MOLECULAR AND CELLULAR MECHANISMS ASSOCIATED WITH CARDIAC HYPERTROPHY FOLLOWING MYOCARDIAL INFARCTION IN RATS: STUDIES ON ION CHANNELS AND INTRACELLULAR CALCIUM

by

ROGER KAPRIELIAN

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy.

Institute of Medical Sciences University of Toronto

© Copyright by Roger Kaprielian (2000)
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

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

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

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

Prolongation of the cardiac action potential duration (APD) is a hallmark feature of cardiac hypertrophy. In my studies, I examined the molecular and ionic basis of this electrical phenotype in different anatomical regions of the heart and examined its functional consequence on calcium handling in rats 8 weeks following ligation of the left anterior descending (LAD) coronary artery. LAD ligation resulted in approximately 40% infarction of the left ventricle, a decrease in left ventricular developed pressure, an increase in left ventricular end-diastolic pressure with no evidence of pulmonary congestion, increased wet heart weight to body weight ratios and elevated cellular capacitance. Following myocardial infarction (MI), the density of the transient outward K⁺ current (Ito) was reduced by about 2 fold in right ventricular myocytes but not septal myocytes. Kv4.2 mRNA and protein and Kv4.3 mRNA were markedly reduced in the right ventricular myocytes but not in septal myocytes, suggesting that reductions in Kv4.2 and Kv4.3 are responsible for the reduced Ito. The recovery from inactivation of Ito was slowed and was associated with an elevation in Kv1.4 mRNA and protein in right ventricular myocytes, but not septal myocytes. Consistent with the changes in Ito density, changes in APD and Ca²⁺ transients following MI varied between the right ventricle and septum. Moreover, the elevations in [Ca²⁺]i were directly linked to prolongation of action potential duration and were not related to changes in L-type Ca²⁺ currents or altered calcium handling in post-MI myocytes. Treatment with the thyroid hormone analog, DITPA, for five weeks restored Ito density by reversing Kv4.2 and Kv1.4 expression and abbreviating action potential duration to levels observed in sham-operated controls. Our
findings demonstrate that LAD ligation in rats is associated with electrical, ionic and genetic changes that can be reversed by thyroid hormone administration.
ACKNOWLEDGMENTS

I would like to take this opportunity to express my sincere appreciation to the people who helped in the preparation of this thesis. I would like to thank my advisory committee; Dr. Peter Backx, Dr. Peter Liu, Dr. Tom Parker and Dr. Christine Bear. These individuals were always easy to approach and more than willing to offer their advice and expertise. In particular, I gratefully acknowledge the assistance and guidance provided by Dr. Backx who acted as my supervisor. I would like to thank the Medical Research Council of Canada for the personnel support through the Doctoral Research Award.

I wish to thank Dr. Fayez Dawood for performing all the required surgeries and Ms. Tin Nguyen for performing the RNase protection assays and the Western blots analyses. Also I would like to express my gratitude to Dr. Robert Tsushima (University of Toronto) who was of great help in all aspects of my studies. Rob was always willing to help around the clock and was indispensable to the laboratory in many ways. Thanks are extended to Dr. Alan Wickenden (Icagen Inc.) for his input on my projects and fellow graduate students that I interacted with on a daily basis (Hee-Cheol Cho, Zam Kassiri, Vito Losito and Rajan Sah). Sincere thanks are extended to Dr. Roger Hajjar (Harvard University) who has been extremely patient during the writing of the Thesis as I begin my post-doctoral studies in his laboratory.

I would like to thank my parents and their constant source of encouragement in the difficult times especially on those long recording days. Finally, this thesis would have not been possible without the support and encouragement of my wife, Vania.

