THE EFFECTS OF EXERCISE TRAINING ON MYOCYTE pH_i REGULATION

by

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in the School
of
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The effects of exercise training on myocyte pHi regulation

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ABSTRACT

Myocardial ischemia, inadequate coronary perfusion relative to demand, is a leading cause of death in North America. Ischemia suppresses the oxidation of glucose and fatty acids and accelerates glycolysis in the cardiomyocytes. One serious consequence of this is the generation of a substantive proton load and the inhibition of transporters which normally serve to negate it in these cells. Epidemiological and experimental evidence indicate that exercise training reduces both the occurrence of ischemia (primary prevention) and the degree of post ischemia myocardial damage (secondary prevention or rehabilitation). The mechanisms for this protective effect, however, are not known. One possibility through which training-induced adaptations may allow the heart to cope better with an hypoxic/ischemic insult is an adaptation in cardiomyocyte H^+ handling. A training-induced increase in H^+ intracellular buffering capacity (β_i) and/or the activity of Na^+-H^+ exchanger, a paramount cardiomyocyte pH regulator, may serve as a protective adaptation. The studies in this thesis, therefore, sought to assess if exercise training can induce adaptations in cardiac H^+ handling. Intracellular buffering capacity (β_i) and the rate of Na^+-H^+ exchange (NHE) using single isolated cardiomyocytes from sedentary control (C) and exercise trained (T) rats were determined. All experiments were conducted in HCO_3^- - free Tyrode's solution (pH_0 7.4 @ 37°C) in myocytes loaded with 15 μM of the pH_i fluorophore C-SNARF-1 AM. Cardiomyocyte pH_i was determined by exciting the cells at 510 nm and measuring the fluorescent emissions at 580 and 640 nm. Intracellular buffering capacity was determined by exposing the cardiomyocytes to a solution of 20 mM NH_4Cl, 5 mM $BaCl_2$ and 1.5 mM amiloride (A) for 120 s. The $[NH_4Cl]_o$ was then sequentially reduced at 120 intervals to 10, 5, 2.5, 1.25, and finally 0 mM NH_4Cl. β_i was calculated using the equation: $\beta_i = \Delta(NH_4)_i/\Delta pH_i$. To study NHE kinetics, the NH_4Cl (20 mM) prepulse technique was used both in the presence (+A) and absence (-A) of 1.5 mM

amiloride, an inhibitor of NHE. The results from all experiments are shown as mean (SEM). β_i of group T was found to be significantly increased. The average $\Delta\beta_i/\Delta pH_i$ slope was -83.3 and -192.9 $mM \cdot pH^{-1}$ in C and T, respectively. At pH_i 7.0, β_i was 22.4 (2.9) and 44.5 (5.9) mM in C and T, respectively. However there were no significant ($p>0.05$) differences between the groups in the initial rate of pH_i recovery in response to the acidosis created by the NH_4Cl prepulse in both the absence of amiloride [0.074 (0.013) and 0.078 (0.009) pH $units \cdot min^{-1}$ in C and T, respectively and the presence of amiloride [0.021 (0.004) and 0.029 (0.003) pH $units \cdot min^{-1}$ in C and T, respectively]. Nevertheless, the amiloride-dependent (-A +A difference) rate of pH_i recovery was significantly ($p<0.05$) less in group T compared to C. The acid efflux (J_{H^+}) rate during the recovery was calculated as the product of β_i and the instantaneous dpH_i/dt. The amiloride-sensitive component of J_{H^+} was significantly greater in group T despite the fact that the rate of pH_i recovery was less. These substantive training-induced adaptations of cardiomyocyte H^+ handling may play a crucial role in the ability of the heart to cope with hypoxic/ischemic insults.

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TABLE OF CONTENTS

Approval ii

Abstract iii

Acknowledgments v

Table of Contents vi

List of Figures x

List of Tables xi

CHAPTER I. REVIEW OF THE LITERATURE AND STATEMENT OF THE PROBLEM 1

A. INTRODUCTION 1

B. ISCHEMIA/REPERFUSION INJURY 3

C. EFFECT OF INTRACELLULAR ACIDOSIS ON CONTRACTILITY 6

D. REGULATION OF pH 7

E. HYPOTHESIS AND OBJECTIVES 9

CHAPTER II. MATERIALS AND METHODS 12

A. ANIMAL CARE AND TRAINING 12

B. CARDIOMYOCYTE ISOLATION AND PLATING 12

1. Solutions for Cell Isolation and Plating 12

2. Procedures for Isolation and Plating 13

3. Myocyte Evaluation Criteria 14

C. MEASUREMENT OF pH_i AND CONTRACTILITY 15

1. Optical Set-up 15

2. Cell Stimulation 16

3. Measurement of Contractility 16

4. Measurement of Intracellular pH 17

a. Solutions 17

b. Loading SNARF-AM into Myocytes 17

c. SNARF-1 Emmissions 17

d. SNARF-1 Calibration 18

-*In vitro* Calibration 18

-*In vivo* Calibration 19

e. pH_i Calculation 20

D. EXPERIMENTAL PROTOCOLS 24

1. Accumulation of Weak Base to Change Intracellular pH (An Introduction) 24

2. Determination of Intracellular H^+ Buffering Capacity (β_i) 26

3. Determination of Contractile Sensitivity to pH_i 28

4. Determination of Na^+/H^+ Exchanger Activity 31

5. Determination of SNARF-1 Compartmentalization 33

6. Data Analyses 33

CHAPTER III. RESULTS 34

A. THE EFFECTS OF NH_4Cl ON pH_i AND THE MAGNITUDE

OF β_i. 34

B. THE EFFECTS OF EXERCISE TARINING ON pH_i AND β_i 38

C. THE EFFECTS OF AMILORIDE ON THE ACTIVITY
OF Na^+/H^+-EXCHANGER 41

D. THE EFFECTS OF EXERCISE TRAINING ON dpH_i/dt AND J_{H^+} 44

E. THE EFFECTS OF EXERCISE TRAINING
ON RESTING MYOCYTE LENGTH 49

CHAPTER IV. DISCUSION 52

A. INFLUENCE OF EXERCISE TRAINING ON β_i 52

B. EXERCISE TRAINING AND J_{H^+} 55

C. INFLUENCE OF EXERCISE TRAINING INDUCED CHANGES
IN pH_i AND β_i ON CONTRACTILITY 58

CHAPTER V. CONCLUSION 62

APPENDICES 64

Appendix 1. Optics 64

1.1. Optical Setup for SNARF-1 64

1.2. Emission Spectra for SNARF-1 65

Appendix 2. Principle Operation of Video Edge Detector 66

2.1. Principles of Video Edge Detector 66

2.2. Video Edge Detector Calibration 67

Appendix 3. Myocyte isolation and plating solution 68

3.1. Calcium-free Solution 68

3.2. Collagenase Solution 68

3.3. Washing Solutions 69

3.4. Solution for cell recovery 69

3.5. Laminin stock solution 69

3.6. Composition of myocyte isolation and plating solution 70

3.7. Composition of M199 and MEM Solutions 71

Appendix 4. Calibration Solutions 73

4.1. *In vitro* calibration solutions 73

4.2. *In vivo* calibration solutions 73

4.3. Composition of *in vitro* calibration solutions 74

4.4. Composition of *in vivo* calibration solutions 74

Appendix 5. pH_i measurements 75

5.1. pH challenge and measurement solutions 75

5.2. Composition of pH measurement solutions 75

LIST OF REFRENCES 76

LIST OF FIGURES

NUMBER PAGE

1. *In vitro* pH calibration of 580nm/640nm SNARF-1 emission ratio 21
2. Calibration of SNARF-1 fluorescence ratio in a resting myocyte 22
3. Conversion of ratio vs time to pH_i vs time 23
4. Principles of pH_i changes upon exposure to NH_4Cl 29
5. An example of β_i determination in rat ventricular myocyte 30
6. An example of β_i calculation in rat ventricular myocyte 36
7. Composite data of β_i over the pH_i range 7.69-6.22 37
8. Dependence of β_i on pH_i within pH_i range 7.26-6.71 40
9. An example of determinationof Na^+/H^+-exchanger ativity 43
10. dpH_i/dt as function of pH_i 45
11. Amiloride sensitive component of dpH_i/dt as a function of pH_i 46
12. J_{H^+} as a function of pH_i 47
13. Amiloride sensitive component of J_{H^+} 48
14. Relationship between changes in pH_i and myocyte length of group C 50
15. Relationship between changes in pH_i and myocyte length of group T 51

LIST OF TABLES

NUMBER PAGE

1. The effects of exercise training on pH_i and β_i 39

2. The mean 'initial' dpH_i/dt and J_{H^+} 42

CHAPTER I

REVIEW OF THE LITERATURE AND STATEMENT OF THE PROBLEM

A. INTRODUCTION

For more than 50 years, coronary heart disease (CHD) has been the prime cause of mortality in the economically developed countries of the western world (Kaplan 1982, Reimer and Jennings 1992). Although the rate of CHD has been declining for the last decade, in Canada it presently accounts for 39% of all deaths (Canadians and Heart Health 1995). Clinically, CHD normally results from one of the following abnormalities in the coronary arteries: atherosclerosis, thrombosis or spasm (Reimer and Jennings 1992). These pathologies can result in myocardial ischemia, defined as inadequate coronary blood flow relative to myocardial demand (Amsterdam 1973), which in turn can result in serious damage or death to cardiac tissue. Since cardiomyocytes are generally post-mitotic, myocardial ischemia can result in loss of irreplaceable tissue, serious cardiac dysfunction and death.

Despite the fact that the effect of regular exercise on CHD has been studied for more than 5 decades, a sedentary lifestyle was first cited as a risk factor for CHD by the American Heart Association only three years ago (Fletcher *et al.* 1992). This is a consequence, undoubtedly, of the multifactorial processes involved in CHD and the difficulty of earlier studies to account for other confounding variables. However, several seminal studies published in the period of 1986-92 (Paffenbarger *et al.* 1986, Powel *et al.* 1987, Salonen, Leon *et al.* 1987, Blair *et al.* 1990, Berlin and Colditz 1990, Haskell *et al.*1992) have resulted in the now generally accepted premise that regular exercise reduces

the morbidity and mortality associated with CHD. Nevertheless, the mechanism(s) of the preventive effects of exercise are not known. Known risk factors for CHD have been studied and it has been suggested that the therapeutic effects of exercise are due to:

- lower body mass index (kg/m^2) and percentage of body fat (Dannenberg *et al.* 1989, Wood *et al.* 1988,1991, Young *et al.* 1993)
- improvement of lipoprotein profiles (primarily a decrease in LDL/HDL) (Dannenberg *et al.* 1989, Folsom *et al.* 1985, Leon *et al.* 1987, Young *et al.* 1993)
- lower plasma fibrinogen concentration (Stratton *et al.* 1991)
- decrease in systolic and diastolic blood pressure levels in both normotensive subjects (Garcia-Palmieri *et al.* 1982, Folsom *et al.* 1985, Reaven, Barret-Connor, Edelstein 1991) and mild to moderate hypertensive subjects (Seals and Hagberg 1984, Hagberg 1988).

While these risk factors are implicated in the etiology of atherosclerosis, the question must also be raised as to whether the heart itself adapts to exercise making it less susceptible to ischemia/reperfusion injury. For example, in clinical studies, endurance exercise not only reduces the occurrence of ischemia (primary prevention of ischemia-infarction heart disease, IHD) but also reduces the degree of myocardial damage that occurs as a complication of ischemia (Seals and Hagberg 1984, Hagberg 1988, Haskell *et al.* 1992, Bittner 1994). Similar findings have also been demonstrated in animal models of ischemia/reperfusion injury (Bersohn and Scheuer 1978, Brinkman *et al.* 1988, Carey, Tipton and Lund 1976, Bowles, Farrar and Starnes 1992, Bowles and Farrar 1994). Cardiac performance, as evaluated by changes in dP/dt, was reported to be less impaired in

trained animals when the animals were subjected to a hypoxic environment (Carey, Tipton and Lund 1976). Two general hypotheses have been suggested as mechanisms:

- alterations in the structure of vascular beds and/or their response to vasoactive substances (Haslam and Cobb 1982, Leon and Bloor 1968, Kramsch *et al.*1981, Cohen *et al.* 1982, Bloor *et al.*1984, Roth *et al.* 1990, Muller et al.1991, Laughlin and McAllister 1992, Pelliccia *et al.* 1990) which serve to reduce the magnitude of the ischemic insult.
- improvements in the response of the myocyte to ischemia/reperfusion insults which reduce tissue damage and hence enhance organ and organismal survival.

There is some evidence for the former training-induced improvement of the coronary vasculature. Both animal histological measurements in rats (Haslam and Cobb 1982, Leon and Bloor 1968) and primates (Kramsch *et al.*1981) and human echocardiographic estimates (Pelliccia *et al.* 1990) of coronary arteries have demonstrated that exercise training-induced enlargement of coronary arteries. These changes could be due to releasing vasodilators and/or an increase in capillary number and collateral development (Cohen *et al.* 1982, Bloor *et al.*1984, Roth *et al.* 1990, Muller *et al.*1991, Laughlin and Mc.Allister 1992). This study, however, will address possible adaptations that are intrinsic to the cardiomyocyte itself that may serve to limit ischemia reperfusion damage.

B. ISCHEMIA/REPERFUSION INJURY

Ischemia results in the deficiency of not only the delivery of oxygen and substrates for energy production (Amsterdam 1973), but also in the removal of catabolites such as hydrogen ions (released during ATP hydrolysis) and lactate. As a consequence, the tissue

manifests intracellular acidosis, reduced [ATP], accumulation of metabolites and many other changes, the magnitude of which depend on the duration and the severity of the ischemic insult. Myocardial reperfusion often results in an exacerbation of cardiac tissue damage (Bond, Herman, and LeMaster 1991, Reimer and Jennings 1986).

Reperfusion injury is associated with the re-establishment of normal blood flow with its attendant reintroduction of molecular oxygen, Ca^{2+} and inflammatory cells such as neutrophils into previously ischemic tissue (Simpson and Lucchesi 1987, McCord and Roy 1982, Karwatowska *et al.*1992, Lucchesi, Werns, and Joseph 1989). While the mechanisms for cell dysfunction in ischemia/reperfusion injury remain controversial, several theories are frequently cited:

- cytosolic Ca^{2+} overload
- generation of oxygen-derived free radicals (ODFR)
- intracellular osmotic overload
- increase in cytosolic free fatty acid levels
- alterations in membrane structure/function
- cytosolic H^+ overload

It must be stated from the outset that these proposed mechanisms are not necessarily mutually exclusive.

Although contractile activity may stop within the first 10-15 seconds after occlusion, ischemic myocytes may be reversibly injured only after as long as 15-30 minutes after the onset of ischemia. Reperfusion in this period of time can result in cells that appear to have normal ultrastructure and high energy phosphate, Ca^{2+} and H^+ levels. However, often normal contractile activity does not recover immediately and dysfunction

may persist for hours or days in experimental animals (Reimer, Jennings and Tatum 1983, Schrader 1985, Reimer and Jennings 1992) resulting in what is referred to as post-ischemic contractile dysfunction or myocardial stunning (Opie 1992, Reimer and Jennings 1992).

A recent (and intriguing) concept to be incorporated into the ischemia/reperfusion literature is "ischemia preconditioning". This term refers to the observation that one or more episodes of brief ischemia, each interrupted by brief reperfusion, paradoxically protect the heart from subsequent sustained coronary artery occlusion (Murry, Jennings, Reimer 1986, Kloner and Przyklenk 1993). Ischemia preconditioning was first documented by Murry, Jennings, Reimer (1986) in the canine model. In these experiments they found that four repeated episodes of five minutes each of coronary occlusion plus five minutes of reperfusion resulted in a markedly reduced infarct size in response to a subsequent 40 minutes of coronary occlusion followed by four days of reflow. Infarct size reduction with ischemic preconditioning has been shown by others to occur in dogs (Ovize *et al.* 1992, Murry, Jennings, Reimer 1986), pigs (Schott *et al.* 1990), rats (Li, Whittaker, Kloner 1992), and rabbits (Cohen, Liu, Downey 1991). A recent angioplasty study suggests that this phenomenon might also occur clinically. Deutsch and colleagues (1990) reported less chest pain, less ST segment elevation, and less lactate production in patients during the second sequential balloon inflation than during the first inflation. The mechanisms responsible for this protection, and the clinical relevance of ischemia preconditioning are unknown.

With respect to factors determining the degree of recovery from ischemia reperfusion injury, pH_i must be considered for several reasons. It has been reported recently that a rapid return of pH_i back to normal level as happens during ischemic

reperfusion may cause myocyte hypercontraction and loss of viability (Bond, Herman and LeMaster 1991, Bond *et al.*1993, Bond *et al.*1994). Conversely, maintenance of acidotic pH_i following reperfusion may offer protection for hypoxic myocytes (Bond, Herman and LeMaster 1991). This observation has been termed the pH paradox and underscores the importance of pH regulation in the normal and ischemic myocardium. Furthermore the interdependence of putative causative factors is shown by the observation that a Ca^{2+} overload can result from changes in pH_i. During ischemia the transsarcolemmal H^+ gradient is abolished and $[H^+]_i$ increases (Nishida *et al.*1993). Reperfusion will result in cellular H^+ efflux and Na^+ influx via Na^+-H^+ exchanger (Nishida *et al.*1993) resulting in elevated $[Na^+]_i$. The latter has been demonstrated to result in Ca^{+2} influx via reverse mode of the Na^+-Ca^{2+} exchanger (Kim, Cragoe, and Smith 1987, Tani and Neely 1989). Thus pH_i regulation appears to be critical and is the focus of this study.

C. EFFECT OF INTRACELLULAR ACIDOSIS ON CONTRACTILITY

It is well documented that acidosis reduces the contractile strength of cardiac myocytes. Although the precise mechanisms by which acidosis affects contractile function are not well understood, they are presumed to be due to multiple sites of action of H^+ on the excitation-contraction (E-C) coupling process. One well documented effect is the acidosis-induced reduction in myofilament sensitivity to Ca^{2+} (Solaro, El-Saleh and Kentish 1989, Orchard and Kentish 1990) and the attendant contractile impairment (Steenbergen *et al.*1987, Marban *et al.* 1987). However reduced pH_i also has strong effects on intracellular calcium homeostasis including: 1) inhibition of calcium currents via the L-type calcium channel (Kaibara and Kameyama, 1988); 2) reduction of SR calcium uptake and

subsequently release to cytoplasm (Fabiato and Fabiato 1978, Fabiato 1985), and 3) displacement of Ca^{2+} from its cytoplasmic buffer sites (Orchard and Kentish 1990). Calcium delivery may be increased or decreased as a consequence of acidosis (Boyett, Kirby and Orchard 1988, Fry and Poole-Wilson 1981, Hecht and Hutter 1965). Clearly the regulation of pH_i is critical in the regulation of contractility.

