellular K^+ efflux and cause the sarcolemmal membrane potential to become more positive. A more positive membrane potential could, in turn, increase reverse Na^+/Ca^{2+} exchange, which has been shown to contribute a significant amount of activator Ca^{2+} at more depolarised membrane potentials in the fish heart (Vornanen 1999). By all accounts, glibenclamide should have facilitated an increase in twitch force development through enhanced Ca^{2+} influx across the sarcolemmal membrane. Further investigations on the membrane events associated with sarc K_{ATP} channel opening are necessary to explain this observation.

Altering K_{ATP} channel activity in cod ventricle strips did not affect force development or resting tension under any of the conditions tested. The data suggest that Atlantic cod do not possess cardiac K_{ATP} channels sensitive to the pharmacological agents used, or that all of the factors needed to alter K_{ATP} channel activity are not present in this tissue. K_{ATP} channel activity is sensitive to [ATP], Mg^{2+} , and other nucleotide concentrations as well as a host of other factors (Terzic et al. 1995). The characteristics of the intracellular environment in cod may lead to differences in the activation state of

K_{ATP} channels, and hence the effectiveness of channel modulators in this animal. Evolutionary differences within teleost fishes, and between fishes and mammals may influence the sensitivity of K_{ATP} channels to pharmacological manipulation which could also have implications when studying these channels in more primitive vertebrate hearts.

This study, along with the investigation presented in Chapter 2, have shown the potential importance of K_{ATP} channels in the modulation of cardiac function in anoxia tolerant fishes. The novel effects of mito K_{ATP} channel modulators in the anoxia tolerant fish heart model suggests substantial differences in the role of these channels over those described for more complex mammalian systems. Future studies should address in more detail the exact mechanisms by which these channels influence contractility in the fish heart as well as their role in hypoxic cardioprotection in other ectothermic vertebrates.

3.5 References

Baker JE, Holman P, and Gross GJ. Preconditioning in immature rabbit hearts: role of K_{ATP} channels. *Circulation* 99: 1249-1254, 1999.

Driedzic WR, and Bailey JR. Isolated perfused fish hearts. In: *Biochemistry and molecular biology of fishes*, vol 3, edited by Hochachka PW, and Mommsen TP. Amsterdam: Elsevier Science B.V., 1994, p. 119-125.

Driedzic WR, and Gesser H. Energy metabolism and contractility in ectothermic vertebrate hearts: hypoxia, acidosis, and low temperature. *Physiol Rev* 74: 221-257, 1994.

Ganim R, Peckol E, Larkin J, Ruchhoeft M, and Cameron J. ATP-sensitive K^+ channels in cardiac muscle from cold-acclimated goldfish: characterisation and altered response to ATP. *Comp Biochem Physiol* 119A: 395-401, 1998.

Gesser H, and Poupa O. Relations between heart muscle enzyme pattern and directly measured tolerance to acute anoxia. *Comp. Biochem. Physiol.* 48A: 97-103, 1974

Gesser H, and Poupa O. The role of intracellular Ca^{2+} under hypercapnic acidosis of cardiac muscle: comparative aspects. *J Comp Physiol* 127: 307-313, 1978.

Gesser H. and Poupa O. Effects of different types of acidosis and Ca^{2+} on cardiac contractility in the flounder (*Pleuronectes flesus*). *J Comp Physiol* 131: 293-296, 1979.

Gross GJ. and RM Fryer. Sarcolemmal versus mitochondrial ATP-sensitive K^+ channels and myocardial preconditioning. *Circ Res* 84: 973-979, 1999.

Hartmund T. and Gesser H. Cardiac force and high-energy phosphates under metabolic inhibition in four ectothermic vertebrates. *Am J Physiol Regulatory Integrative Comp Physiol* 271: R946-R954, 1996.

Hoglund L. and Gesser H. Electrical and mechanical activity in heart tissue of flounder and rainbow trout during acidosis. *Comp Biochem Physiol* 87A: 543-546, 1987.

Holmuhamedov EL, Jovanovic S, Dzeja PP, Jovanovic A, and Terzic A. Mitochondrial ATP-sensitive K^+ channels modulate cardiac mitochondrial function. *Am J Physiol Heart Circ Physiol* 275: H1567-H1576, 1998.

Holmuhamedov EL, Wang L, and Terzic A. ATP-sensitive K^+ channel openers prevent Ca^{2+} overload in rat cardiac mitochondria. *J Physiol* 519.2: 347-360, 1999.