Roger Kaprielian, November 1999
THE WAY OF AN INVESTIGATOR

In the past four years, I learned a great deal on the process of scientific inquiry. I realized that the process of scientific investigation is universal in all the different disciplines of science. First, one must identify a problem or phenotype that can be readily quantified followed by formulation of specific hypotheses that may explain the mechanism of the observed phenotype. Second, one must test the proposed mechanism in given experimental model. Third, upon identification of the mechanism, strategize models that can rescue the phenotype. This thinking mode allowed me to learn and pose pertinent questions about the mechanisms of disease. In the past 10 years or so, many molecular and biochemical abnormalities have been identified implicated in the onset and progression of cardiac disease that have provided us a conceptual framework to test the mechanisms of heart disease. Advances in the field of transgenesis and gene therapy have enabled us to learn the genetic and cellular mechanisms by which heart cells undergo hypertrophy and ultimately failure. This knowledge will hopefully help us to find a cure for this terrible disease. Dr. Eugene Braunwald once said: “It is now clear that abnormalities of molecular processes may be the basis of many cardiovascular diseases and that genetic influences play critical roles in the development of these abnormalities. (Dr. Eugene Braunwald, Boston, Massachusetts From Heart Disease: A Textbook of Cardiovascular Medicine, 5th edition).
STATEMENT OF CONTRIBUTION

As mentioned in Chapter 2, the group technician (Ms. Tin Nguyen) performed all the molecular assays such as the ribonuclease protection assays and the Western blot analyses. Dr. Alan Wickenden performed the transfections and recordings on the tsa-201 cells. The left anterior descending ligation had been previously developed and characterized in the Department by the laboratory of Dr. Liu. His technician (Dr. Fayez Dawood) performed all the required surgeries. I performed the myocyte isolations, the electrophysiological and fluorescence experiments arising from this Thesis.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>2-3</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>4</td>
</tr>
<tr>
<td>THE WAY OF AN INVESTIGATOR</td>
<td>5</td>
</tr>
<tr>
<td>STATEMENT OF CONTRIBUTION</td>
<td>6</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>7-8</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>9</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>10-12</td>
</tr>
<tr>
<td>CHAPTER 1. INTRODUCTION</td>
<td>13-63</td>
</tr>
<tr>
<td>1.1 Purpose and Specific Hypotheses</td>
<td></td>
</tr>
<tr>
<td>1.2 Synopsis</td>
<td></td>
</tr>
<tr>
<td>1.3 Literature Review</td>
<td></td>
</tr>
<tr>
<td>1.3.1 The Heart</td>
<td></td>
</tr>
<tr>
<td>1.3.2 Ion Channels</td>
<td></td>
</tr>
<tr>
<td>1.3.3 The Ventricular Action Potential</td>
<td></td>
</tr>
<tr>
<td>1.3.4 Depolarizing currents provided by sodium channels and L-type calcium channels</td>
<td></td>
</tr>
<tr>
<td>1.3.5 Repolarizing currents provided by potassium channels</td>
<td></td>
</tr>
<tr>
<td>1.3.5A The delayed rectifier potassium channel</td>
<td></td>
</tr>
<tr>
<td>1.3.5B The inward rectifier potassium channel</td>
<td></td>
</tr>
<tr>
<td>1.3.5C The transient outward potassium channel</td>
<td></td>
</tr>
<tr>
<td>1.3.5D Nomenclature and molecular correlate of the transient outward potassium channel</td>
<td></td>
</tr>
<tr>
<td>1.3.5E Biophysical characteristics of the transient outward potassium channel</td>
<td></td>
</tr>
<tr>
<td>1.3.5F The transient outward potassium channel in cardiac development and hypertrophy</td>
<td></td>
</tr>
<tr>
<td>1.3.5G Regional differences in the transient outward potassium channel function and expression</td>
<td></td>
</tr>
<tr>
<td>1.3.6 Excitation-contraction coupling in the normal myocardium</td>
<td></td>
</tr>
<tr>
<td>1.3.7 Rat models of cardiac hypertrophy and failure</td>
<td></td>
</tr>
<tr>
<td>1.3.8 Mechanism of cardiac hypertrophy</td>
<td></td>
</tr>
<tr>
<td>1.3.9 Alterations in electrical phenotype: Action potential prolongation</td>
<td></td>
</tr>
<tr>
<td>1.3.10A Mechanism of action potential prolongation</td>
<td></td>
</tr>
<tr>
<td>1.3.10 Calcium handling in human cardiac failure</td>
<td></td>
</tr>
<tr>
<td>1.3.11 Calcium handling in animal models of cardiac hypertrophy and failure</td>
<td></td>
</tr>
<tr>
<td>1.3.12 Regional modulation of gene expression in hypertrophic myocytes</td>
<td></td>
</tr>
</tbody>
</table>
1.3.13 Connection between action potential prolongation, $[\text{Ca}^{2+}]_i$ and cardiac hypertrophy