D. REGULATION OF pH

Both intracellular and vascular pH levels are very tightly regulated in mammals. For example, in rat ventricles at temperatures of 30-37°C, pH_o is normally in the range of 7.25-7.42, and pH_i 7.02-7.30 (Roos and Boron 1981). However even under normal physiological conditions protons are continuously generated by the intense metabolic activity of cardiac cells (Lazdunski, Frelin, and Vigne 1985). A number of metabolic reactions which involve or produce inorganic phosphate (P_i), H^+ and lactate that subsequently create acidosis are : 1) ATP$\leftrightarrow$ ADP + P_i + H^+ , 2) PCr $\leftrightarrow$ Cr + P_i + H^+, and 3) glucose $\leftrightarrow$ 2 lactate + $2H^+$ (Allen and Orchard 1987, Dennis, Gevers, and Opie 1991). Furthermore, at a pH_o of 7.4 and pH_i of 7.0, the H^+ equilibrium potential is ~-23 mV. Thus at a resting membrane potential of -70 mV, there is a very strong inwardly-directed proton electrochemical gradient. For H^+ to be at equilibrium at -70 mV, pH_i would have to be ~6.2, therefore the cytosol is almost one pH unit more alkaline than would be expected if H^+ were in equilibrium. Hence, in cardiac cells, like in all other cells, in addition to intracellular H^+ buffering, active processes must be present in the plasma membrane to remove H^+ from the cytosol. These membrane transporters include the Na^+-H^+ exchanger and HCO_3^- transporters (Murer *et al.* 1976, Deitmer and Ellis 1980;

Piwnica-Worms, Jacob, Horres and Lieberman 1985, Frelin, Vigne, Ladoux and Lazdunski 1988, Liu *et al.* 1990). The Na^+-H^+ exchanger/antiporter catalyzes the reversible and electroneutral exchange of Na^+ for H^+. The driving force for H^+ extrusion is provided by the inwardly-directed Na^+ electrochemical gradient across the membrane, therefore, the system is secondarily dependent on ATP hydrolysis by the (Na^+-K^+) ATPase (Frelin, Vigne, Ladoux and Lazdunski 1988, Orchard and Kentish 1990) and experimental evidence suggests that this is the predominant regulator of pH_i in cardiomyocytes in response to a proton load. Amiloride (3,5-diamino-6-chloro-N-(diaminomethylene) pyrazine carboxamide) and its derivatives are known to inhibit the Na^+-H^+-exchange (NHE), making them useful for studying the kinetics of this exchanger.

Although the role of HCO_3^- dependent mechanisms in pH_i regulation in mammalian hearts has attracted less attention than the role of the Na^+-H^+ exchanger, they have been postulated recently to play significant role in regulation of pH_i (Frelin, Vigne, Ladoux and Lazdunski 1988, Lagadic-Gossmann, Buckler and Vaughan-Jones 1992). A net influx of HCO_3^- contributes to cell alkalization, whereas a net efflux leads to cell acidification. Regulation of pH_i by bicarbonate requires both the presence of a membrane bicarbonate-transport system, a high membrane permeability to CO_2 and the enzyme carbonic anhydrase (CA). CA catalyzes the reaction : $HCO_3^- + H^+ \leftrightarrow CO_2 + H_2O$ (Frelin, Vigne, Ladoux and Lazdunski 1988). Three major bicarbonate-dependent mechanisms have been identified in experiments that combine pH_i recovery, and Na^+ and Cl^- flux measurements (Lagadic-Gossmann, Buckler and Vaughan-Jones 1992; Frelin, Vigne, Ladoux and Lazdunski 1988). These are : 1) an electroneutral Na^+-dependent HCO_3^-/Cl^- antiporter for cell alkalinization, 2) an electroneutral Na^+-independent HCO_3^-/Cl^- antiporter for cell

acidification, and 3) an electrogenic Na^+/HCO_3^- symporter as an uptake mechanism for HCO_3^- and hence for cell alkalinization (Frelin, Vigne, Ladoux and Lazdunski 1988).

Although H^+ buffering systems in ventricular myocytes are poorly identified, during the early restriction of coronary blood flow, protons are thought to be firstly buffered by histidine residues and by inorganic phosphate (Dennis, Gevers and Opie 1991). The intracellular buffering capacity (β_i) of most ventricular tissue varies with pH_i. In guinea pig myocytes, β_i was found to be 23 mM at pH 7.0 and this value is consistent with that found in the hearts of other mammalian species at this pH (Lagadic-Gossmann, Buckler and Vaughan-Jones 1992). Values for the β_i/pH_i slope have been observed to vary according to investigated pH_i range. According to Puceat *et al.* (1993) this value was 60 mM/pH unit over the pH_i range of 6.8-7.2. Walert and Frohlich (1989) found this to be 94 mM/pH unit and 122 mM/pH over the pH_i ranges of 7.0-7.2 and 6.4-6.8, respectively, for rat ventricular myocytes. Inorganic phosphate (P_i) is probably the major intracellular buffer and especially important in ischemic tissue (Allen and Orchard 1987). As phosphocreatinine (PCr) is broken down to help maintain ATP levels, P_i accumulation increases β_i, and depending on the pH, rapid P_i release may even produce a transient alkalosis (Dennis, Gevers and Opie 1991).

E. HYPOTHESIS AND OBJECTIVES

One possible mechanism through which training-induced adaptations may allow myocytes to cope better with an hypoxic/ischemic insult is by increasing the H^+ intracellular buffering capacity and/or the activity of specific sarcolemmal transports mechanisms to extrude excess protons from the cell. It is well known that the regulation

of intracellular pH in cardiac cells in response to a proton load is achieved by a combination of both of these processes (Vaughan-Jones 1988). A decrease in pH_i is well known to be a causal factor in the hypoxia/ischemia associated increase in cytosolic calcium. Moreover, accumulated H^+ decreases the myofilament sensitivity to Ca^{2+} that subsequently impairs the contractility. Although in heart muscle their role as a pH buffering system has not been elucidated, intracellular dipeptides (imidazole-ring contained histidines: L-carnosine and L-homocarnosine) have been recently reported to increase the sensitivity of myofilaments to Ca^{2+} in both skeletal and cardiac muscles (Lamont and Miller 1992). It is not known whether exercise training can alter the quantity of these dipeptides in the heart, however, in fast twitch skeletal muscle fibers of elite sprinters, carnosine and imidazole concentrations were found to be increased concomitant with an increased pH buffering capacity (McKenzie *et al.* 1983, Parkhouse *et al.* 1983) in comparison to other elite athletes. The principal mechanism identified for acid extrusion from cardiac cells is the Na^+-H^+ exchanger (Deitmer and Ellis 1980; Piwnica-worms, Jacob, Horres and Lieberman 1985) and this will also be studied.

1. Hypotheses

The experiments in this proposal were designed to test the following hypotheses:

Endurance training increases the ability of the cardiomyocytes to limit the degree of cytosolic acidification in response to a given proton load. This is implemented by:

1. increasing the cytosolic buffering capacity (β_i)
2. increasing the extrusion of H^+ by the Na^+/H^+ exchanger

2. Objectives

1. to determine the relationship between β_i and pH_i in the cardiomyocytes from the hearts of trained and sedentary rats. The literature values for the normal rat heart β_i determined by different techniques vary by more than three fold. In these experiments β_i will be determined from the following relationship:

$$\beta_i = \Delta\,[NH_4]_i / \Delta\,pH_i$$

2. to determine the relationship between cell (and sarcomere) length and pH_i in the cardiomyocytes of trained and sedentary rats. This parameter will be used to determine the contractile sensitivity (λ) to pH_i.
3. to determine the efficacy of the Na^+/H^+ exchanger in reversing cytosolic acidification in the cardiomyocytes of trained and sedentary rats. This will be determined as the rate of amiloride-dependent, HCO_3^- -independent change in pH in response to cytosolic acidification.

CHAPTER II

MATERIALS AND METHODS

A. ANIMAL CARE AND TRAINING

Female Sprague-Dawley rats, 8-10 weeks old, were obtained from the University of British Columbia (UBC) and housed in cages (4 per cage) at Simon Fraser University Animal Care Facility, on a 12h light-dark cycle. They were fed *ad libitum* a commercial rat diet with free access to drinking water. Rats were randomly divided into two groups C (sedentary control) and T (trained). Rats in group T participated in a progressive treadmill endurance protocol for 10-15 weeks, 5 days/wk, between 07.00 to 11.00 a.m. By 10 weeks, group T was running at 30 m/min, 5% grade, for 60 min/day. This final intensity and duration were maintained for another 5-wk period until all rats were sacrificed. All rats had a recovery period of 48-72 hr from the last exercise bout prior to sacrifice.

B. CARDIOMYOCYTE ISOLATION AND PLATING

1. Solutions for Cell Isolation and Plating

The myocyte isolation required 4 different types of solutions. These were: 1) calcium-free solution used to remove extracellular calcium, clear the coronary arteries and its branches, and cleave desmosomal and intermediate junctions, 2) collagenase solution used to digest connective tissue, 3) washing solutions (A, B and C) with progressively higher [Ca^{2+}] for the isolated myocytes, and 4) recovery solution. All solutions were made with Milli-Q grade water and the composition of each is shown in Appendix 3. For

myocyte plating, 20 μg laminin from Sigma (cat no.L2020)/ml Medium 199 was used and the procedure of dissolving of laminin in Medium 199 (stock solution) is in Appendix 3.5.

2. Procedures for Isolation and Plating

Ventricular myocytes were enzymatically dissociated with minor modifications of a technique previously described (Powell *et al.* 1980, Spitzer and Bridge 1992). Briefly, rats were subjected to narcosis with an intraperitoneal injection of sodium pentobarbital (Somnotol™ 6.5 mg/100g body weight). In order to prevent blood clotting, heparin was given simultaneously at a dose of 1.5 mg/100g body weight. After ensuring that the rat was anesthetized (no pain reflexes), the heart was rapidly excised after a midsternal thoracotomy, and the excess tissue was cut off. This isolated heart was immediately placed in a sterile 65 mm diameter Petri-dish containing 15 ml aerated Minimal Essential Medium (MEM) on ice. The heart was rapidly attached to an aortic canula in a laminar flow hood and then retrogradely perfused in a Langendorff manner for 3-4 minutes at 37°C. The perfusate contained calcium-free solution (50 ml non-recirculating MEM stock) that was continuously gassed with 100%O_2 and maintained at pH 7.40. In order to disrupt the extracellular matrix, the perfusate was then switched to 50 ml of the collagenase solution (Appendix 3.2). After 20-30 minutes of perfusion with this medium, perfusion was terminated and the heart was removed from Langendorff apparatus. The heart was placed in a large Petri dish containing collagenase solution to keep the heart moist. The ventricles were isolated by removing the atria, connective tissues and fat. The tissue was then mechanically dissociated by mincing it gently with a scalpel and then putting it back into the reservoir containing fresh collagenase solution to be aerated for 5-15 minutes. At

the same time, this solution was pipetted and released gently several times, every two minutes, for further mechanical disruption. The obtained myocytes then were filtered through coarse nylon mesh (200 μm size) into 50 ml Corning disposable sterile centrifuge tubes and centrifuged at 7 x g (setting no.2) in a IEC clinical centrifuge. The supernatant containing dead myocytes and debris was discarded and the pellet containing living single cardiac myocytes was washed in each of the three MEM washing solutions (buffer A, B. and C respectively). Finally, the pellet was resuspended in Medium 199/5%FBS (recovery solution) and allowed to recover for 2-3 hours at room temperature.

The technique for myocyte plating follows that of Haddad *et al.* 1988. Prior to cell plating, 25 mm round glass cover slips were etched in concentrated nitric acid, rinsed thoroughly with distilled water, and then put in Petri dishes (Corning 35x10 mm polystyrene dish). Cell adhesion to the coverslip was improved by spreading a thin film of the laminin stock solution (500 μl) on the coverslip for 1 hour at room temperature. After this time, the cell suspension was pipetted into 1 ml aliquots and plated onto these laminin-prepared surfaces at the density of approximately 2500 cells/ Petridish, to ensure the sufficient space between myocytes. After 30-60 min, the cells were then washed twice with and preincubated in Medium 199/5% FBS (cell recovery solution) for another hour. After another hour these cells were washed three times and finally incubated with the same solution.

3. Myocyte Evaluation Criteria.

Only myocytes which met the following criteria were used:

- rod shaped in appearance
- clear cytoplasm with well-defined striations
- defined edges with no sarcolemmal blebbing
- no spontaneous contractions
- minimal dimensions of 80 μm by 10 μm
- when stimulated must contract at least 5% of the quiescent length and show no bending during contraction

C. MEASUREMENT OF pH_i AND CONTRACTILITY

1. Optical Set-up

The optical setup for these measurements is shown in Appendix 1.1. The plated cells were mounted on the stage of a Nikon TMD inverted microscope at room temperature (~25°C). The pH fluorophore SNARF-1 was excited with 75 watt Xenon arc lamp and the excitation beam of collimated light was directed through a monochromator tuned to 515 $\pm$ 1 nm. The exposure of the cells to the excitation beam was controlled by a Uniblitz electronically-operated shutter which was governed by computer. The output from the monochromator was connected to a Nikon TMD Diaphot inverted microscope through a fibre optic cable. The 515 nm excitation beam was then reflected into the sample by a 540 nm dichroic mirror (DM) that was housed below a Nikon 40x quartz fluor oil immersion objective (NA 1.3) to excite the SNARF-1. The fluorescence emission from the cell was reflected out the microscope sideport to a 700 nm DM in a dual optical path tube (Nikon). This allowed wavelengths <700 nm to be reflected to a PTI photometer.

This photometer contains a pair of photon-counting photomultiplier tubes (Hamamatsu). This emission was directed to a 610 nm DM in the photometer that has 580 $\pm$ 10 nm (A) and 640 $\pm$ 10 nm (B) band pass filters. The output of each PMT was stored on a computer (586 PC) and the ratio of these fluorescence emissions (580:640, A/B ratio) was used to determine pH_i as described. Autofluorescence of the cell and the background fluorescence at each wavelength (normally $\leq$1% of the fluorescence signal from SNARF-1) were determined from an unloaded cell and subtracted from the SNARF-1 signal.

2. Cell Stimulation

In most of the experiments, the myocytes were not stimulated and remained therefore, quiescent. In those experiments in which cells were electrically stimulated, the following protocol were used. Single myocytes were field stimulated at a frequency of 0.5 Hz, 5-10 V (1.2 times threshold), with a duration of 5 ms. These electrical stimuli were delivered to the myocytes by a Grass S11 stimulator (Grass Medical Instruments, Quincy MA.) via a set of insulated platinum wires (0.25 mm tip diameter, Morton Thiokol Inc., Danvers MA.) positioned ~0.5 mm from the myocyte.

3. Measurement of Contractility

In order to determine contractility simultaneously with pH_i, the coverslip was also trans-illuminated with light from the lamp housing of the inverted microscope. This light was filtered with a 695 nm long band pass filter (Omega Optical). This near infrared (IR) output passed through the 700 nm DM to a black and white charge-coupled device (CCD) video camera (COHU CCD 4810 Series, San Diego, CA) that was attached to the

microscope side port, allowing the myocytes to be video imaged. The output from the video camera was analyzed by video edge detection (VED) instrumentation similar to that described by Steadman *et al.* (1988) (Appendix 2.1) and continuously visualized using a 27 inch television monitor (Sony). The myocyte cell length was displayed on line on a Tektronix oscilloscope.

4. Measurement of Intracellular pH

a. Solutions

For intracellular pH measurements, a variety of modified Tyrode's solutions were used and are described in Appendix 5. These solutions were used for bathing the cells, loading the cells with SNARF-AM, and in the experiments in which pH_i was challenged.

b. Loading SNARF-AM into Myocytes

The procedure for loading the myocytes with SNARF-AM (Molecular Probes, Eugene, OR) followed that of Spitzer and Bridge (1992) and Blank *et al.* (1992) with slight modifications. Briefly, after plating, cells were washed gently with (CO_2-HCO_3^-)-free Tyrode solution 2-3 times until the debris (dead cells) was removed. The plated cells then were equilibrated at 25°C with the same solution containing 15 μM SNARF-AM for another 10 minutes. The (CO_2-HCO_3^-)-free Tyrode's solution (without SNARF-AM) was then continuously applied to the cell bath to remove extracellular dye. Measurements of pH_i from these cells began no less than 10 minutes later.

c. SNARF-1 Emissions

The fluorescence spectra of SNARF-1 are shown in Appendix 1.2 and show a clear pH-dependent shift in emission wave-length. The emission spectrum of SNARF-1 when it is excited at 514 nm exhibits two well-separated peaks at approximately 580 nm (yellow-orange) and 640 nm (deep red), corresponding to the acidic and basic forms of the indicator, respectively. This spectral feature allows the indicator to be used in single excitation, dual emission ratio mode. SNARF-1 has a pK_a of about 7.6 (K_d ~25 nM) at room temperature and 7.3-7.4 at 37°C, making it useful for measuring pH changes in the 6.3 to 8.6 range (Whitaker, Haughland and Prendergast, 1991).

d. SNARF-1 Calibration

Calibration of the SNARF-1 signals was performed both without (*in vitro* calibration) and with myocytes (*in vivo* calibration). For the *in vitro* calibration, the unesterified form of SNARF-1 was used. This type of SNARF is hydrophilic and therefore cannot readily permeate cell membranes and is dissolved in water. Since the esterified form (acetoxymethyl ester - SNARF-AM) is hydrophobic, it is suitable for membrane permeation, and is dissolved in DMSO. In the cell, non-specific esterases cleave off the AM groups rendering the dye suitable for pH_i measurement. SNARF-AM has been widely used as an indicator of cytosolic pH in cardiomyocytes (Blank *et al.* 1992, Spitzer and Bridge 1992, Martinez-Zaguilan *et al.* 1991, Bassnett, Reisnisch and Beebe, 1990).

***In vitro* Calibration.** To characterize the spectral properties of the microspectrofluorometer, an *in vitro* calibration was conducted using 5 µM SNARF-1 and

seven different Tyrode's solutions of varying pH (6.50, 6.75, 7.00, 7.20, 7.40, 7.60, and 7.80 Appendix 4.3), at temperature 25°C, as described above. About 500 μl of each of these solutions was injected into a round glass coverslip and then mounted on the stage of a Nikon Diaphot TMD inverted microscope to determine emission ratios. A typical *in vitro* calibration from our optical setup is shown in **Figure 1** on the next page.