Hu H, Sato T, Seharaseyon J, Liu Y, Johns DC, O'Rourke B, and Marbán E. Pharmacological and histochemical distinctions between molecularly defined

sarcolemmal K_{ATP} channels and native cardiac mitochondrial K_{ATP} channels. *Mol Pharmacol* 55: 1000-1005, 1999.

Inoue I, Nagase K, Kishi K, and Higuti T. ATP-sensitive K^+ channel in the mitochondrial inner membrane. *Nature* 352: 244-247, 1991.

Kantor PF, Coetzee WA, Carmeliet EE, Dennis SC, and Opie LH. Reduction of ischemic K^+ loss and arrhythmias in rat hearts. Effect of glibenclamide, a sulfonylurea. *Circ Res* 66: 478-485, 1990.

Noma A. ATP-regulated K^+ channels in cardiac muscle. *Nature* 305: 147-148, 1983.

Poupa O, and Johansen K. Adaptive tolerance of fish myocardium to hypercapnic acidosis. *Am J Physiol* 228(3): 684-688, 1975.

Sato T, Sasaki N, Seharaseyon J, O'Rourke B, and Marbán E. Selective pharmacological agents implicate mitochondrial but not sarcolemmal K_{ATP} channels in ischemic cardioprotection. *Circulation* 101: 2418-2423, 2000.

Terzic A, Jahangir A, and Kurachi Y. Cardiac ATP-sensitive K^+ channels: regulation by intracellular nucleotides and K^+ channel-opening drugs. *Am J Physiol Cell Physiol* 269: C525-C545, 1995.

Tibbits GF, Hove-Madsen L, and Bers DM. Calcium transport and the regulation of cardiac contractility in teleosts: a comparison with higher vertebrates. *Can J Zool* 69: 2014-2019, 1991.

Venkatesh N, Lamp ST, and Weiss JN. Sulfonyleureas, ATP-sensitive K^+ channels, and cellular K^+ loss during hypoxia, ischemia, and metabolic inhibition in mammalian ventricle. *Circ Res* 69: 623-637, 1991.

Vornanen M. L-type Ca^{2+} current in fish cardiac myocytes: effects of thermal acclimation and β -adrenergic stimulation. *J Exp Biol* 201: 533-547, 1998.

Vornanen M. Na^+/Ca^{2+} exchange current in ventricular myocytes of fish heart: contribution to sarcolemmal Ca^{2+} influx. *J Exp Biol* 202:1763-1775, 1999.

Wilde AA, Escande D, Schumacher CA, Thuringer D, Mestre M, Fiolet JW, and Janse MJ. Potassium accumulation in the globally ischemic mammalian heart. A role for the ATP-sensitive potassium channel. *Circ Res* 67: 835-843, 1990.

4 Summary

The research within this thesis focuses on the effects of low oxygen levels on ion trafficking in the fish heart. Cardiac muscle in fishes has been well studied; however, many details concerning ion flux in cardiomyocytes during anoxia are unknown. Over the past decade clinical evidence has accumulated showing ATP-sensitive K^+ channels (K_{ATP}) play a major role in protecting mammalian hearts from injury during periods of hypoxia or ischemia yet almost nothing is known of these channels in fish. Ganim et al. (1998) studied K_{ATP} channels in goldfish cardiomyocytes but their focus was on the contribution of these channels to the physiology of temperature acclimation. K_{ATP} channels open when intracellular [ATP] falls, rendering them most likely to contribute to heart function under conditions of metabolic stress, such as hypoxia. The clinical knowledge base, along with the above mentioned study prompted my research into K_{ATP} channels in the anoxic fish heart.

Chapter 1 provides background knowledge on anatomy and ion cycling in the fish heart which is required to understand the cytological environment in which K_{ATP} channels function and how they interact with other cellular processes. In chapter 2, mito K_{ATP} channel activity was considered in acari-bodo, a species of fish living at high temperatures and showing extreme cardiac anoxia tolerance. Since acari-bodo inhabits waters of 25 °C or higher, the processes underlying contractility were likely to be much quicker and more pronounced than expected for north temperate zone fishes, which are generally acclimated to waters below ~ 15 °C.

Chapter 2 focussed on mito K_{ATP} activity due to the emerging consensus in the literature that this channel was more important to mammalian cardioprotection than the sarcolemmal channel. Mito K_{ATP} channels had also never been studied in the fish heart and it seemed important to establish their presence before continuing on to more detailed investigations. It was also logistically more difficult to study sarc K_{ATP} channels due to time constraints in the field and because specific agents for manipulating sarc K_{ATP} activity were not readily available.