1.3.14 Thyroid Hormones
1.3.14 A. Effect of thyroid hormones on potassium channels

CHAPTER 2. METHODS..........................................................................................64-82

2.1 Left anterior descending coronary artery ligation procedure
2.2 Treatment with 3,5-diiodothyropropionic acid (DITPA)
2.3 In vivo monophasic action potential and hemodynamic studies
2.4 Isolation of ventricular myocytes
2.5 Basic electrophysiological circuitry
2.6 Whole-cell electrophysiological evaluation
2.7 tsa-201 culture, transfection and electrophysiological recordings
2.8 Electrophysiological recordings in ventricular myocytes
2.9 Fluorescence microscopy in contracting myocytes
2.10 Sarcoplasmic reticulum calcium content in ventricular myocytes
2.11 Molecular assays to study ion channel function and expression
2.11.1 RNA extraction and RNAse protection assay
2.11.2 Protein extraction and Western blotting

CHAPTER 3. Relationship between $K^+$ channel down-regulation and $[\text{Ca}^{2+}]_i$ in rat ventricular myocytes following myocardial infarction.................................83-115

CHAPTER 4. Regional differences in $Kv1.4$, $Kv4.2$ and $Kv4.3$ to the transient outward $K^+$ current in the normal and infarcted rat heart ........................................116-145

CHAPTER 5. The thyroid hormone analog, 3,5-diiodothyropropionic acid (DITPA) restores the transient outward potassium channel in rats following myocardial infarction........................................146-171

CHAPTER 6. DISCUSSION....................................................................................172-194

LIMITATIONS OF THE STUDY........................................................................195-197
The advantages and disadvantages of using the rat animal model Experimental conditions

RECENT PROGRESS, PROSPECTS AND CHALLENGES..............................197-204

REFERENCES.................................................................................................205-227

PUBLICATIONS ARISING FROM THIS THESIS..........................................228-229
REPRINTS FOR SELECTED PUBLICATIONS.............................................230-239
## LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Potassium currents in cardiac hypertrophy and failure</td>
<td>54</td>
</tr>
<tr>
<td>1</td>
<td>Changes associated with myocardial infarction</td>
<td>104</td>
</tr>
<tr>
<td>2</td>
<td>Effect of action potential duration on the L-type calcium current</td>
<td>104</td>
</tr>
<tr>
<td>3</td>
<td>Changes associated with myocardial infarction in right ventricular and septal myocytes</td>
<td>136</td>
</tr>
<tr>
<td>4</td>
<td>Regional differences in the characteristics of the transient outward current in right ventricular and septal myocytes following myocardial infarction</td>
<td>137</td>
</tr>
<tr>
<td>5</td>
<td>General characteristics and hemodynamic parameters following myocardial infarction and DITPA treatment</td>
<td>163</td>
</tr>
<tr>
<td>6</td>
<td>Characteristics of the transient outward current following myocardial infarction and DITPA treatment</td>
<td>164</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

FIGURE I. Cartoon representation of a voltage-gated ion channel (adapted from *ionic channels in excitable membranes*, By B. Hille, 1991). 20

FIGURE II. General structure of the α subunit of cardiac ion channels. The potassium channel genes along with their respective currents in the heart are listed. (adapted from Structure and function of cardiac sodium and potassium channels. By D.M. Roden and A.L George-invited review. American Physiological Society, 1997). 24

FIGURE III. Schematic of the depolarizing and repolarizing currents that underlie the cardiac action potential in the rat (modified from *ion channels in the cardiovascular system: function and dysfunction*. By H. Fozzard, 1994) 27

FIGURE IV. General scheme of the calcium handling in a cardiac myocyte (Adapted from *excitation-contraction coupling and cardiac contractile force*. By D. Bers 1991) 40

FIGURE V. Circuitry in electrophysiological studies. 69

FIGURE VI. The big picture. The role of potassium channels in cardiac hypertrophy and disease progression. (Modified from Wickenden et al., 1998). 182