***In vivo* Calibration**. Because the K_{app} and emission spectra of many of the dye indicators vary when bound to intracellular proteins, an *in vivo* or *in situ* calibration was necessary in order to get an accurate determination of pH_i from the emission ratios. *In situ* calibration of SNARF-1 in myocytes was performed as described by Spitzer and Bridge (1992) on control rats in pilot studies prior to the exercise training study. The emission ratio (580 nm/640 nm) from each cell in the present study was calibrated by exposing the SNARF-AM loaded cell to (CO_2-HCO_3^-) free Tyrode's solution with varying pH_o (6.50, 6.75, 7.00, 7.20, 7.40, 7.60, and 7.80- Appendix 4.4). The polyether ionophore nigericin (10 μM) with its properties to couple K^+ and H^+ gradients across plasma membrane, was used to equilibrate the pH inside and outside the cell and was included in each of the pH solutions. Each solution also contained 140 mM KCl to keep K^+ constant between internal and external cell. By keeping the internal and external $[K^+]$ equal, nigericin was able to equalize pH_o and pH_i. In each experiment, 2 mM EGTA was applied to reduce $[Ca^{2+}]_o$ and thus prevent cell contracture during the application of the calibrating solutions. Before applying the calibrating solutions, the cell was first bathed for 1-2 min in zero-Ca^{2+} Tyrode's solution to remove extracellular Ca^{2+}. Even with these procedures, some cells elicited large contractures especially in the high pH solutions ($pH \geq 7.4$, Spitzer & Bridge 1992). Thus, to minimize these changes in cell length, 15 mM BDM (Lagadic-Grossmann

and Feuvray 1990 using microelectrode, Spitzer and Bridge 1992) was also included. A typical pH emission ratio calibration relationship from a rat cardiomyocyte is shown in **Figure 2** (using slightly different pH values). From these data, the K_{app} of the SNARF-1 for H^+ under *in vivo* conditions was calculated. Under *in vitro* conditions the K_{app} was 65 nM but it was expected to be higher *in vivo* and was calculated from our pilot experiments to be 80 nM.

e. **pH_i Calculation**

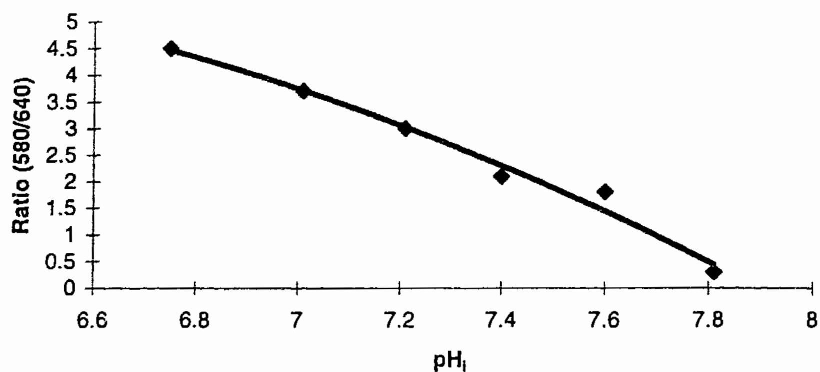
Intracellular pH was calculated using nigericin calibration technique. At the end of each experiment, the R_{max} and R_{min} of each myocyte were determined using Tyrode's solutions identical to that for the *in vivo* calibration (described above) except that for R_{max} the solution was pH 6.00 and for R_{min} the solution was pH 8.20. Intracellular pH was obtained from the equation:

$$[H^+]_i = K_d (R-R_{min}/R_{max}-R)$$

or

$$pH_i = pK_{app} + \log [(R_{min} - R)/(R - R_{max})]$$

This conversion was performed using Felix (v 1.1) software from PTI using a K_d of 80 nM. **Figure 3** shows an example of the conversion of ratio vs time to pH_i vs time, resulting from the application of the above equation.



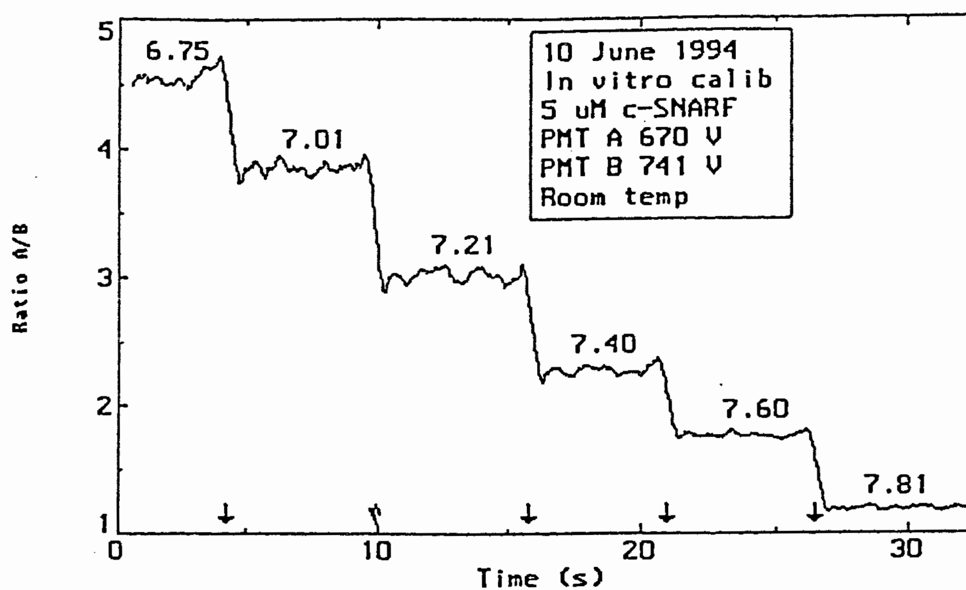
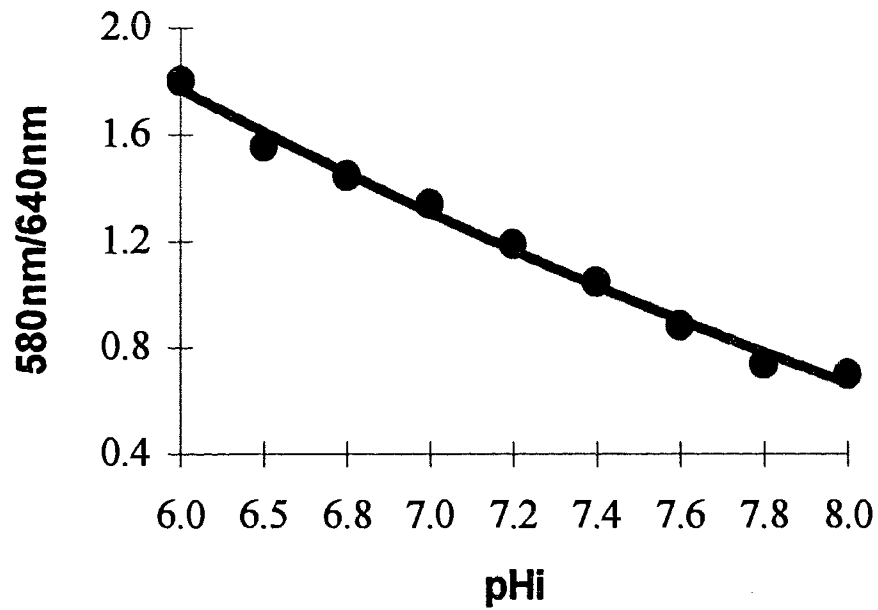


Figure 1. *In vitro* pH calibration of the 580/640 nm SNARF-1 emission ratio. Excitation was performed at 515 nm with 5 μM SNARF-1 (unesterified form).



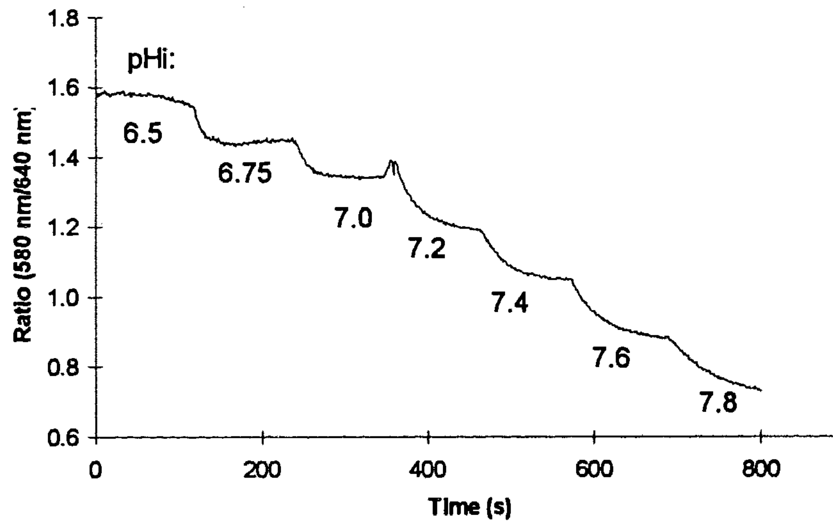
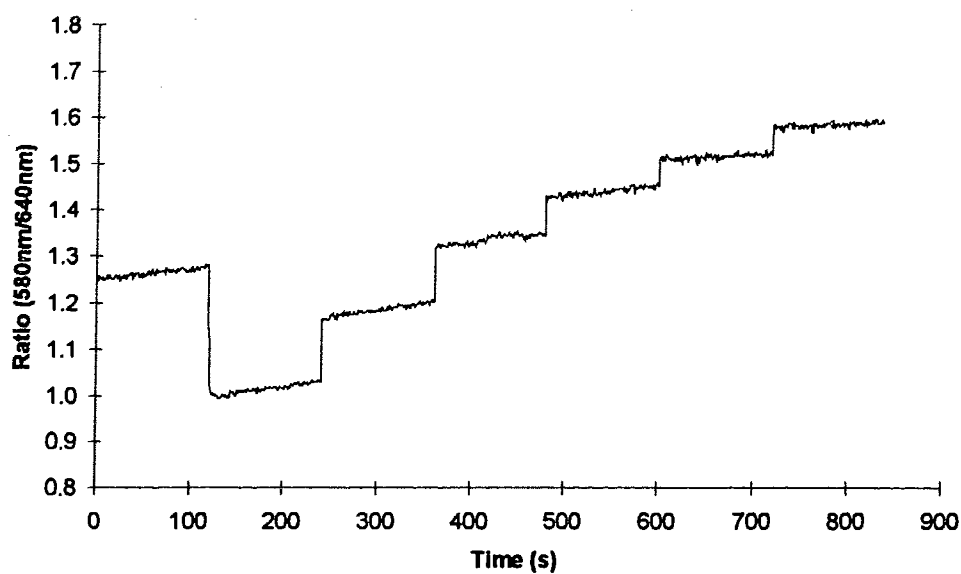


Figure 2. Calibration of the SNARF-1 fluorescence ratio (580/640 nm) in a resting rat ventricular myocyte. Inset: Ratio vs pH_i to determine pK_a (K_d).



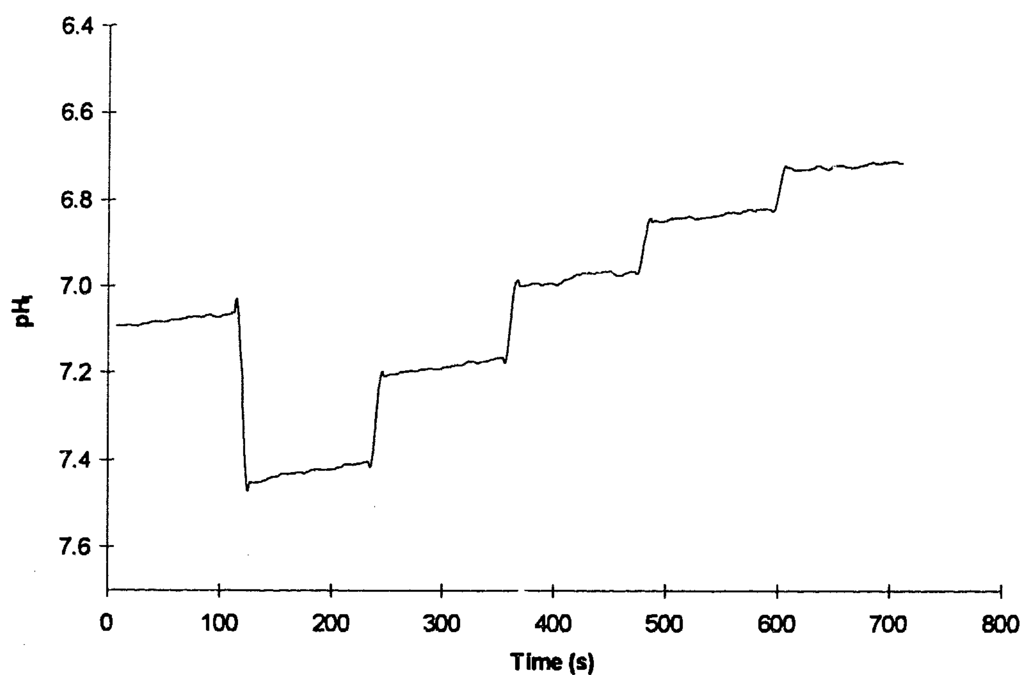


Figure 3. An example of the conversion of ratio vs time to pH_i vs time, resulting from the application of the equation : $pH_i = pK_{app} + \log [(R_{min} - R)/(R - R_{max})]$ with pK_{app} of 7.1.

D. EXPERIMENTAL PROTOCOLS

1. Accumulation of Weak Base to Change Intracellular pH: An Introduction.

This section provides a brief account of the NH_4Cl acid pulse technique, which was introduced and popularized by Roos, Boron and de Weer (1976, 1981), to achieve a rapid acidification of the myocyte. These experiments determined the magnitude of intracellular buffering capacity (β_i) and the ability of the myocyte to respond to a proton overload through the Na^+/H^+ exchanger. To induce changes in pH_i, without changing pH_o, myocytes were incubated with (CO_2-HCO_3^-)-free Tyrode's solution with and without NH_4Cl, a permeant weak base. This technique is based on the ability of the neutral NH_3 to diffuse rapidly through lipid bilayer, while NH_4^+ moves only slowly through unspecified proteins (e.g. in mouse medullary thick limb of henle of kidney, NH_4^+ moves through Ba-sensitive $NH_4^+(K^+)_{ATP}$-channel or $Na^+/K^+(NH_4^+)/2Cl^-$ cotransport (Kikeri, Sun Zeidel, Hebert 1992). There are 4 phases to this process (Frochlich and Wallert, 1995) as shown diagramatically in **Figure 4**. In the first phase, the cell is suddenly exposed to a solution containing NH_4Cl. The NH_3 equilibrates across the membrane and once inside the cell, consumes an intracellular H^+ while establishing a new intracellular NH_3/NH_4^+ equilibrium. The overall result is a rapid cytoplasmic alkalinization, the magnitude of which depends on the initial extracellular NH_4Cl concentration and the cytoplasmic buffering capacity. This first phase takes only a few seconds and depends on how rapidly the added NH_4Cl reaches the myocyte in the experimental chamber. The second phase can extend over a long time, typically a period of 5-30 minutes (Frochlich and Wallert, 1995). During this period, pH_i returns to normal and can even become acidic with prolonged exposure to NH_4Cl. Several

different mechanisms contribute to this, including entry of NH_4^+ through potassium transporting pathways and exit of bicarbonate via chloride-bicarbonate exchange (Kikeri *et al.* 1992, Frochlich and Wallert, 1995). This phase is the actual acid loading phase but the extent of loading goes beyond what one can see from the pH trace. Much of the entering acid is not visible because it is buffered by the intracellular NH_3/NH_4^+ system. This hidden acid load becomes evident, however, during the third phase in the form of a rapid acidification to a pH of 6.5 or even lower (Frochlich and Wallert, 1995). To initiate this phase, the extracellular NH_4Cl solution is switched to a NH_4Cl-free solution. This phase, like the first phase, lasts only a few seconds. Then all of the accumulated ammonium ions leave the cell in the form of neutral NH_3, leaving behind the H^+ that had been consumed. The last phase is the fourth phase, during which the pH_i returns to normal during which the kinetics of the pH regulatory transport mechanisms (i.e. NHE) were studied.

Thus, the usefulness of this acid pulse protocol is that it provides a rapid acidification without changing pH_o. Thus it permits one to study the fairly rapid recovery rates through NHE and other contributing acid extruding mechanisms because during the acidification period (step 3), there is no extraneous buffer (from NH_3/NH_4^+ system) remaining which might interfere with the measurement of pH recovery rates (dpH_i/dt) and the acid extrusion (J_H^+) (Frochlich and Wallert, 1995).

Modifying this technique by step reducing the extracellular NH_4Cl concentration was also done to induce a step wise increase in acid load. By doing so, we were able to study the intracellular buffering capacity (β_i) in this experiment. Data from this type of β_i experiment are shown in **Figure 5.**

2. Determination of Intracellular H^+ Buffering Capacity (β_i)

The value for β_i was determined in a manner similar to that described by Boyarsky, Ganz, Sterzel and Boron (1988); Lagadic-Gossmann, Buckler and Vaughan-Jones (1992) and Vaughan-Jones and Wu 1990. The principle of these methods was to induce pH_i changes in the cardiomyocytes by using externally applied weak bases such as NH_4Cl and trimethylamine chloride or weak acid such as sodium propionate (Vaughan-Jones and Wu 1990). These agents freely permeate the cell membrane in their uncharged form (i.e. NH_3, trimethylamine/TMA and propionic acid) but they exist mainly in ionized form once inside the cell. The ionization of weak base absorbs H^+ ions while that of the weak acid liberates H^+ ions. Both types of agent can be used therefore to induce changes of pH_i which then can be used to estimate β_i. Thus β_i can be defined as

$$\beta_i = \Delta A/\Delta pH_i \qquad = \Delta B/\Delta pH_i \qquad (1)$$

where ΔA is the amount of intracellular acid load ($[H^+]_i$ in mM); ΔB is the amount (in mM) of intracellular base (NH_4 or TMA) introduced into the cell, and ΔpH_i is the resultant change in intracellular pH. The $[H^+]_i$ is assumed to equal $[NH_4]_i$ or $[TMA]_i$ at the moment of their removal from the external solution or $[propionate^-]_i$ at peak acidosis induced by addition of external sodium propionate. For the experiments in this thesis, NH_4Cl (pK_a=8.9 @37°C, Boyarsky, Ganz, Sterzel and Boron 1988) was used. In brief, the cells were exposed to solutions sequentially containing 0, 20, 10, 5, 2.5 and 1.25 mM NH_4Cl for 2 minutes each as shown in **Figure 5**. The NH_4Cl containing solutions also contained 5 mM Ba^{2+} and 1.5 mM amiloride HCl (Sigma, Lot No. 82H0012) to reduce NH_4^+ efflux

through potassium channels and H^+ through the Na^+/H^+-exchanger, respectively. Introducing 20mM NH_4Cl solution caused pH_i to rapidly increase (*ab*), due to the influx of NH_3, which then combines with H^+ to yield NH_4^+. The intracellular pH then stabilizes during the plateau phase (*bc*) at a relatively high value.

The relative stability of pH_i during the plateau phase suggests that background acid loading or acid consuming processes are minimal (Boyarsky, Ganz, Sterzel and Boron, 1988). In some experiments in which higher concentrations (e.g. 40-60 mM) of NH_4Cl were used to drive pH_i to even higher levels, there were substantial decreases in pH_i during the segment -*bc* plateau phase, suggesting the activation of acid-loading processes at very high pH_i (Boron and deWeer 1976). Thus, in my experiments it was preferable to use NH_4Cl concentrations of not more than 20 mM (i.e at pH_i values below ~ 7.7) to determine the buffering capacities, since the calculations required that pH_i not to be affected by processes other than NH_3 fluxes. Lowering total NH_4^+-NH_3 from 20 to 10 mM caused pH_i to decrease rapidly (in this example, by 0.238 pH units from 7.402 *(bc)* to 7.164 *(cd)*), reflecting the dissociation of intracellular NH_4^+ into NH_3 (which diffuses from the cell) and H^+ (which is trapped within). Virtually all of the newly formed H^+ is consumed by intrinsic intracellular buffers. Because one intracellular H^+ is formed for every NH_4^+ that is consumed between *c* and *d*, the magnitude of acid load (ΔA) is numerically the same as $-\Delta[NH_4^+]_i$ or $[NH_4^+]_i$ at point d - $[NH_4^+]_i$ at point c (Boyarsky, Ganz, Sterzel and Boron, 1988). Thus, the apparent value of β_i (mM per pH unit) was then calculated as a function of pH_i from this equation:

$$\beta_i = \Delta[NH_4^+]_i/\Delta pH_i \qquad (2)$$

where β_i is the mean intrinsic buffering power of all non- NH_3-NH_4^+ intracellular buffers,

includes physicochemical buffering by endogenous buffers, metabolic processes and possible proton sequestration by organelles (Roos and Boron, 1981). Assuming that $[NH_3]_o = [NH_3]_i$ at points *c* and *d*, $\Delta[NH_4^+]_i$ was calculated using these following equations:

$$NH_4^+ \Leftrightarrow NH_3 + H^+ \qquad (3)$$

The rate of dissociation of NH_4^+ is described by: $k_1 \bullet [NH_4^+]$; while the rate of association of NH_4^+ is described by $k_2 \bullet [NH_3] \bullet [H^+]$. Since under equilibrium conditions association rates are equal to dissociation rates, and assuming the association constant of this reaction to be identical on both sides of the membrane, $[H^+]_o/[NH_4^+]_o = [H^+]_i/[NH_4^+]_i$. Thus, $[NH_4^+]_i$ in equation 2 is calculated from:

$$[NH_4^+]_i = [NH_4^+]_o \bullet [10^{pHo-pHi}] \qquad (4)$$

where: $[NH_4^+]_o = C/[10^{(pHo-pKa)}+1]$ and C is the total concentration of external NH_4Cl (Boyarsky, Ganz, Sterzel and Boron 1988). Thus in this example, the average β_i in the small pH_i range between *c* and *d* (average pH_i 7.283) at pH_o=7.4 (@37°C) is:

$$\beta_i = (16.690-19.296) / (7.164-7.402) \quad = 10.95 \text{ mM / pH}$$

Similar decreases in NH_4^+-NH_3 from 10 to 5 to 2.5 to 1.25 mM caused further pH_i decreases from which the calculations of the average β_i values at their average pH_i were done.