In Chapter 3 an attempt was made to address both mitochondrial and sarcolemmal K_{ATP} channel activity in two species of cold-water marine teleosts. Yellowtail flounder, like acari-bodo, is thought to have good whole animal and cardiac hypoxia tolerance. As described in chapter 3, most flatfish also exhibit a non-typical cardiac response to anoxia relative to other species studied. Based on the results of the first study and because K_{ATP} channels are so intimately involved in hypoxic cardioprotection in mammals, I felt it would be interesting to compare cardiac muscle from an anoxia tolerant species (yellowtail flounder), with that from a less hardy animal. Atlantic cod was chosen for the comparison since previous investigations have shown cod to have rather poor cardiac anoxia tolerance. The experiment was designed to compliment the first study in that it provided further evidence for the presence of mito K_{ATP} channels in the fish heart and extended our knowledge concerning the implications of channel opening under physiologically relevant conditions.

The results presented in Chapter 2 showed that mito K_{ATP} channels were important in acari-bodo heart, and they pointed toward a clear function of these channels under physiological conditions. When myocardium from acari-bodo was subjected to

anoxia, mitochondrial K_{ATP} channels contributed to a down regulation of cardiac contractility: an important function which would preserve energy reserves and allow the heart to survive long bouts hypoxia typically incurred by this animal. Mito K_{ATP} channels also contributed to a reduction in Ca^{2+}_i loading in the acari-bodo heart during reoxygenation. Reoxygenation following extended hypoxia often results in much more extensive cardiac injury than hypoxia alone, a component of which is due to Ca^{2+}_i loading. As such, limiting Ca^{2+}_i loading and reoxygenation injury may be an important part of overall cardiac hypoxia tolerance.

Chapter 3 presented evidence of interspecies differences in the ability of K_{ATP} channel modulators to affect contractility in the fish heart. Though altering mito K_{ATP} channel activity in the yellowtail flounder heart affected contractility, a clear role for these channels under physiological conditions was not clear. Blocking mito K_{ATP} channels reduced the decline in force production observed during oxygenated conditions but had little effect on anoxic contractility in the flounder heart. Evidence was presented that sarc K_{ATP} channels may play a role in the regulation of contractility on a beat to beat basis in flounder heart, although further study with more specific pharmacological agents would be useful in clarifying this. Interestingly, no evidence of K_{ATP} channels could be found in Atlantic cod heart.

Flounder are generally thought to be more tolerant to anoxia than cod, and the presence of K_{ATP} activity in the yellowtail flounder heart is consistent with the hypothesis that these channels contribute to hypoxia tolerance in the fish heart. Unfortunately the results presented in Chapter 3 did not point to a clear role for these channels in cardiac anoxia tolerance. Results from experiments involving the mito K_{ATP} blocker 5HD were

somewhat consistent with those gathered from acari-bodo heart in Chapter 2; however, the extreme interspecies differences between these fish make it difficult to draw comparisons. Differences in the characteristics of the whole animal response to hypoxia in these fish may lead to misleading results using an *in vitro* model. Yellowtail flounder are normally sessile, spending much of their time laying inactive on the bottom, while Atlantic cod are more active, schooling fish, better able to escape hypoxic waters than the flounder. Unfortunately, it is unclear how behavioral differences may influence hypoxic contractility at the cellular level in the fish heart.

The data presented within this report leads to additional questions regarding K_{ATP} channels in the fish heart. The influence of pharmacological channel blockers on contractility during oxygenated conditions suggest that in some fish, either mitochondrial or sarc K_{ATP} channels may be active on a beat to beat basis. With the exceptions mentioned earlier, mammalian studies have generally found that K_{ATP} channels are not active under normal conditions. Elucidating the contribution of K_{ATP} channels in the normoxic fish heart may help to clarify processes involved in E-C coupling and explain the apparent differences between fish and mammals. Moreover, additional study is required on the relationship between mito K_{ATP} channels and cardiac hypoxia tolerance in fish. Hypoxia tolerance has been extensively studied in fish; however, the information presented within reveals a new, previously undescribed mechanism by which protection may be achieved in the fish heart. The description of a novel means of increasing cardiac hypoxia survival in fish emphasises the need for continued investigation into this field.