FIGURE VII. The effect of varying the duration of cellular depolarization on calcium handling. 198

FIGURE VIII. The role of trigger action potential under controlled loading conditions on calcium handling. 199

FIGURE IX. Time course of down-regulation of the transient outward current following myocardial infarction. 204

FIGURE 3.1 Action potential characteristics in right ventricular myocytes following myocardial infarction. 105

FIGURE 3.2. The inward rectifier (I_K1) in right ventricular myocytes following myocardial infarction. 106

FIGURE 3.3. The transient outward (I_o) and sustained currents (I_{sus}) in right ventricular myocytes following myocardial infarction. 107

FIGURE 3.4. Biophysical properties of I_o in right ventricular myocytes following myocardial infarction. 108
FIGURE 3.5. Representative comparison of candidate genes encoding the transient outward current in the right ventricle following myocardial infarction.

FIGURE 3.6. Action potential and $[Ca^{2+}]_i$ characteristics recorded from sham and post-MI right ventricular myocytes.

FIGURE 3.7. Effect of action potential prolongation on $[Ca^{2+}]_i$ in right ventricular myocytes.

FIGURE 3.8. Effect of short voltage-clamp pulses on the $[Ca^{2+}]_i$ transient in sham and post-MI right ventricular myocytes.

FIGURE 3.9. The L-type calcium current ($I_{Ca,L}$) in right ventricular myocytes following myocardial infarction.

FIGURE 3.10. Effect of action potential duration on the L-type calcium current ($I_{Ca,L}$).

FIGURE 3.11. $Ca^{2+}$ content in the sarcoplasmic reticulum in right ventricular myocytes following myocardial infarction.

FIGURE 4.1. Action potential characteristics in right ventricular and septal myocytes following myocardial infarction.

FIGURE 4.2. The transient outward ($I_{to}$) and sustained currents ($I_{sus}$) in right ventricular and septal myocytes following myocardial infarction.

FIGURE 4.3. Biophysical properties of $I_{to}$ in right ventricular and septal myocytes following myocardial infarction.

FIGURE 4.4. Representative comparison of candidate $K^+$ channel $\alpha$-subunits encoding the transient outward current in the right ventricle and septum following myocardial infarction.

FIGURE 4.5. The inward rectifier ($I_{K1}$) in right ventricular and septal myocytes following myocardial infarction.

FIGURE 4.6. The L-type calcium current ($I_{Ca,L}$) in right ventricular and septal myocytes following myocardial infarction.

FIGURE 4.7. Action potentials and $[Ca^{2+}]_i$ in right ventricular and septal myocytes following myocardial infarction.

FIGURE 4.8. Voltage-clamp stimulated $[Ca^{2+}]_i$ in right ventricular and septal myocytes following myocardial infarction.
FIGURE 5.1. Effect of DITPA on the transient outward ($I_{to}$) and sustained currents ($I_{sus}$) in right ventricular myocytes following myocardial infarction. 165

FIGURE 5.2. Effect of DITPA on genes encoding the transient outward current and myosin heavy chain in the right ventricle. 166

FIGURE 5.3. Effect of DITPA on Kv channel subunit immunoreactive proteins encoding the transient outward current in the right ventricle following myocardial infarction. 167

FIGURE 5.4. Effect of DITPA on the inward rectifier ($I_{k1}$) current in right ventricular myocytes following myocardial infarction. 168

FIGURE 5.5. Effect of DITPA on the L-type calcium current ($I_{Ca,L}$) in right ventricular myocytes following myocardial infarction. 169

FIGURE 5.6. Effect of DITPA on action potentials in right ventricular myocytes following myocardial infarction. 170

FIGURE 5.7. Effect of DITPA on monophasic action potentials in the right ventricular free wall following myocardial infarction. 171

Please note: The figures labelled as x.y were done to satisfy thesis requirements where x represents the Chapter, whereas y represents the number of the figure.
1.1 PURPOSE AND SPECIFIC HYPOTHESES