3. Determination of Contractile Sensitivity (λ) to pH_i

Contractile sensitivity was determined in the same experiment by monitoring changes in cell length in response to changes in pH_i.

$$\lambda = \Delta \text{ myocyte length } (\mu m) / \Delta pH_i \qquad (5)$$

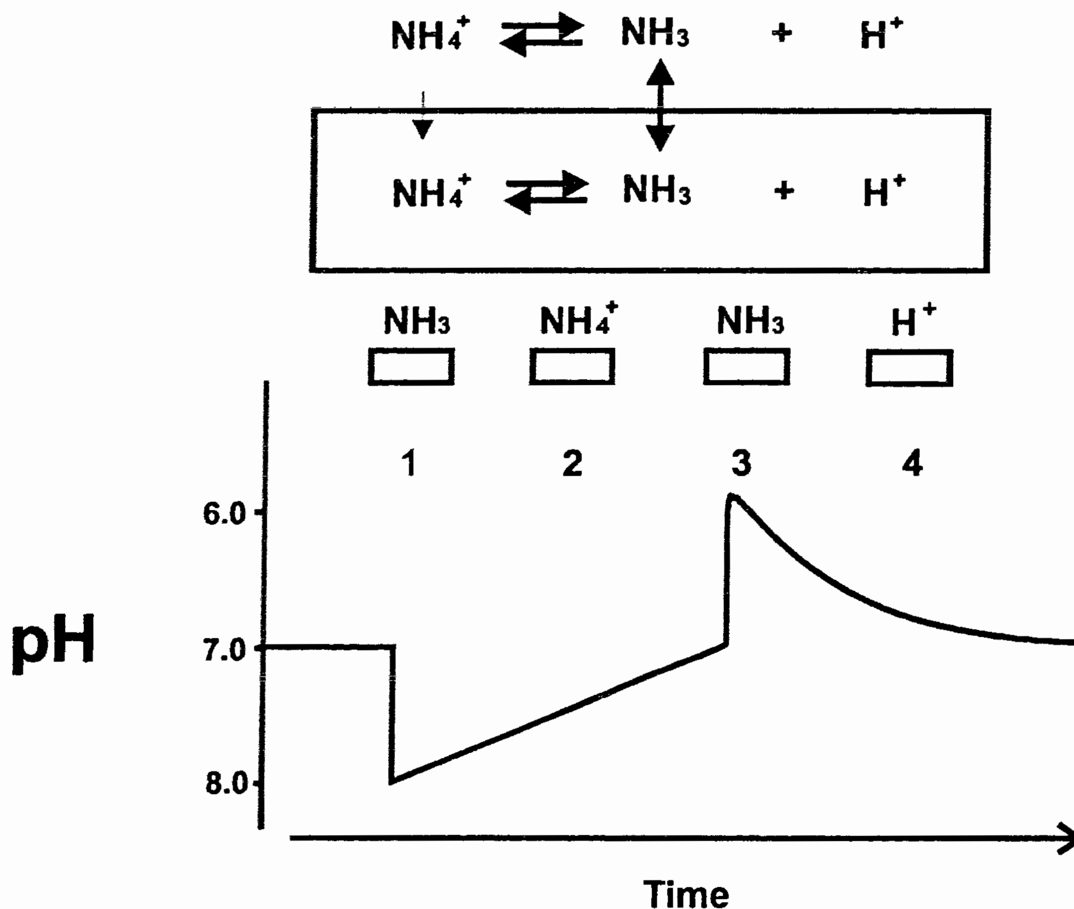


Figure 4. A schematic representation of the principles that underlie the intracellular pH changes upon exposure to NH_4Cl. **Top**: Only NH_3 the neutral form can move freely through the membrane lipid bilayer. The ammonium ion (NH_4^+) has a much lower permeability and moves mainly through unspecified transport proteins. Coupled to this process are the dissociation and association of NH_4^+ in which protonation and unprotonation occur. These protonation and unprotonation reactions cause changes in intracellular pH. **Bottom**: Time course of events during the acid loading process with NH_4Cl. During phase 1, NH_3 rapidly enters the cell and subsequent production of NH_4^+ alkalinizes the cytosol. During phase 2, NH_4^+ ions enter carrying with them the H^+ ions that make the pH_i return back to its "normal" basal level. These accumulated hydrogen ions are left behind in phase 3 when NH_3 is rapidly washed out. This step (step 3) marks the actual experimental phase during which the kinetics of pH regulatory transport mechanisms (ex. Na-H exchange) are studied.

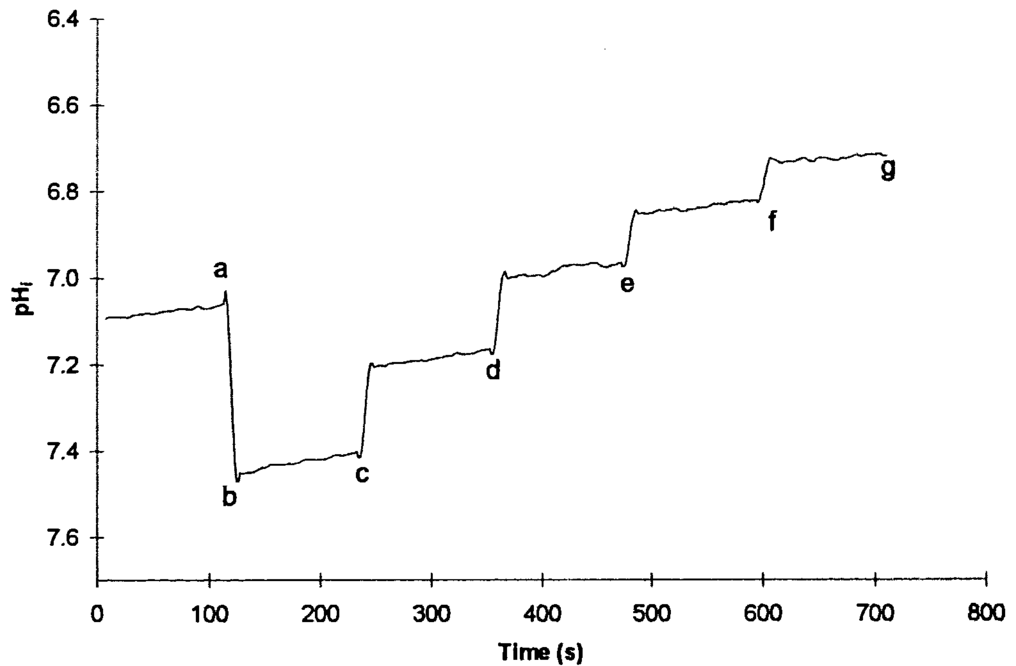


Figure 5. An example of NH_4Cl induced pH_i changes to determine β_i in rat single cardiomyocytes. During the first two minutes, the cell was bathed with HCO_3^--free Tyrode solution (made at pH_o=7.4, @ 37°C) (*a*). Then it was sequentially perfused with HCO_3^--free Tyrode solution containing NH_4Cl in mM : 20, 10, 5, 2.5, 1.25 for 2 minutes each. 20 mM NH_4Cl produced alkalinization (*bc* segment), whereas decreasing its level from 20 to 10→5→2.5→1.25 produced increasing acidification (*cd, de, ef* and *fg* segments).

4. Determination of Na^+/H^+ Exchanger Activity

The involvement of the sarcolemmal Na^+/H^+ exchanger as the paramount regulator of pH_i in cardiac muscle has been described in a variety of species (Kleyman and Cragoe 1988, Lazdunski, Frelin and Vigne 1985, Frelin *et al.* 1988, Lagadic-Gossmann, Buckler and Vaughan-Jones, 1992 and Bertoni *et al.* 1993). A possible adaptation of the exchanger in response to exercise training was evaluated in the following manner. Myocytes were exposed to 20 mM NH_4Cl for a period of 2.5 minutes and then to 0 mM NH_4Cl for a period of fifteen minutes. An experiment of this type is principally similar to that previously described in the section D.1 (An introduction) and was shown diagramatically in **Figure 4.** The rate of recovery of pH_i to higher values during the washout should be due primarily to the Na^+/H^+ exchanger because the role of HCO_3^- transporters was minimized in these media. After full recovery, the protocol included a second 2.5 min exposure to 20 mM NH_4Cl and 1.5 mM amiloride and a 15 min washout with 0 mM NH_4Cl and 1.5 mM amiloride. Amiloride is a pyrazinoyl-guanidine and a weak base (pK_a 8.7) consisting of protonated and unprotonated forms. Amiloride bears amino groups on the 3- and 5-positions and chloro group on the 6-position of the pyrazine ring making it quite lipid soluble. Only its protonated form interacts with the Na^+-H^+ exchanger, while the unprotonated form may bind to and easily cross the cell membrane, accumulate within cells, and alter a number of cellular processes (Kleyman and Cragoe 1988). It becomes a selective and potent inhibitor of Na^+-H^+ exchange with an IC_{50} of 3-7 μM in the presence of low concentrations of external Na^+ and a weak inhibitor of the Na^+-Ca^{2+} exchanger with an IC_{50} of 1 mM (Kleyman and Cragoe,1988). In the presence of high concentration of external Na^+ (at concentrations similar to those of the solutions that

were used in these experiments) however, the IC_{50} for Na^+-H^+ exchanger is as high as 1 mM (Kleyman and Cragoe, 1988). The amiloride-sensitive component of the rate of pH_i recovery was taken as the contribution of the Na^+-H^+ exchanger. Two parameters were used to quantify the rate of recovery from the acid load: 1) the rate of pH_i recovery (described below) and 2) the rate of acid efflux (J_{H^+}) which was calculated as the product of β_i and dpH_i/dt, where β was estimated at pH_i points along the curve :

$$J_{H^+} = [dpH_i/dt] \bullet [\beta_i] \qquad (6)$$

where : $[dpH_i/dt]$ is the instantaneous rate of recovery of pH_i and $[\beta_i]$ is the intracellular buffering capacity at this pH_i.

The relationship of pH_i recovery after a proton load as a function of time (t) was determined by : 1) taking the slope of the initial linear (dpH_i/dt) component of the recovery and/or 2) fitting the entire 15 min recovery period with a 2nd order polynomial equation. The emission data were collected at a rate of 1 Hz using the Felix software. The first method was performed by highlighting the initial rate period (by eye) and fitting the slope over this range using the derivative function in Felix software. Although much more time consuming, the second method is more accurate as it does not require a judgment as to the range linearity of the recovery. For this analyses, the data were imported into an Excel 7.0 spreadsheet. The recovery data were then fit with a 2nd order polynomial (where $pH_i = at^2 + bt + c$) using the least squares method within Excel, resulting in fits with $r^2 \geq 0.9$. From the 2nd order polynomial, the instantaneous derivatives (dpH_i/dt at any pH_i during the recovery) were determined as: $f'(t) = 2at + b$. From the two perturbations (+A, -A) of the NH_4Cl prepulse technique, the pH_i ranges for +A and -A were 6.5-6.9 and 6.7-7.1, respectively. Thus the amiloride-sensitive recoveries were analyzed over the intercept pH_i

range of 6.7-6.9 and the instantaneous dpH_i/dt values were calculated at 0.01 pH unit intervals using the calculated expression of time as a function of pH_i ($t = a \bullet pH_i^2 + b \bullet pH_i + c$).

5. Determination of SNARF-1 Compartmentalization

Although it is preferable that the SNARF-1 is localized exclusively within the cytosol, some of the SNARF-AM may enter mitochondria where it may also be deesterified. To estimate the extent of mitochondrial SNARF-1 accumulation, loaded cells were rapidly exposed to 10 μM digitonin similar to the technique described by Spitzer and Bridge (1992). For the data to be accepted, the decline in fluorescence signals at both wavelengths (580 nm and 640 nm) after the application of digitonin had to be ≥ 90%.

6. Data Analyses

The parameters determined in this study were resting pH_i, β_i, λ, slope (dpH_i/dt) and J_{H^+} as described above. The data from the two groups were compared and the results were expressed as the means (SEM). Unless indicated otherwise, statistical significance was evaluated by a one tailed Student's *t* test and p values of ≤ 0.05 were considered significant.

CHAPTER III

RESULTS

A. THE EFFECTS OF NH_4Cl ON pH_i AND THE MAGNITUDE OF β_i.

The cells were perfused with CO_2/HCO_3^--free HEPES buffered Tyrode's solution to inhibit HCO_3^--dependent pH_i regulatory systems and minimize intracellular buffering from CO_2/HCO_3^- (i.e. $\beta_{Tyrode}=\beta_i$). The mean resting (basal) pH_i (@ pH_o 7.4; 37°C) in quiescent myocytes of C group was 7.051 (SEM 0.033, n=24). Step reducing in $[NH_4Cl]_o$ produced a decline in pH_i. As pH_i declined, β_i (using the equations 1, 2 and 4) tended to increase in a roughly linear fashion. An example of a β_i determination in a rat ventricular myocyte of the control group is shown in **Figure 6A** and the result of single cell β_i calculations at each NH_4Cl step is presented as a linear relationship in **Figure 6B**. β_i values in this curve were categorized according to their pH_i groups resulting from the different levels of NH_4Cl treatment. In this example, reducing $[NH_4Cl]_o$ from 20 to 10→5→2.5→1.25 mM produced a total decrease in pH_i of almost 0.7 units (pH_i dropped from 7.4 to 6.7).

The results from 24 cells are summarized in **Figure 7.** Upon simple inspection, it appears that within the pH_i range of ~ 7.69-6.22 (ΔpH_i = 1.47 units), β_i increases from ~ -5 mM to 60 mM ($\Delta\beta_i$ = 65 mM). Thus, for all cells in group C, the change ($d\beta_i /dpH_i$) was ~44.217 mM/pH as the best estimation of β_i within this pH_i range. The least squares linear regression of the pH_i-β_i relationship resulted in the following: β_i = -44.802 pH_i +

339.95 for group C. The scatter of all the data taken collectively as shown in this figure is reflected in the fact that the regression coefficient was low ($R^2 \sim 0.3$). This is probably due in part to individual variations of β_i and pH_i among the 24 different myocytes that were used. Thus, this traditional linear regression is not suitable to describe the results. Another approach was used to describe the results. From each cell there were four different pH changes from which β_i was calculated. The data from each pH_i perturbation from the different levels of NH_4Cl were pooled and the results are shown in **Figure 8.** The linear regression of this pH_i-β_i relationship is expressed by $\beta_i = -83.268\ pH_i + 607.54$ for group C. As can be seen, the goodness-of-fit is superior to the previous linear regression line (in figure 7) with R^2 being 0.99. From this figure it can be seen that β_i increases from 3.48 mM (±SEM 0.04, mean of 24 cells, 24 measurements) to 50.43 mM (±SEM 0.05, n=25) within the pH_i range of 7.268-6.714. Using this pooled data approach, $d\beta_i/dpH_i$ was 84.76 mM/pH as the best estimation of β_i within this pH_i range. Using the regression equation from figure 8, the β_i of group C at the basal pH_i of 7.055 is 22.4 mM which is virtually identical to that derived from in figure 7 (23.87 mM). Thus in order to study the effects of exercise training on β_i, either procedure could be applied to predict the difference between two groups.

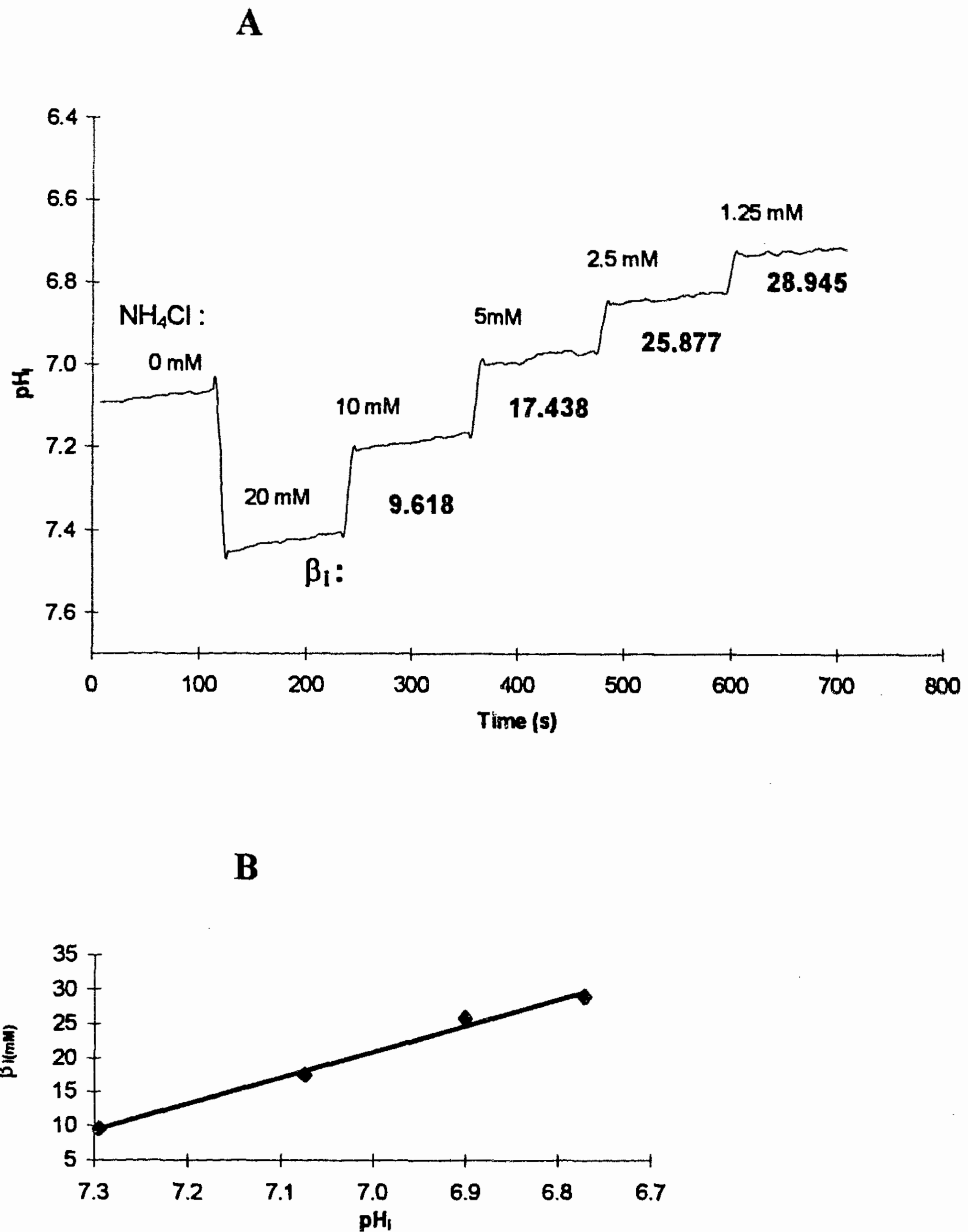


Figure 6. Intracellular buffering capacity β_i in rat ventricular myocyte. pH_i changes elicited by the sequential reduction of $[NH_4Cl]_o$ were used to calculate β_i. **(A)** Example of β_i determination in a single cell (pH_o 7.4). Calculated β_i values are indicated adjacent to pH_i signal. **(B)** Relationship between β_i and pH_i where pH_i was taken at midpoint of each pH_i step. Line fit by least squares, linear regression: $\beta_i = -38.31\ pH_i + 289.04$. The slopes and intercepts from this type of equation of all cells in both groups C and T were pooled and the comparison between two groups was done.