Heart disease is a major cause of death in Canada and 23,000 individuals die each year as the result of an acute myocardial infarction (Heart and Stroke Foundation of Canada website at www.hsf.ca/research). Through research, we are able to understand some of the targets and mechanisms of disease with the ultimate goal to use this information to tailor therapy and treatment of this terrible disease. The mechanism by which cardiac hypertrophy occurs is multifactorial and no single mechanism can explain the chain of events leading up to cardiac hypertrophy. In this thesis, I will examine one of the abnormalities that is associated with cardiac hypertrophy. The so-called electrical abnormality is a prolongation of the action potential duration is present in several animal models of cardiac hypertrophy. This electrical phenotype also exists in animal models of cardiac failure and in end-stage human hearts. Therefore, the purpose of these studies were to 1) examine whether action potential prolongation occurs in a rat model of myocardial infarction 2) examine the molecular and ionic mechanism that explain the observed electrical phenotype and the consequence on calcium handling 3) examine the regional modulation of ionic currents and intracellular calcium 4) examine the effect of thyroid hormone on potassium channel function and expression. To this end, I combined molecular and electrophysiologic approaches to study these changes in rat hearts 8 weeks following ligation of the left anterior descending coronary artery ligation. With the specific aims that were set out for this study, my working hypothesis was that prolongation of action potential duration occurs due to a reduction of repolarizing $K^+$ channel expression resulting in changes in calcium handling. Due to the heterogeneity of $K^+$ channel expression in mammalian ventricular myocardium, myocytes from different
anatomical regions will be separated to allow for meaningful comparisons to be made. An earlier study by Dr. Wickenden in the laboratory showed that thyroid hormone can increase K⁺ channel expression in neonatal ventricular myocytes (Wickenden et al., 1997). With this background, I postulated that thyroid hormone treatment in rats subjected to heart disease may increase expression of K⁺ channels and thereby abbreviate action potential duration.

1.2 SYNOPSIS

Animal models of hypertension, cardiac hypertrophy, and heart failure show that pressure overload in a variety of species leads to hypertrophy and then loss of contractile function which can be due to changes in the Ca²⁺ transient or altered expression of myosin isoenzymes, or both. Similarly, coronary artery ligation in the rat show compensated cardiac hypertrophy up to 8 weeks following myocardial infarction leading to decompensated cardiac hypertrophy and failure after 12 weeks (DeFelice et al., 1997; Yoshiyama et al., 1997; Zarain-Herzberg et al., 1996). My studies included rats at 8 weeks following coronary artery ligation and is more representative of cardiac hypertrophy than failure. In this thesis, I will be focusing on the genetic and cellular changes occurring in rat models of cardiac hypertrophy with a brief overview of cardiac hypertrophy in other animal species for the sake of completeness. The literature on ion channels is exhaustive since a number of voltage-gated ion channels and exchangers are modulated in cardiac hypertrophy. In this Thesis, I will only introduce the ion channels that exist in the rat myocardium. To this end, I will review the function and expression of potassium, sodium and calcium channels which exist in the rat. I will review the molecular and cellular mechanisms of excitation-contraction coupling that distinguish (or
appear) in rat models of cardiac hypertrophy. Again for the sake of completeness, I will relate some of the findings in the rat to other animal models of cardiac hypertrophy since some of the features of calcium handling are similar. Finally, I will present some features of calcium handling in animal models of failure and human heart failure that distinguish from models of cardiac hypertrophy.

In Chapter 2, I consolidated all the methods from the four manuscripts that I published into one comprehensive section highlighting the background and experimental details of the various techniques that were used. In Chapter 3, I provide experimental evidence for reduction of K+ currents and changes in calcium handling following myocardial infarction. In this regard, I show the role of specific ion channels and the regulation of intracellular calcium. The normal heart has considerable anatomic and functional heterogeneity. Highly regulated patterns of expression of ion channels determine the normal, ordered transmission of the cardiac action potential. I show experimental evidence for the heterogeneity of potassium channel function and expression in the normal heart (Chapter 4) and a loss of this heterogeneity following myocardial infarction (Chapter 5). Since thyroid hormones can modulate potassium channel expression, we examined whether thyroid hormone treatment can increase potassium channel expression and thereby rescue the electrical phenotype observed in hypertrophied myocardium (Chapter 6). The results will be presented and discussed in each Chapter, separately. Every chapter will be accompanied with its abstract, results, discussion and the tables and figures will be placed at the end of the discussion for easy reference. In Chapter 7, some of the results will be summarized and discussed along with
discussions on the limitations of my studies, recent progress and recommendations for future work.
1.3 LITERATURE REVIEW

1.3.1 The Heart

The Heart is composed of specialized cell types ranging from sinoatrial (SA) nodal pacing cells with few contractile elements to cardiac myocytes packed with contractile proteins. Each cardiac myocyte is enclosed by an approximately 80 Ångstrom thick bimolecular lipid membrane which serves as an excellent electrical insulator. Numerous ion channels, pumps exchanger proteins, receptors and gap junctions are embedded in the membrane to allow the propagation of electrical signals and passage of ions with each cardiac cycle (Bers, 1991). To efficiently pump blood these proteins must act together to produce normal electrical signals, to propagate that electricity throughout the heart and to convert the electrical signals into mechanical contraction (Bers, 1991).

The heart must contract continuously from the moment that the fetal circulatory system is completed until death and accomplishes this task with minimal cell replacement after development. In heart disease, the terminally differentiated myocytes can sustain cardiac function by altering gene expression in response to mechanical load, classical growth hormones and various neuroendocrine factors to "rejuvenate" (more commonly referred to as remodeling) the compromised heart. Interestingly, the hypertrophied myocyte re-expresses a variety of embryonic genes which can be energy-sparing in several ways.

For example, increased expression of a low ATPase isoform of the myosin heavy chain (Izumo et al., 1988, Izumo et al., 1987) and decreased expression of sarcoplasmic reticulum calcium pump ATPase molecules (Arai et al., 1996; Arai et al., 1994) resembles closely the fetal gene program and leading to reductions in the hydrolysis of cellular ATP. In this regard, a re-expression of the fetal program may be favorable to the
energy-deprived hypertrophic cell. Unfortunately, there are drawbacks including a slowing of the cross-bridge turnover and an impairment of cardiac relaxation.

The molecular mechanisms by which gene expression is altered remain unclear, but activation of several stress or signaling pathways are probably involved (Force et al., 1999; Hunter and Chien 1999). A common finding observed in the remodeled myocardium is a down-regulation of K⁺ channel function and expression (Tomaselli and Marban, 1999; Nuss et al., 1999; Wickenden et al., 1998) which will be the subject of this Thesis. Similar to findings on a number of other genes, the reduction in K⁺ channel gene expression is consistent with a re-expression of a fetal program (Wickenden et al., 1997; Shimoni et al., 1997).

1.3.2 Ion Channels

Cardiac myocytes expend energy to maintain concentration gradients for inorganic ions between their interior and exterior. Typically, potassium ion is at higher concentration in the cytoplasm than in extracellular fluid; whereas as sodium, chloride, and calcium ions exhibit the opposite distribution. These ionic gradients are then used up by proteins embedded in the cell membrane such as channels and exchangers for electrical signaling (Hille 1992). These charged ions pass through ion channels and carry an electrical current. Ion channels have three essential functional properties: (1) a central tunnel or pore, 2) a selectivity filter that dictates which ion (s) are allowed to cross the pore, and 3) a gating structure that controls switching between open and closed conformations and determines whether permeation occurs (Hille et al., 1992). Figure I is a cartoon representation of an ion channel demonstrating the structure of these functional
properties. Channels are very efficient and can transport ions at rates of $1 \times 10^6$ to $100 \times 10^6$ ions per second. This flow of ions creates electrical current on the order of $1 \times 10^{-12}$ to $1 \times 10^{-10}$ amperes per channel. Despite the large permeation, the channels themselves are regulated or gated so that the pores are only open when the proper chemical or electrical signal is present. Ion channels are capable of making quick transitions between conducting and non-conducting conformations and may be induced by extracellular ligands, intracellular second messengers and metabolites, protein-protein interaction and phosphorylation. Ion channels co-localized in discrete subcellular compartments function together as signaling elements in excitable cells. These elements amplify weak signals and propagate signals to other regions of the cell as will be discussed in the section on excitation contraction coupling. Many ion channels involved in excitability are subject to direct modulation by G proteins or second messengers and/or to post-translational modification by kinases and/or phosphatases. These modulatory changes are important in cell signaling in normal and diseased myocardium. For example β-adrenergic stimulation leads to activation of stimulatory G proteins, elaboration of cAMP and stimulation of protein kinase A. These effects can alter excitability by a variety of mechanisms including up-regulation of L-type calcium channels.
Figure I shows the working hypothesis of an ion channel which contain an aqueous pore, a selectivity filter and a gating structure. Ion channels are proteins embedded in the lipid bilayer which can sense small changes in transmembrane voltage (voltage sensor) physically opening the pore via the gate to allow ion fluxes to pass. The anchor protein which are also known as β-subunits may be involved in the process of gating. Several putative sites of glycosylation (labelled as globular structures) and of phosphorylation (labelled as -P-). The phosphorylation sites involve protein kinase C and protein kinase A and are believed to be involved in channel regulation.