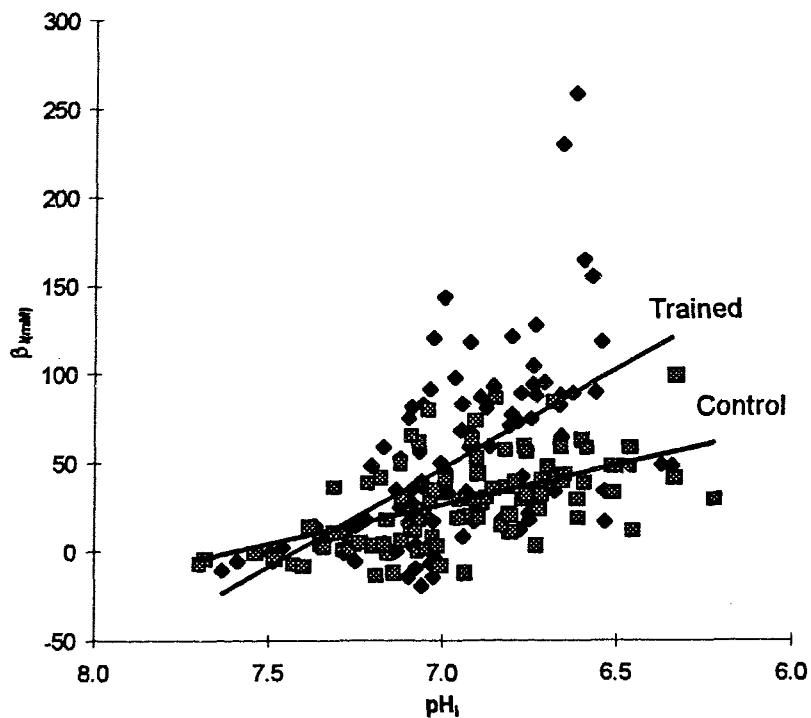


Figure 7. Composite data of β_i over the pH_i range ~ 7.69-6.22. The relationship can be expressed as : β_i = -44.802 pH_i + 339.95 for group C and β_i: -111.27 pH_i + 825.53 for group T (R^2 ~0.30 for both groups). Measurements were done in total 48 cells: C (n=24, open squares) and T (n=24, closed diamonds) in which one measurement was taken from one cell. The β_i/pH_i was significantly greater ($p<0.005$) in group T compared to group C.

B. THE EFFECTS OF EXERCISE TRAINING ON BASAL pH_i AND β_i

In both β_i and NHE experiments, exercise training did not seem to influence the resting pH_i. As previously mentioned the resting pH_i of group C was 7.051 (± SEM 0.033, n=24). For group T the resting pH_i was 7.013 (± SEM 0.007, n=24). The slight difference in basal pH_i (group T mean is 0.038 pH units less than group C) is not significant ($p > 0.05$, Student's *t*-test, one tail). Similarly, exercise training did not effect the magnitude of the pH_i decrease induced by $[NH_4Cl]_o$ removal. As shown in Table 1, the overall pH_i decrease is not significantly different between groups C and T ($p > 0.5$, Anova: two factors with replication). The decrease in pH_i of both groups is ~0.4 units (decreasing the $[NH_4Cl]_o$ from 20 to 10→5→2.5→1.25 mM, resulted in declining the average pH_i from 7.26 to 6.71 and 7.23 to 6.77 of group C and T respectively).

The effect of exercise training on β_i is presented in **Figure 8.** In the presence of amiloride and Ba^{2+}, both groups showed an increase in β_i as pH_i decreased. However, during this period of intracellular acidosis induced by step reducing NH_4Cl, the magnitude of β_i increase was ~2 times greater in group T in comparison to group C. Using the equations from the individual pooled data approach (similar to that in figure 6.B), the average β_i values at pH_i 7.00 were 22.4 (2.9) and 44.5 (5.9) mM for groups C and T, respectively. These values are virtually identical to the values roughly calculated using the equation in figure 8 (24.6 and 37.1 mM for groups C and T respectively). The mean β_i/pH_i slopes of group T was increased ~2 times ($p<0.001$) in comparison to that of group C (90.79 vs. 206.41 $mM \bullet pH^{-1}$ over the investigated pH_i range of 6.71-7.26). In addition, the intercepts also increased ~3 times (657.10 vs. 1545.42 in C and T, respectively).

$[NH_4Cl]_o$ (mM)	Average pH_i (SEM) group C	Average β_i (SEM) group C	Average pH_i (SEM) group T	Average β_i (SEM) group T
20 - 10	7.268 (0.039)	3.48 (2.7)	7.232 (0.039)	-0.02 (2.03)
10 - 5	7.010 (0.037)	22.40 (2.9)	6.990 (0.037)	44.58 (5.77)
5 - 2.5	6.834 (0.040)	36.83 (3.7)	6.861 (0.039)	69.32 (9.43)
2.5 - 1.25	6.714 (0.046)	50.43 (4.6)	6.774 (0.040)	88.93 (10.88)
at pH_i 7.0		22.4 (2.9)		44.5 (5.9)
Basal	7.055 (0.033)		7.013 (0.006)	

Table 1. The effects of exercise training on pH_i and β_i. The pH_i of both groups C and T are not significantly different ($p \geq 0.5$, Anova: two factors with replication), however the β_i of group T is significantly greater than group C ($p \leq 0.005$, Anova: two factors with replication). Resting (basal) pH_i measured at pH_o 7.4 (37°C) of group C and T are also not significantly different. N=24 for each group.

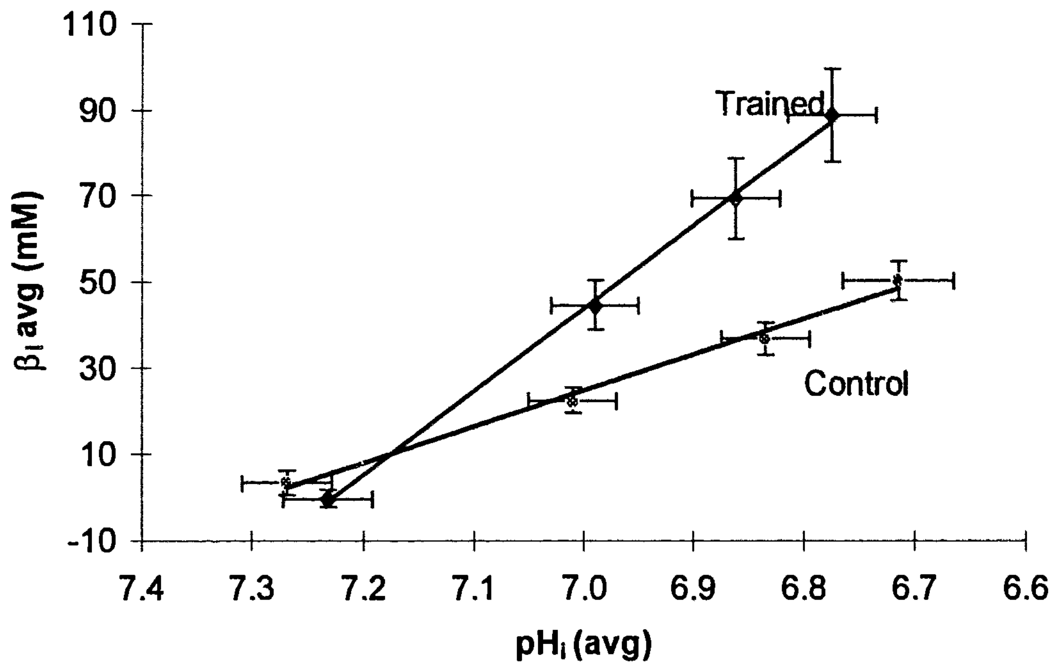


Figure 8. Dependence of intracellular buffering capacity (β_i) on intracellular pH (pH_i) within the pH_i interval ~7.26-6.71), in both control (C) and trained (T) groups. This figure is derived from the same data as in figure 7, however, they were pooled and averaged by the $[NH_4Cl]_o$ steps as described. The relationships can be expressed as : $\beta_i = -83.268\ pH_i + 607.54$ for C group (r^2=0.99, n=24 cells) and β_i= $-192.93pH_i + 1394.2$ for T group (r^2 =0.99, n=24 cells). As in figure 7, there is a significant difference in the slopes ($p< 0.001$) and intercepts ($p< 0.0002$) (Student's *t*-test, one tail).

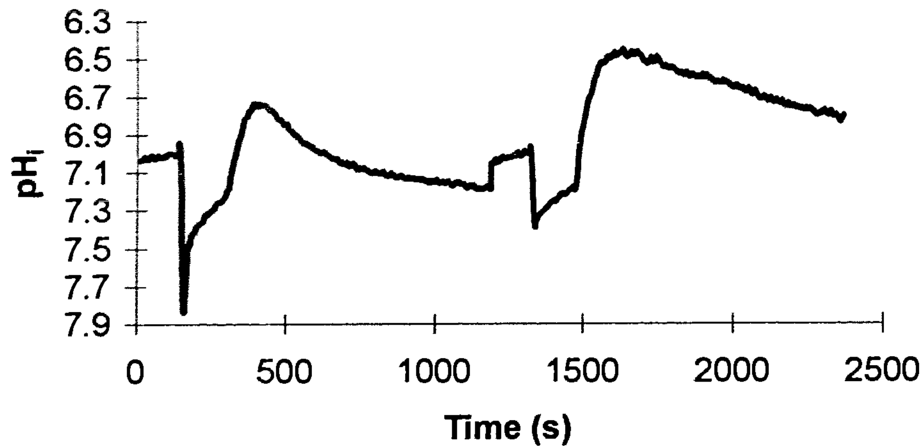
C. THE EFFECTS OF AMILORIDE ON THE ACTIVITY OF Na^+/H^+-EXCHANGER

The ability of amiloride to inhibit the activity of the Na^+-H^+ exchanger has been shown in the experiments in this thesis. The application of 20 mM NH_4Cl produced an immediate alkalinization, whereas the subsequent removal of NH_4Cl induced an acidosis **(Figure 9)**. The half-recovery time from acidosis of non amiloride treated cells was 2.52 (± SEM 0.24) minutes while that of amiloride-treated cells was 4.91 (± SEM 0.54) ($p < 0.0002$, one-tail student's *t* test, n=20 cells, 20 measurements). In some cases, in the presence of amiloride, the cell doesn't completely recover but remains acidotic (data not shown).

The initial rates of pH_i recovery from acidosis (dpH_i/dt) were also used to determine the effectiveness of amiloride on blocking the Na^+-H^+ exchanger. In the nominal absence of CO_2 and HCO_3^- and in the presence of 126 mM Na^+ and 4.4 mM K^+, mean initial dpH_i/dt of amiloride (+A) treated and the non-amiloride (-A) treated cells were significantly different in both groups. In group C (n=20), mean initial dpH_i/dt were 0.074 (0.013) pH units•min^{-1} and 0.022 (0.004) pH units•min^{-1} for (-A) and (+A), respectively. In group T (n=20), the mean initial dpH_i/dt were 0.078 (0.009) pH units•min^{-1} and 0.029 (0.003) pH units•min^{-1} for (-A) and (+A) respectively. Thus the rate was much higher in the absence of amiloride. Moreover, the J_{H+} in the presence of amiloride of both groups was also significantly less than that in the absence of amiloride. This statistically significant decrease in dpH_i/dt and subsequently J_{H+} in the presence of amiloride of both groups ($p<0.0001$, One-tail Student's *t* test), indicates that amiloride successfully blocked the exchanger. **Table 2** shows the mean '***initial***' dpH_i/dt and J_H+ of group C and T.

	SLOPE (pH/min) (-A)	SLOPE (pH/min) (+A)	Starting pH_i (-A)	ΔJ_{H+} mM/min (-A)	Starting pH_i (+A)	ΔJ_{H+} mM/min (+A)
Group C (n=20)						
Mean	-0.074	-0.021	6.56	1.77	6.53	0.27
SD	0.060	0.020	0.27	1.83	0.25	0.48
SEM	0.013	0.004	0.06	0.41	0.06	0.11
Group T (n=20)						
Mean	-0.078	-0.029	6.63	7.93	6.61	1.43
SD	0.038	0.015	0.15	6.87	0.18	1.21
SEM	0.009	0.003	0.04	1.58	0.04	0.28

Table 2. The mean 'initial' dpH_i/dt and $J_{H}+$ of both groups with (+A) and without (-A) 1.5 mM amiloride. The mean starting pH_i were also indicated for these initial dpH_i/dt and $J_{H}+$ values.



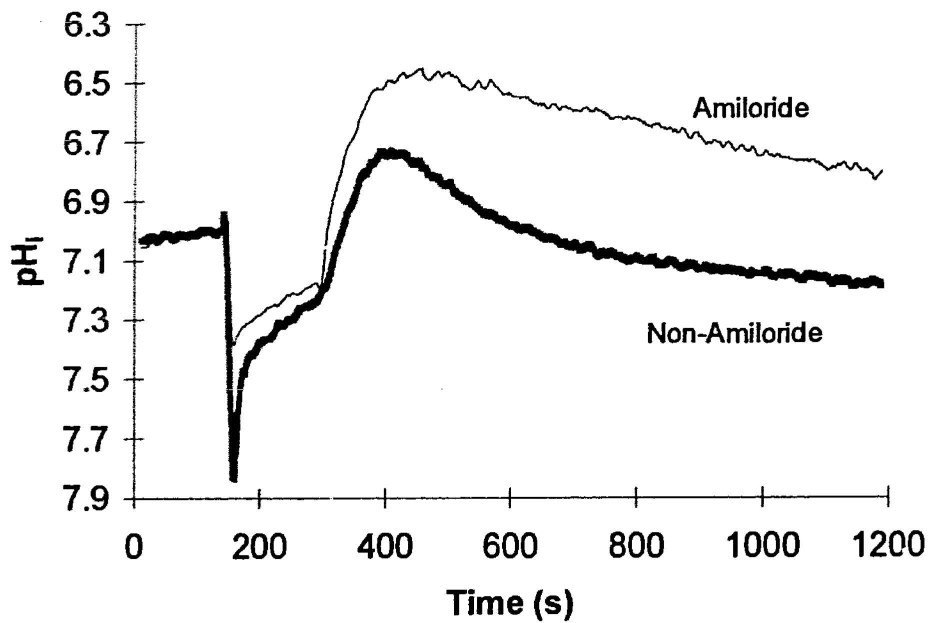


Figure 9. Typical experiment for the determination of the rate of pH_i change and the role of the Na^+/H^+-exchanger in the recovery from a proton load in a rat ventricular myocyte. At 150 seconds, 20 mM NH_4Cl was added and then removed at 300 seconds. The addition of 1.5 mM amiloride markedly slowed the recovery of pH_i as expected. The role of the Na^+/H^+ exchanger was taken as the amiloride-sensitive component of this response. The entire record is shown at the top panel and the overlay is shown at the lower panel.

D. THE EFFECTS OF EXERCISE ON RATE OF pH_i RECOVERY FROM A PROTON LOAD (dpH_i/dt) AND THE NET ACID EXTRUSION (J_{H^+}) *via* Na^+/H^+-EXCHANGER

As can be seen in **Table 2**, there were no significant ($p>0.05$) differences between the groups in the initial rate of pH_i recovery (dpH_i/dt) from 20 mM NH_4Cl prepulse induced acidosis. In the absence of amiloride, the initial dpH_i/dt were 0.074 (0.013) and 0.078 (0.009) pH units•min^{-1} in C and T, respectively and in the presence of amiloride were 0.021 (0.004) and 0.029 (0.003) pH units•min^{-1} in C and T, respectively.

In order to determine the H^+ flux (J_H^+) by the Na^+/H^+ exchanger, the pH_i recovery profiles were fit with a 2nd order polynomial equation. From this equation, the instantaneous derivative (dpH_i/dt) could be determined. The results of a total of 40 cells are displayed in **Figures 10** and **11**. The amiloride-dependent rates of pH_i recovery [(-A - +A) dpH_i/dt] was significantly less in group T compared to group C. From the relationship shown in **Figure 8**, the β_i at all pH_i values during the recovery could be determined for both trained and control myocytes. The J_{H^+} and the amiloride-dependent J_{H^+} were then calculated as the product of dpH_i/dt and β_i for any given pH_i within the range of recovery and are displayed in **Figure 12 and Figure 13,** respectively. Over this range, J_H^+ for the Na^+/H^+ exchanger was significantly greater for the Group T despite the fact that the rates of pH_i recovery were less. This is due, in large part, to the significantly greater β_i values in Group T compared to C over this range of pH_i values.

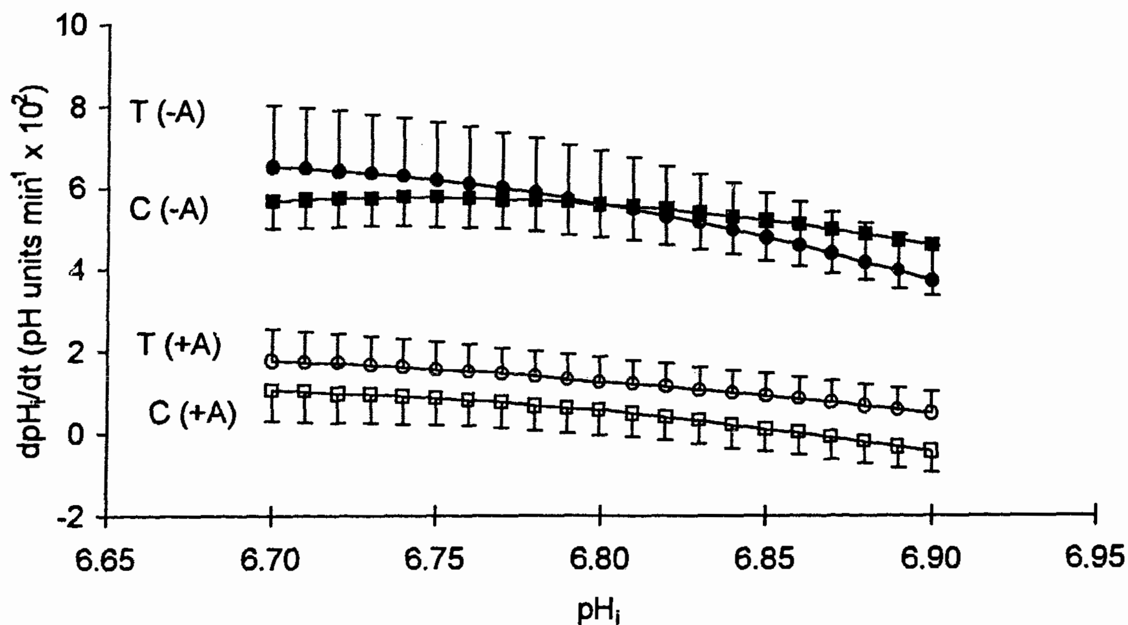


Figure 10. The rate of pH_i recovery from acid load (dpH_i/dt) as function of pH_i in the presence (+A) and in the absence (-A) of amiloride of each group C and T. The relationship of pH_i recovery after a proton load as a function of time (t) was fit with a 2nd order polynomial equation. From the general equation : $pH_i = a \bullet (t)^2 + b \bullet (t) + c$, the instantaneous derivatives- the dpH_i/dt - was resolved as : $f'(t) = 2 \bullet a \bullet (t) + b$. Over this pH_i range (6.7-6.9), time was resolved using the general 2nd order polynomial equation taken from the relationship of time (t) as a function of pH_i : $t = a \bullet (pH_i)^2 + b \bullet (pH_i) + c$, at any given pH_i.

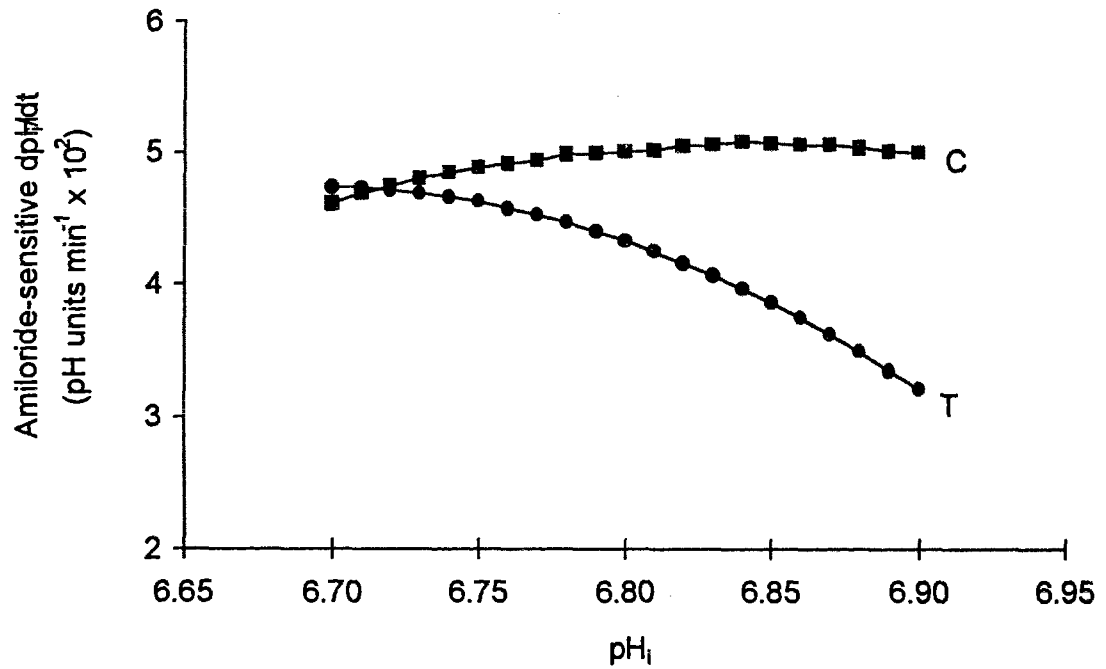


Figure 11. The amiloride-sensitive component of the rate of pH_i recovery from acid load (dpH_i/dt) as function of pH_i. For both groups C and T (n=24 each), this component was calculated by subtracting dpH_i/dt (+A) from the dpH_i/dt (-A).

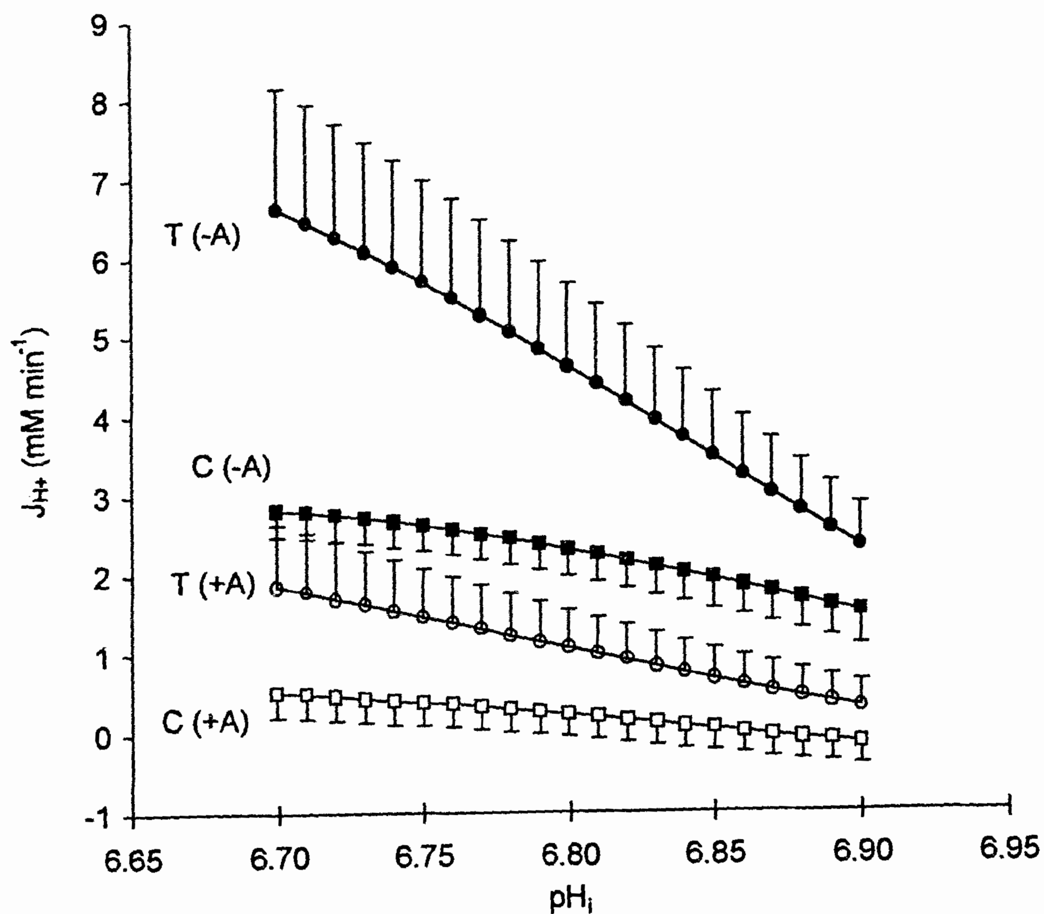


Figure 12. The net acid extrusion (J_H^+) as function of pH_i in the presence (+A) and in the absence (-A) of amiloride for groups C and T. J_H^+ was calculated as the product of dpH_i/dt and β_i for any given pH_i within the range of recovery (6.7-6.9).

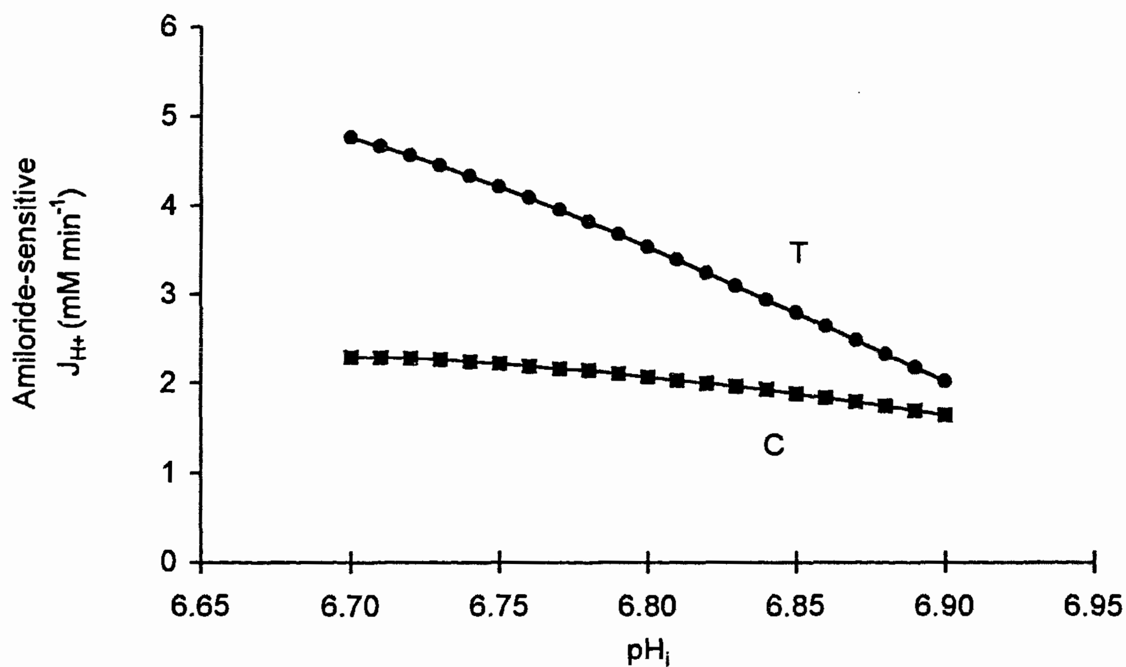
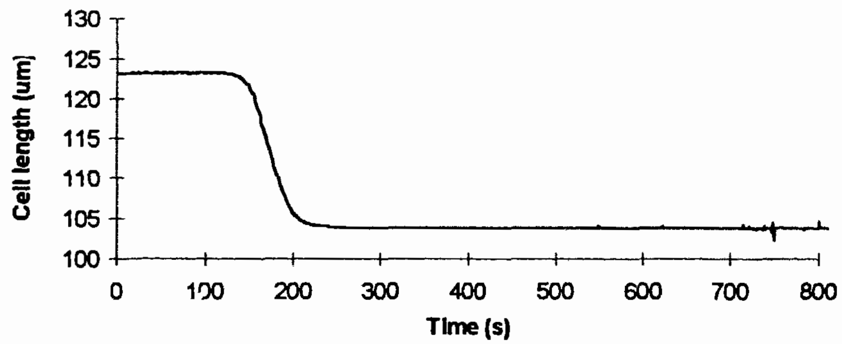


Figure 13. The Na^+-H^+ exchanger (amiloride sensitive component) dependent H^+ flux (J_{H^+}) as a function of pH_i within the range of recovery (6.7-6.9) for both groups C (n=20) and T (n=20).

E. THE INFLUENCE OF EXERCISE TRAINING ON RESTING MYOCYTE LENGTH (CHANGES IN CELL LENGTH IN RESPONSE TO CHANGES IN pH_i)

From the experiments in which β_i was determined, it was found that resting myocyte length (L) of group T was increased ~ 5 % to compare to that of group C. The resting L were 124.0 ± 2.0 μm and 130.1 ± 2.04 μm for group C and T respectively (n = 24 cells for each group). This exercise training elicited increase in resting L is statistically significant ($p < 0.02$). In response to the changes in pH_i induced by changing in the $[NH_4Cl]_o$, however, most quiescent myocytes in both groups: the C (13 out of 24 cells) and T (17 out of 24 cells), showed no changes in their lengths (data not shown). Thus, these cells maintained their lengths at all pH_i. The rest of the cells (11 cells from group C and 7 cells from group T) showed changes in their lengths in response to changes in pH_i. These 11 cells elicited a decrease in their lengths in alkalosis condition induced by 20 mM NH_4Cl exposure. These decreased cell lengths persisted even after the acidosis condition - induced by step reducing the $[NH_4Cl]_o$- was introduced. Data from a single cell from C group is shown in **Figure 14.** The other 7 cells in group T however showed a differential response in length to changes in pH_i. In alkalosis, all of these cells showed a decrease in lengths, whereas in the acidosis condition, cells showed an increase in lengths. Data from a single cell from T group is shown in **Figure 15**. Because of these different responses, the magnitude of ΔL and λ which is $\Delta L/\Delta pH_i$ (sensitivity) of both groups C and T were not calculated. During the NHE experiments, myocytes in both C and T groups showed no changes in their cell lengths in response to the changes in their pH_i (data not shown).



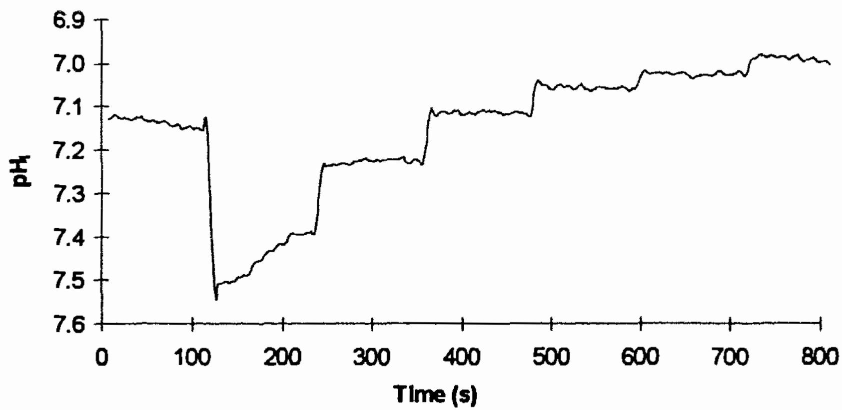
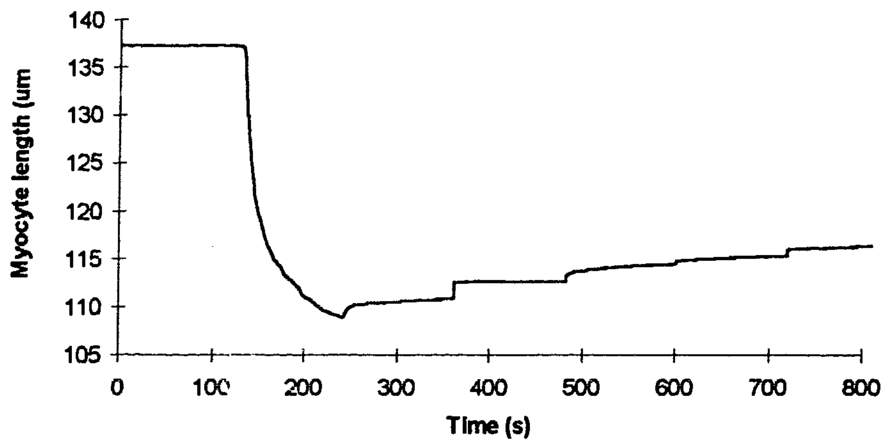


Figure 14. Relationships between changes in pH_i and changes in myocyte length (μm) of a cell in group C.



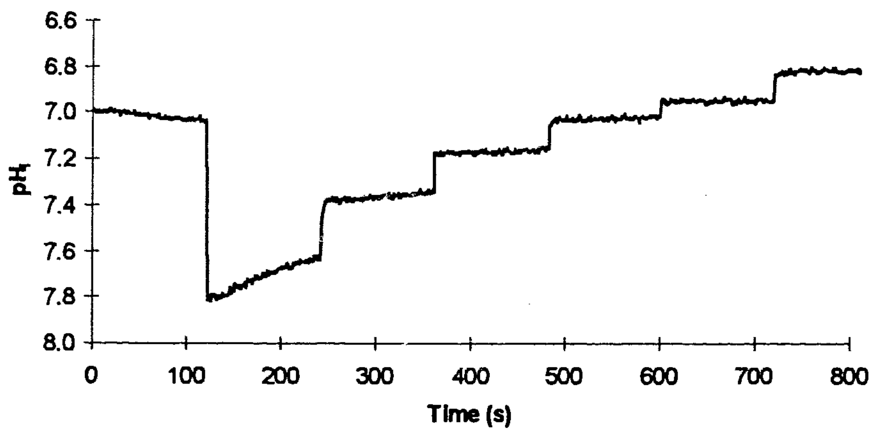


Figure 15. Relationship between changes in pH_i and changes in resting myocyte length (µm) of a cell in group T.

CHAPTER IV

DISCUSSION

A. INFLUENCE OF EXERCISE ON INTRACELLULAR BUFFERING CAPACITY (β_i)

One of the main findings in the present study was that the trained rats had a significantly higher cardiomyocyte H^+ buffering capacity than the control-sedentary rats. Exercise training however, had no influence on the resting (basal) pH_i ($p=0.079$, Student's *t*-test, one tail). The mean resting pH_i (@ pH_o 7.4, 37°C) in quiescent myocytes of C group was 7.051 (SEM 0.033, n=24). This value is well within the previously observed range for mammalian hearts under these conditions (Wallert and Frolich 1989, $pH_i=7.16 \pm 0.11$ for adult rats; Puceat, Clement-Chomienne, Terzic, and Vassort 1993, $pH_i=7.17 \pm 0.01$ for adult wistar rats; Xu and Spitzer 1994, $pH_i=7.05$; Bountra, Powell and Vaughan Jones 1989, $pH_i=7.0$ for guinea pig; Matsui, Barry, Livsey and Spitzer 1995, $pH_i=7.12$ for adult rabbit). For group T the resting pH_i was 7.013 ($\pm$ SEM 0.0065, n=24). As pH_i declined due to the step reduction of $[NH_4Cl]_o$, β_i increased; and this inverse relationship between β_i and pH_i of myocytes is in agreement with observations from other cell types (Kikeri, Sun, Zeidel and Hebert 1992, Roos and Boron 1981; Boyarsky, Ganz, Sterzel and Boron 1988; Xu and Spitzer 1994; Bountra, Powell and Vaughan-Jones 1990; Vaughan-Jones and Wu 1990).

Intracellular buffering in muscle is an important factor in offsetting the effects of an increased H^+ load during intense exercise (e.g. skeletal muscle) and in pathological

conditions such as myocardial ischemia and infarction. Only a few studies evaluating the effects of exercise on β_i in muscles have been performed and these are mostly on skeletal muscle preparations. The literature reports that in skeletal muscle, intracellular acidosis plays a prominent role in the development of fatigue during intensive muscle contraction (Hainaut and Duchateau, 1989). Accordingly, the decline in intracellular pH with intense exercise is thought to contribute to fatigue by inhibiting: 1) Ca^{2+} binding to troponin (Fabiato and Fabiato 1978), 2) the sarcoplasmic reticulum ATPase (Baldwin *et al.* 1972, Inesi and Hill 1983), 3) phosphofructokinase, the rate limiting enzyme of glycolysis (Danforth 1965), and 4) cross bridge cycling (Edman and Mattiazzi 1981). In cross-sectional studies of skeletal muscle from three athletic species : thoroughbred race horse (Harris *et al.* 1990), greyhound dog (Harris *et al.* 1990) and elite human sprinters (Harris *et al.* 1990, Parkhouse and Mc.Kenzie 1984, Parkhouse *et al.* 1985, Sahlin and Henriksson 1984), a high H^+ buffering capacity was found and became an important determinant of high-intensity exercise performance. The major conclusion from these studies is that muscle buffering capacity is strongly correlated with the ability of muscles to function anaerobically. Accordingly a higher muscle buffer value could allow more prolonged utilization of anaerobic glycolysis (Parkhouse and Mc.Kenzie 1984), but did not permit the accumulation of a higher muscle lactate concentration (Mannion *et al.* 1995, Sahlin and Hendriksson 1984). An elevated β_i is also associated with lesser decrement in muscle pH during intensive exercise (Mannion *et al.* 1995, Sahlin and Hendriksson 1984). It should be noted that these skeletal muscle studies are cross-sectional and not longitudinal and so it is not clear whether these differences in β_i are training- or genetically-induced.