(Adapted from B. Hille. Ionic channels in excitable membranes)
Many ion channels that will be discussed in this thesis are gated by the membrane potential itself and are referred to as \textit{voltage-gated ion channels}. In other words, ion channels can sense small changes in transmembrane voltage and can physically open the pore to allow ions to pass. This process is referred to as activation and involves large conformational changes in the channel protein. Some but not all voltage-gated ion channels have an additional property, called inactivation which causes them to close even in the presence of a continuing activating level of membrane potential. Activation and inactivation processes have been studied for decades based on electrical measurements with the voltage-clamp technique which is described below, but mutagenesis experiments have identified protein regions involved in inactivation (Isacoff et al., 1991; Hoshi et al., 1990). The ion channels discussed in this Thesis are composed of \( \alpha \) and \( \beta \) subunits. The so-called "business portion" of these ion channels resides in the \( \alpha \) subunit which contain the structural elements for permeation, selectivity and gating (review: Marban et al., 1998; Kukuljan et al., 1995). Also drug (Li et al., 1997) and toxin (Backx et al., 1992) binding is confined to the \( \alpha \) sub-unit. Recent technical advances in x-ray crystallography (Doyle et al., 1998), combined with site-directed mutagenesis (Tsushima et al., 1997) experiments have provided new detailed glimpses on ion channel permeation and selectivity. The functional role of \( \beta \)-sub-unit is uncertain at the present time. In the case of potassium channels, they may involved in ion channel gating related to inactivation (Rettig et al., 1994) and serve a role as molecular chaperones (Nagaya and Papazian 1997).
In the case of sodium and calcium channels, the α subunit consists of four internally homologous domains (I to IV), each of which contains six transmembrane spanning segments (see figure II). The NH₂ and COOH termini of the α subunit are located on the intracellular side, whereas the middle core region contains six membrane-spanning segments (designated S1 through S6). The S4 transmembrane segment contains positively charged arginine and lysine residues that represent the "voltage-sensor", a charged region that responds to membrane depolarization by opening the channel. A structure referred as the H5 linker (between S5 and S6 transmembrane segments) is believed to form a portion of the channel pore. In the case of sodium and calcium channels, four domains fold together so as to create a central pore whose structures determine the selectivity and conductance of the channel as mentioned above. On the other hand, K⁺ channels are generally accepted to be multimers composed of four separate α subunits but unlike the sodium and calcium channels the four domains are not linked covalently. Only compatible α subunits from a subfamily of closely related proteins are able to coassemble to form tetramers (Li et al., 1992). The amino terminus is responsible for the oligomerization of α subunits belonging to the Shaker family (Li et al., 1992) (reviewed in Section 1.4.5D). The α subunit structure in potassium channels such as the transient outward channel and delayed rectifier channels share the six transmembrane spanning segment motif seen in a single domain of the sodium or calcium channel. Some classes of potassium channels such as the inward rectifier family, ATP sensitive potassium channels and acetylcholine gated potassium channel contain only two transmembrane segments. Recent data suggest that ATP sensitive potassium channels can form octamers containing four pore-forming Kir6.2 subunits and four sulfonyl urea
receptor molecules (Babenko et al., 1998). Since unicellular organisms express K\(^+\) and Ca\(^{2+}\) channels, it is believed that the simpler K\(^+\) channels were primordial, with the subsequent evolution of Ca\(^{2+}\) channel by gene duplication. Na\(^+\) channels might have arisen in an analogous manner or, more likely, from mutations in a primitive Ca\(^{2+}\) channel. Multiple approaches have been successfully used to clone ion channel genes and some of the genes along with their ion currents are listed in Figure II. More details on the nomenclature of potassium channel genes is provided in a later section.
Figure II. Schematic representation of the α subunit of various ion channels. The six membrane spanning segment of Kv4.x and Kv1.x known to encode for the transient outward K⁺ channel have possibly evolved by gene duplication of more primitive K⁺ channel genes. Four α subunits from a given family (Kv1.x or Kv4.x series) must co-assemble together to form a functional transient outward K⁺ channel.