Unlike skeletal muscle, one would not expect an exercise-induced acidosis in the myocardium. However, the greater β_i found in group T is a crucial factor in handling the excess proton load produced during the course of ischemia/infarction. Alternatively it may reduce the degree of acidosis that is known to disrupt many intracellular metabolic processes such as Ca^{2+} metabolism and enzymatically process that contribute to the contractile performance and cell viability.

Potential buffers to H^+ accumulation in mammalian skeletal muscle include: proton uptake by proteins, dipeptides and phosphates (Harris *et al.* 1990, Burton 1978, Somero 1981, Hultman and Sahlin 1980). In ventricular myocytes, protons are thought to be firstly buffered by histidines residue and then inorganic phosphate (P_i) probably becomes an important intracellular buffer especially for ischemic tissue (Allen and Orchard 1987, Dennis, Gevers and Opie 1991). In land-based mammals, the major muscle dipeptides are carnosine (β-alanyl-L-histidine) with a pK_a of 6.83 (Tanokura *et al.* 1976) and anserine (β-alanyl-(1-methyl)-L-histidine) with a pK_a of 7.04 (Merck Index 1983). This imidazole ring containing peptide: carnosine is particularly abundant (at millimolar levels) in skeletal muscles. These two histidines can provide for ~ 40 % of pH-buffering capacity of muscle tissue (Boldyrev 1990). In fast twitch skeletal muscle fibers of elite sprinters, carnosine and imidazole concentrations were found to be higher in comparison to controls. Furthermore, this increase was concomitant with higher pH buffering capacity (Mc.Kenzie *et al.* 1983, Parkhouse *et al.* 1983). Although it is not known whether exercise training can increase the quantity of imidazole dipeptides in cardiac muscle, these imidazoles containing substances have been found in the heart of rats, dogs and guinea pigs (O'Dowd,

Robins, Miller 1988, House, Miller and O'Dowd 1989) in concentrations (~10 mM) similar to that of the skeletal muscle. In addition to this, L-carnosine and L-homocarnosine have been recently reported to increase the sensitivity of myofilaments to Ca^{2+} in both skeletal and cardiac muscle (Lamont and Miller 1992). Calcium loading of the SR and mitochondria in skinned cardiac fibres is also strongly facilitated by carnosine (Harrisson , Lamont and Miller, 1986) and could be a secondary result of carnosine in buffering H^+. If in the trained heart carnosine levels increase, then it is expected that: 1) binding competition between H^+ and Ca^{2+} may be less, which subsequently may allow Ca^{2+} to bind to its binding sites or 2) it may significantly alter the sensitivity of the contractile apparatus to calcium ions. Thus, it is clear that further study (especially measuring the concentration of dipeptides and other potential intracellular buffers) has to be done in order to elucidate the responsible buffering moieties.

B. EXERCISE TRAINING AND NET H^+ EXTRUSION (J_{H^+}) THROUGH THE Na^+-H^+ EXCHANGER

As previously mentioned, Na^+-H^+ exchange plays a key role in regulating the cardiac intracellular pH (pH_i). The bottom trace of **Figure 9** showed that the acidosis induced by washing out the $[NH_4Cl]_o$ was not sustained and the cell recovered to its basal pH_i because a fall of pH_i stimulates acid extrusion via Na^+/H^+-exchanger. Forward exchange (1 external Na^+ exchange for 1 internal H^+) mediates pH_i recovery from intracellular acidosis and help maintain normal steady state pH_i. The present finding showed that after the drop in pH_i, in the absence of amiloride the cell recovered to its basal pH_i within < 8 minutes which in agreement with that reported by Puceat, Clement-

Chomienne, Terzic, and Vassort (1993). In the presence of 1.5 mM amiloride, the time required to recover from acidosis was significantly prolonged and in some cells pH_i did not completely return to the basal pH_i but stayed in acidotic condition. In this persistent acidosis, the possible contribution of Na^+ independent HCO_3^--Cl^- exchanger for H^+ loading is unlikely since the solution is free from CO_2/HCO_3^-. Thus this persistent acidosis, which has been reported in a previous study (Puceat, Clement-Chomienne, Terzic, and Vassort 1993) in HCO_3^- -free solutions, suggests that the amiloride successfully blocked the Na^+/H^+-exchanger under these experimental conditions. It should be noted that a non selective alkali cation-proton exchanger which has a similar affinity for both Na^+ and K^+ and thus responsible also for the process of H^+ extrusion has been described in cardiac sarcolemmal vesicles (Periyasamy *et al.* 1990). The experimental conditions in my studies therefore were set to minimize the role of this exchanger. The solution was contained low $[K^+]_o$ and normal $[Na^+]_o$ and as a consequence H^+ would be exchanged for Na^+ in preference to K^+. Thus, the data reported here mainly reflect the activity of Na^+/H^+-exchanger. The partial recovery seen in 1.5 mM amiloride could also reflect the fact that amiloride is weak base and will increase pH_i or that it is a competitive inhibitor of the Na^+/H^+-exchanger and is less effective in high $[Na]_o$. However the concentration of amiloride that was used was 1.5 mM which is higher than the predicted IC_{50} for amiloride under these experimental conditions.

In the nominal absence of CO_2 and HCO_3^-, the difference between amiloride (+A) treated and the non-amiloride (-A) treated cells in the rates of pH_i recovery (dpH_i/dt) after an NH_4Cl prepulse in both groups T and C were significant different over the investigated pH_i range 6.7 - 6.9. This suggests that amiloride (as expected) successfully blocked the

exchanger. It is surprising however, that the amiloride-sensitive component of dpH_i/dt of trained group was significantly decreased in comparison to that of control group. This result is unexpected and the mechanism responsible for this is not known. There are two possible ways: first, since β_i in group T was increased, it could be that the ability of Na^+-H^+ exchanger to extrude the excess H^+ out the cells is reduced relative to the increase intracellular buffering capacity. Therefore, virtually all of the intracellular proton removal capacity in trained cells appears to be taken over by training-induced activated intracellular buffering systems resulting in less dpH_i/dt (-A +A difference) found in group T. Secondly, the greater increase in β_i of group T represents greater activities of the exchanger and capacity of the cytoplasmic buffer systems. Despite the fact that the amiloride-sensitive dpH_i/dt during recovery was less in the trained group, the greater J_{H^+} of T group must be a reflection of an up regulation of the Na^+/H^+ exchanger extruding activity.

It is well known that catecholamines are released during exercise. Although it is not known whether circulating catecholamines can modify the expression of the sarcolemmal Na^+/H^+-exchanger, adrenergic stimulation on this exchanger activity should be considered, since it is known that α_1 and β adrenergic stimulation play a crucial role in regulating pH_i via protein kinase C-mediated activation of Na^+-H^+ exchange (Gambassi, Spurgeon, Lakatta, Blank and Capogrossi 1992). For example, α_1 adrenergic agonists such as phenylephrine are known to stimulate the Na^+/H^+-exchanger thus stimulating pH_i recovery from acidosis (Lagadic-Gossmann and Vaughan-Jones 1993; Wallert and Frohlich 1992; Puceat, Clement-Chomienne, Terzic and Vassort 1993); while β–adrenergic agonists such as isoprenaline inhibit this exchanger and thus inhibit pH_i recovery from acidosis (Lagadic-Gossmann and Vaughan-Jones 1993).

Increasing J_{H^+} is not without a potential negative impacts on Ca^{2+}_i and contraction. It is possible that the activation of NHE mechanism will lead to an increase in $[Na^+]_i$ and secondarily to increased $[Ca^{2+}]_i$ and the twitch due to reverse mode Na^+-Ca^{2+} exchange. Thus a Ca^{2+} overload and subsequent contracture might occur in this condition. It was reported that 1 mM amiloride completely inhibited the rise of Na^+_i (Meng and Pierce 1991, Harrison *et al.* 1992) resulted from ischemia and subsequent reperfusion. This observation is thought to account for the protective effects of amiloride and its derivatives from acidosis-induced cardiac contractile dysfunction (Meng and Pierce 1991, Harrison *et al.* 1992, Harper *et al.* 1993, Myers *et al.* 1995, Duff 1995) and infarction (Bugge and Ytrehus (1995).

C. INFLUENCE OF TRAINING-INDUCED CHANGES IN pH_i, AND β_i ON CONTRACTILITY.

As previously mentioned it was found that exercise training elicited an ~ 5 % ($p < 0.02$) increase in resting myocyte length (124.0 ± 2.0 vs 130.1 ± 2.0 μm in C and T, respectively). This is in agreement with earlier findings from our laboratory and others (Moore *et al.* 1993). It has been suggested that this longitudinal myocyte growth may contribute to an increased left ventricular end-diastolic dimension/volume and therefore may improve the intrinsic contractile function of the myocardium (Moore *et al.* 1993).

It is well documented that changes in intracellular pH_i has strong influence on intracellular Ca^{+2} homeostasis (Solaro, El-Saleh and Kentish 1989, Orchard and Kentish

1990, Kaibara and Kameyama 1988, Fabiato and Fabiato 1978, Fabiato 1985, Boyett, Kirby and Orchard 1988, Fry and Poole-Wilson 1981, Hecht and Hutter 1965) and subsequently on the contractile strength of cardiac myocytes (Harrison *et al.* 1992, Steenbergen *et al.* 1987, Marban *et al.*1987). In an experiment using the NH_4Cl prepulse technique (Spitzer and Bridge 1992), a rapid decrease in intracellular pH was reported to accompany an abrupt small decline in resting tension in papillary muscles and an increased resting cell length in unloaded myocytes. Increased pH_i (alkalosis) on the other hand, caused a much larger increase in resting tension and decrease in resting cell length. These changes in resting length and tension however, were small compared to the changes during twitch contraction. Excessive intracellular H^+ ions are known to impair the contractility by decreasing myofilament sensitivity to Ca^{2+} and by decreasing the Ca^{2+} delivery to myofilament by either : 1) inhibiting the Ca^{2+} current via L-type Ca^{+2} channel (Kaibara and Kameyama 1988), or 2) reducing SR Ca^{2+} uptake and subsequent release to cytoplasma (Fabiato and Fabiato 1978, Fabiato 1985), or 3) directly displacing Ca^{2+} from its cytoplasmic buffer sites (Orchard and Kentish 1990). In this thesis, pH_i and resting cell length were simultaneously measured. In accordance with an earlier finding (Spitzer and Bridge 1992), myocytes from the β_i experiment in cells from group T showed a similar response. An NH_4Cl-induced alkalosis elicited a decrease in cell length whereas step reductions of $[NH_4Cl]_o$ induced acidosis conversely elicited step increases in cell lengths (7 out of 24 cells measured). The alkalosis eliciting a decrease in cell lengths was similarly found in group C (11 out of 24 cells measured). However, in response to acidosis, decreasing pH_i effected the length of these 11 cells in different manner. A decrease in pH_i in the presence of amiloride did not coincide with an increase in cell length. Conversely,

the resting myocyte lengths persisted after exposure with acidotic pH_i ("alkalosis induced contracture"). Thus, the acidosis that was supposed to correct the increased pH_i and thus correct the decreased cell length as observed in group T, did not occur in group C. The reason for this is not understood. The phenomena of alkalosis-induced hypercontraction, however, has been reported previously (Harper *et al.* 1993, Bond *et al.* 1993). In those experiments, returning the pH_i after ischemia-induced acidosis (pH_i <6.5) in the alkalosis direction (pH_i 7.4, in the presence of monensin, a Na^+-H^+ ionophore) resulted in blebbing, hypercontraction and eventually cell death. Conversely, maintaining acidosis (in the presence of amiloride) protected the cells from those detrimental effects. Accordingly, reperfusion (washing out the intracellular H^+, thus returning the pH_i toward the "normal" - alkalotic direction) stimulates the exchange of intracellular H^+ for extracellular Na^+ via the Na^+-H^+ exchanger (Harper *et al.* 1993, Bond *et al.* 1993). The resulting increase of intracellular Na^+ stimulates exchange of intracellular Na^+ for extracellular Ca^{2+}, which may result in Ca^{2+} overload, activation of Ca^{2+}- dependent degradative enzymes and inhibition of mitochondrial oxidative phosphorylation. These Ca^{2+}- dependent effects may therefore account for the pH paradox. Apart from this theoretical basis, protection against hypercontraction (= decreased in cell length) or reperfusion injury in general by acidotic pH_i seems also to be mediated directly by pH_i itself. This is because in simulated ischemia where free Ca^{2+} was progressively increased and pH_i was kept acidotic (pH_i<6.2), it was found that blebbing, hypercontraction and cell death did not occur (Bond *et al.* 1993). The mechanism responsible for this is still not elucidated. The mechanism of amiloride induced acidosis preventing contracture is unlikely to play a role in the experiment of group C reported here. As previously mentioned some cells in group T were able to return

their cell lengths toward normal while in group C were not during exposure to acidosis pH_i. The mechanism behind this is unknown. Greater β_i could possibly contribute to this, since the more β_i the better the intracellular buffer systems to sequester the H^+ ions. Therefore a greater β_i in group T could better handle the excessive proton load, thus preventing Na^+ overload by reducing Na^+ influx through Na^+/H^+ exchanger, and secondarily prevents Ca^{2+} overload eliciting contracture. The failure of the cells to relax following the alkaline contracture could also be related to use of laminin. Weak restoring force found in group C may not overcome laminin binding of the cells (Dr. KW Spitzer, personal communication).

CHAPTER V

CONCLUSIONS

- The mean resting (basal) pH_i in quiescent myocytes of groups C (7.051) and T (7.013) were not significantly different from each other and well within the previously observed range for mammalian hearts under these conditions.

- The intracellular buffering capacity (β_i) increased as pH_i decreased in both the T and C groups. However, the magnitude of increase in β_i of the T group was found to be greater than that of the C group.

- The rate of pH_i recovery from a imposed proton load was found not to be different between the trained and control groups. Unexpectedly, the amiloride-dependent rate of pH_i recovery, however was found to be significantly **less** in T compared to C.

- The net H^+ extrusion (J_{H^+}) rate during the recovery from a imposed proton load was found to be greater in group T compared to C. Furthermore, the amiloride-sensitive or Na^+-H^+ exchanger-dependent J_{H^+} was significantly greater in group T.

- By increasing β_i and subsequently J_{H^+}, exercise training may provide the cardiomyocytes with the ability to handle better an intracellular excess of H^+ generated during hypoxia/ischemic insults and may serve in a cardio-protective role. This

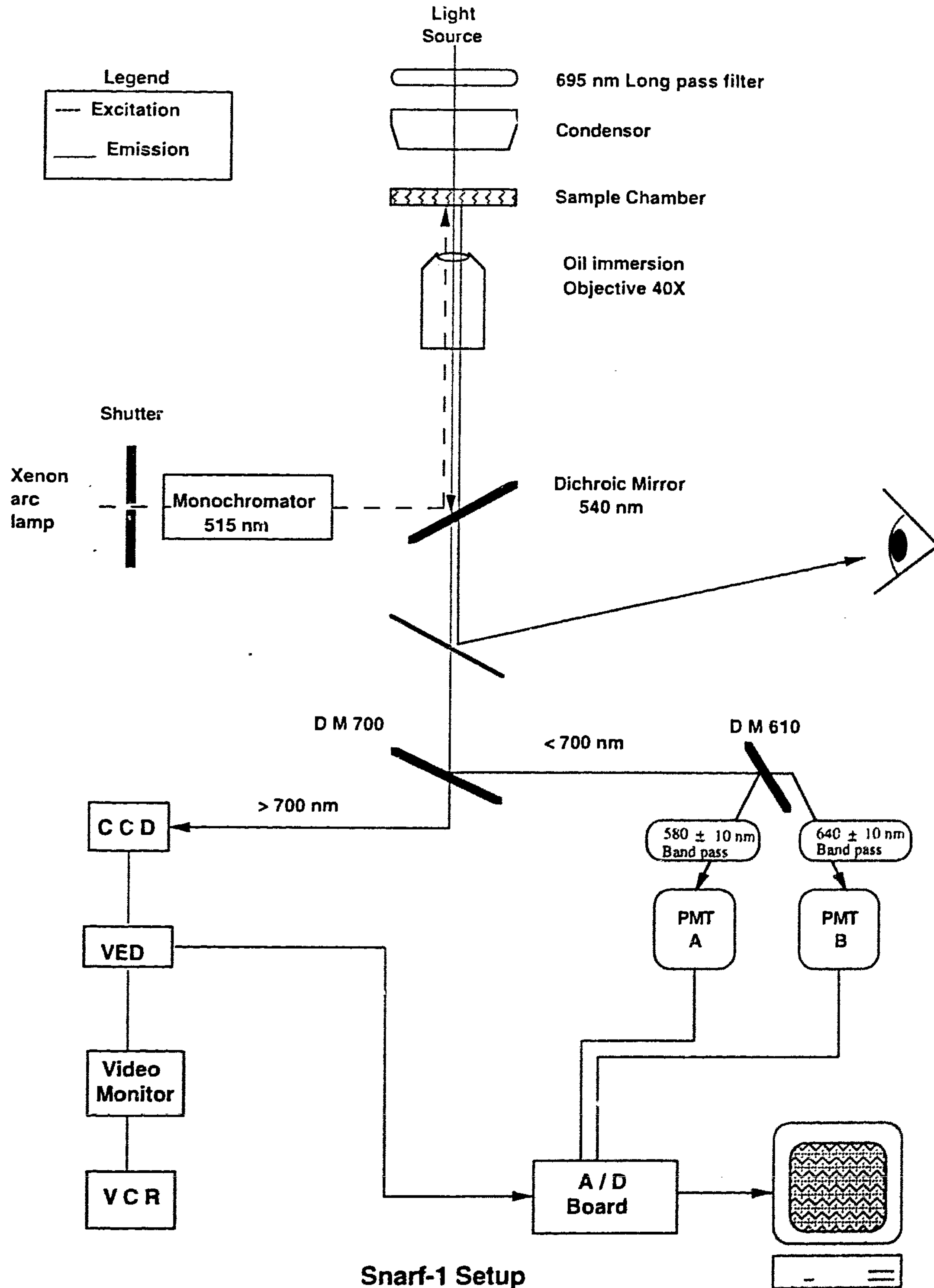
potential protective mechanism could act by subsequently limiting the degree of myocardial damage previously described by others in epidemiological studies.

- Alkalosis elicited a decrease in cell length (μm) in both groups T and C. Conversely acidosis induced by subsequently reducing the $[NH_4Cl]_o$ elicited an increase in cell lengths of some cells in group T. In group C, some cells responded differently to acidosis. Decreasing pH_i after the period of 20 mM NH_4Cl induced alkalosis did not produce an increase in cell lengths as seen in group T. The mechanism responsible for this is unknown.

- The results from this study may be useful in explaining the exercise-induced increase in myocardial contractility observed in several laboratories. In these studies, myocardial contractility was enhanced by training while myocyte $[Ca^{2+}]_o$ was found to be lower or unchanged. The mechanism for this enhanced contractile sensitivity is not known but may be impacted by changes in β_i observed in this study.

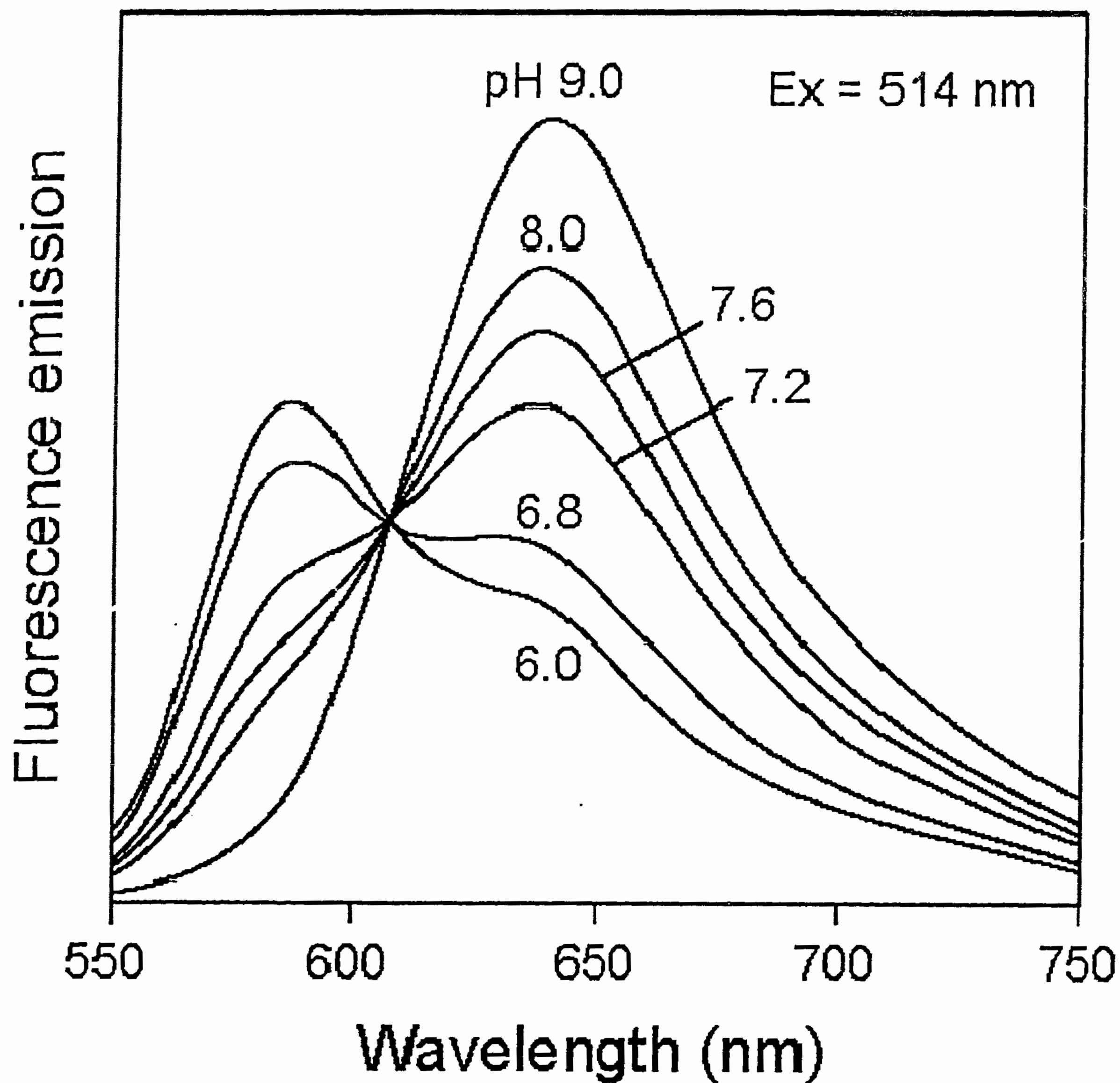
APPENDIX 1.
OPTICS

1.1 Optical Setup for SNARF-1



Snarf-1 Setup

1.2 Emission Spectra for SNARF-1



The pH-dependent emission spectra of carboxy SNARF-1 when it is excited at 514 nm (copied from Molecular Probes: Handbook of Fluorescent Probes and Research Chemicals by Richard P.Haughland 1992-1994)

APPENDIX 2.

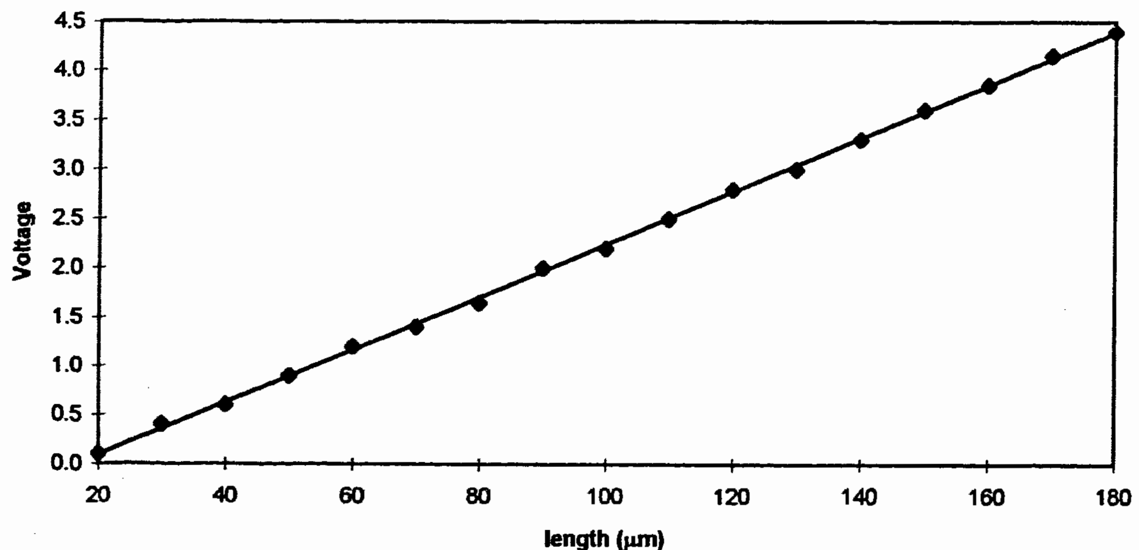
PRINCIPLE OPERATION OF VIDEO EDGE DETECTOR

2.1 Principles of the Video Edge Detector

A video image was produced by focusing the myocyte on the light-sensitive element of the high resolution CCD video camera. This video camera rapidly scans the image area with 256 parallel horizontal lines from top to bottom in every 16.7 ms, corresponding to a typical vertical sweep rate (60 Hz). The image of a single myocyte was positioned so that the cell longitudinal axis is oriented parallel to the horizontal scan lines (raster lines). Every time it scans, the single raster line sweeps from left to right and the voltage along this line varies in response to changes in light intensity on the element. To identify each end of the cell, the edge detection system depends on these voltage changes. The ramp and the sample and hold (S&H) circuits are activated at the start of the sweep and the linearly rising voltage ramp serves as the reference signal for the left and right sample and hold circuits. Adjusted by the user, a raster line was selected to pass through the longitudinal axis of the myocyte. Left and right windows were selected on this raster line to cover the left and right ends of the cell, respectively. The tresholds of the tracking dot in each window were adjusted until the dot locked on to the edge. The edge transition (black to white or white to black) of the right and left tracking dots were also individually adjusted until the user saw appropriate tracking. The ramp voltage is retained by the left S&H circuit and this left threshold voltage (ΔV_l), representing the difference signal between the two dots, is recorded by VED. The voltage ramp continuously increases linearly until the raster line sweep attains the right tracking dot of the right cell edge,

reaching its threshold voltage (right threshold voltage, ΔV_2), whereby the S&H circuit retains this ramp voltage. During the sweep, the cell length is displayed as (ΔV_2-ΔV_1) and continuously updated with each vertical sweep. Thus, the output from the edge detector (ΔV_2-ΔV_1) is proportionally related to myocyte length and represents as a function of time ($V_{(t)}$). The relationship between (ΔV_2-ΔV_1) and length was determined by mounting a graticule in 10 μm increments (shown below) on the stage of the microscope. The (ΔV_2-ΔV_1) output was digitized and stored on the 486 DX2 computer. $V_{(t)}$ was digitally (Felix, PTI) differentiated with respect to time for the analysis of the rate of change of cell length ($d(V_{(t)})/dt$). $V_{(t)}$ was displayed on an analog oscilloscope (Tektronics).

2.2 Video Edge Detector Calibration



APPENDIX 3

MYOCYTE ISOLATION AND PLATING SOLUTIONS

Since the myocyte pH_o is 7.40 at 37°C but the experiments will be conducted at 25°C, we modified pH_o based on the α-stat relationship. From this we used a pH_o of 7.6 at 25°C.

3.1. Calcium-free solution. The stock solution (one litre) was made up by dissolving 1 package of MEM (Minimal Essential Medium -Joklik Modified) from Gibco Laboratory (Cat. no.410-2300 EA) and buffered with TES and sodium bicarbonate. The solution was continuously gassed with 100%O_2 and equilibrated with 1 N NaOH at 25°C to give a pH 7.60. This stock solution was a standard solution and it was stored at refrigerator until further use. The compositions of the solution are given in table 1 and MEM in table 3.

3.2. Collagenase Solution. MEM solution was used to dissolve the enzyme collagenase. 1-2 mg/ml MEM solution of crude collagenase (Type II, Worthington Biochemical corporation, Freehold, NJ. Cat.no.M5C 314) was used in each myocyte isolation and small amount of Ca^{2+}, in the form of $CaCl_2$, was added in order to ensure proper tissue digestion. In addition, bovine serum albumin (fatty acid free BSA, fraction V, Boehringer Mannheim GmbH) was also added to this solution. The final solution was then filtered using a sterile filter : 0.22 mm cellulose acetate syringe filters (Nalgene), prior to use. The composition of this solution is given in Appendix 3.6.

3.3. Washing Solutions. There were 3 different MEM solutions (A,B and C solutions) used for washing cells. Each of the three solutions differed in the concentrations of $CaCl_2$ content which was gradually increased to reach the normal extracellular calcium concentration of 1 mM. The composition of these solutions are given in Appendix 3.6.

3.4. Solution for cell recovery. The stock solution (2L) that was used for cell recovery was prepared by dissolving 1 package of Medium 199 from Gibco laboratory (cat. no 400-1100 EB) that contains 1.8 mM Ca^{2+} (in the form of $CaCl_2$ anhydrous). Since the calcium requirement for this cell recovery period in this experiment was set up to 1 mM, in this 2 L solution was then added 0.1 mM $CaCl_2$ to bring this [Ca] up to that level. Na^+ and K^+ were kept at their original concentrations i.e 116.35 mM and 5.365 mM respectively. It was also buffered with TES 10 mM and gassed with 100% O_2 at 25°C to give a pH of 7.60. The antibiotics Penicillin G and Streptomycin were added to prevent bacterial contamination. The solution was stored in the refrigerator until further use. At time of use, 5 ml of FBS (Fetal Bovine Serum, Gibco lab. Cat.no 26140-012, Lot no. 32P3237) was added to 95 ml of this M199 stock solution. Before use, the FBS was activated by heating at 56°C for 30 minutes, then 5 ml aliquots per tube were stored at 4°C. The composition of this solution is given in Appendix 3.6. and that of M199 in Appendix 3.7.

3.5. Laminin stock solution

Before use, laminin which was stored at -70°C, was thawed slowly overnight at 4°C. One ml of laminin was diluted in 49 ml of Medium 199 to give a 20 μg/ml of laminin stock solution which was stored at -70°C in 1 ml aliquots until used.

3.6. Composition of myocyte isolation and plating solutions

Solution	1.1	1.2	1.3. A	1.3.B	1.3.C	1.4	1.5
MEM (1 pkg)	*	*	*	*	*	-	-
M199 (1 pkg)	-	-	-	-	-	*	*
TES (10 mM)	*	*	*	*	*	*	*
$NaHCO_3$ (4.4 mM)	*	*	*	*	*	-	-
pH 7.60 at 25°C with 1N NaOH	*	*	*	*	*	*	*
Collagenase(mg/ml MEM)	-	1-2	-	-	-	-	-
$CaCl_2$ (μM)	-	50	200	500	1,000	1,000	1,000
BSA (4 mg/ml MEM)	-	*	-	-	-	-	-
FBS (5 ml/100 ml M199)	-	-	-	-	-	*	*
Penicillin G (0.1 mg/ml)	-	-	-	-	-	*	*
Streptomycin (0.1 mg/ml)	-	-	-	-	-	*	*
Laminin (20 μg/ml M199)	-	-	-	-	-	-	*
100% O_2	*	*	*	*	*	*	*

3.7 Composition of M199 and MEM Solutions (mg/L)

	Medium 199	MEM
Inorganic Salts		
$CaCl_2$ (anhyd)	200	-
$Fe(NO)_3.9H_2O$	0.72	-
KCl	400	400
$MgSO_4$ (anhyd)	97.67	-
$MgCl_2.6H_2O$	-	200
NaCl	6,800	6,500
$NaH_2PO_4.H_2O^a$	140	1,327
Amino Acids		
DL-Alanine	50	-
L-Arginine.HCl	70	126
DL-Aspartic acid	60	-
L-Cysteine.HCl.$2H_2O$	0.11	-
L-Cysteine.2HCl	26.06	32.40
DL-glutamic acid.H_2O	150	-
L-glutamine	100	294
Glycine	50	-
L-Histidine.HCl.H_2O	21.88	-
L-Histidine	-	31
L-Hydroxyproline	10	-
L-Isoleucine	40	52
DL-Leucine	120	-
L-Leucine	-	52
L-Lysine.HCL	70	-
L-Lysine	-	58
DL-Methionine	30	-
L-Methionine	-	15
L-Phenylanine	50	32
L-Proline	40	-
DL-Serine	50	-
DL-Threonine	60	-
L-Threonine	-	48
L-Tyrptophan	20	10
L-Tyrosine.2Na..$2H_2O$	57.66	54.52
L-Valine	50	46

3.7. Composition of M199 and MEM Solutions (mg/L) (continued)

	Medium 199	MEM
Other components		
Adenine Sulfate	10	-
Adenosinetriphosphate.2Na	1	-
Adenylic acid	0.20	-
Cholesterol	0.20	-
Deoxyribose	0.50	-
D-glucose	1,000	2,000
Glutathione (reduced)	0.05	-
Guanine.HCl	0.30	-
Hypoxanthine.Na	0.354	-
Phenol red	20	10
Ribose	0.50	-
Sodium acetate	50	-
Thymine	0.30	-
TWEEN 80	20	-
Uracil	0.30	-
Xanthine.Na	0.344	-
Vitamins		
Ascorbic acid	0.05	-
a-Tocopherol phosphate (Sodium salt)	0.01	-
Biotin	0.01	-
Calciferol	0.10	-
D-Ca pantothenate	0.01	1
Choline chloride	0.05	1
Folic acid	0.01	1
i-Inositol	0.05	2
Menadione	0.01	-
Niacin	0.025	-
Niacinamide	0.025	1
Para-aminobenzoic acid	0.05	-
Pyridoxal.HCl	0.025	1
Riboflavin	0.01	0.10
Thiamine.HCl	0.01	1
Vitamin A (acetate)[a]	0.14	-

APPENDIX 4.

CALIBRATION SOLUTIONS

4.1. ***In vitro*** **Calibration Solutions.** For the *in vitro* calibration, Tyrode solutions with pH values of 6.50, 6.75 and 7.00 were buffered with PIPES while the remaining solutions (pH 7.20, 7.40, 7.60 and 7.80) were buffered with HEPES. pH was adjusted using 1.0 N KOH and KCl was added to maintain $[K^+]$ at 140.0 mM, at 25°C. The final solution also contained $MgCl_2$ and was ready to use with 5 μM SNARF-1. Prior to use, the unesterified SNARF-1 (Molecular Probes, Eugene, OR., USA. Cat. no C-1270, MW 453.45) was prepared by dissolving it intoTyrode's solution to yield a 1 mM stock solution of SNARF-1.

4.2. ***In vivo*** **Calibration Solutions.**

A 1 mM stock solution of SNARF-AM (Molecular Probes, Eugene, OR., USA. Cat.no. C-1272, M.W.: 567.55. 1 vial contains 50 μg SNARF) was prepared by adding DMSO to the vial. The stock solution was stored in the dark at -25°C until required. In order to load the cells with this fluorescence dye, the SNARF-AM was diluted to a final concentration of 15 μM with the pH measurement solution described in Appendix 5.

For the *in vivo* calibration, solutions were made at various pH levels (pH: 6.50, 6.75, 7.00, 7.20, 7.40, 7.60, and 7.80) as described in Appendix 4.4. These were titrated with 1N KOH. EGTA and BDM was added to prevent contractures during the calibration.

4.3 Composition of *in vitro* pH Calibration Solutions

pH	6.50	6.75	7.00	7.20	7.40	7.60	7.80
PIPES (12mM)	*	*	*	-	-	-	-
HEPES (12mM)	-	-	-	*	*	*	*
KCl (140 mM)	*	*	*	*	*	*	*
$MgCl_2$ (10mM)	*	*	*	*	*	*	*
SNARF-1 (5mM)	*	*	*	*	*	*	*

4.4. Composition of *in vivo* pH Calibration Solutions

	R_{max}								R_{min}
pH	6.00	6.50	6.75	7.00	7.20	7.40	7.60	7.80	8.20
PIPES (12mM)	*	*	*	*	-	-	-	-	-
HEPES (12mM)	-	-	-	-	*	*	*	*	*
KCl (140 mM)	*	*	*	*	*	*	*	*	*
$MgCl_2$ (1 mM)	*	*	*	*	*	*	*	*	*
Dextrose (11 mM)	*	*	*	*	*	*	*	*	*
EGTA (2 mM)	*	*	*	*	*	*	*	*	*
BDM (15 mM)	*	*	*	*	*	*	*	*	*
Nigericin (10 mM)	*	*	*	*	*	*	*	*	*

APPENDIX 5.

pH_i MEASUREMENTS

5.1 pH Challenge and Measurement Solutions. For the actual measurement of pH_i, using NH_4Cl, the Tyrode solutions were made at a pH 7.60 at 25°C by titrating with 13 mM NaOH. The composition of each solution is shown below.

5.2 Composition of pH Measurement Solutions

NH_4Cl (mM)	0	20	10	5	2.5	1.25
pH 7.60 @ 25°C	*	*	*	*	*	*
HEPES (12 mM)	*	*	*	*	*	*
KCl (4.4 mM)	*	*	*	*	*	*
$MgCl_2$ (1 mM)	*	*	*	*	*	*
NaCl (126 mM)	*	*	*	*	*	*
Dextrose (11 mM)	*	*	*	*	*	*
$CaCl_2$ (1 mM)	*	*	*	*	*	*

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