The positively-charged segment known as the voltage sensor and the channel pore are indicated.
1.3.3 The Ventricular Action Potential

An elementary and distinctive feature of cardiac tissue is its action potential. The action potential is described as a waveform of depolarization followed by repolarization and occurs during each cardiac cycle. Because different parts of the heart have different complements or densities of channels and pumps, the action potentials can differ substantially between different mammalian species and within the same species; between different myocardial regions. Figure III shows a rat action potential with the approximate time course of the depolarizing and repolarizing currents carried by the different ion channels. The clones for the different $K^+$ channels identified in the rat species is provided. While other ion channels exist in rat ventricular tissue such as chloride channels, ATP-sensitive potassium channels and Na$^+$/K$^+$ ATPase pump, they will not be discussed in this Thesis. It must be remembered that larger mammals such as rabbits and dogs as well as humans exhibit longer action potential durations mainly due to a decreased expression of a specific class of potassium channels known as the transient outward channel ($I_{to}$). As mentioned above, electrical excitation and action potential conduction in the heart depend on the ion conductance activity of specific voltage-gated ion channels which mediate rapid, voltage-dependent changes in ion permeability causing a change in the membrane potential. In the heart as in most excitable cells, the action potential consists of three phases. A rapid increase in Na$^+$ permeability mediated by voltage-sensitive Na$^+$ channels causes rapid depolarization during the initial phase of the action potential. The cell remains depolarized during the plateau phase of the action potential owing to the inward movement of Ca$^{2+}$ ions through voltage-sensitive Ca$^{2+}$
channels. Ca^{2+} that enter the cells during this phase of the action potential serves as a primary intracellular second messenger for the electrical signals generated in the plasma membrane and initiates excitation-contraction coupling. The opening of potassium channels and the closing of sodium or calcium channels favor repolarization of the cell back to the resting potential. Voltage-sensitive potassium channels mediate the outward movement of K^+ ions to repolarize the cell. Under physiological conditions, three K^+ currents participate in repolarization, including the transient outward current (I_{to}) which participate in the initial phase of repolarization, the delayed rectifier current (I_{K} or I_{sus}) and the inward rectifier (I_{K1}) which plays a role in the terminal phase of repolarization. In native rat ventricular myocytes, two major outward current components are regularly detected including the 4-aminopyridyne sensitive K^+ current I_{to} and the sustained tetraethylammonium-sensitive K^+ current I_{sus} (Clark et al., 1993; Apkon and Nerbonne, 1991). Recently, four different components have been shown to contribute to outward currents in rat ventricular myocytes including the peak current (I_{peak} or transient outward current I_{to}), the delayed rectifier current (I_{K}), the steady state current (I_{ss} or sustained current I_{sus} as referred in this thesis) and a less well characterized novel current (I_{Ks}) (Himmel et al., 1999). I_{to} is a rapidly inactivating current whereas I_{K}, I_{ss} and I_{Ks} are slowly activating and non-inactivating currents that belong to the delayed rectifier family. While the role of these currents on the action potential waveform is uncertain, they all contribute to cellular repolarization.
Figure III. Ion channels activated during an action potential in the rat species (modified from Fozzard, 1994). The action potential was recorded from a right ventricular rat ventricular myocyte by R. Kaprielian with 5 mM ATP and no calcium buffer (EGTA). The approximate time courses of the currents associated with the channels are symbolized by the variation in line weight. The molecular correlate of the various currents are indicated. Symbols below and below the line (—) indicate depolarizing and repolarizing currents, respectively.
1.3.4 Depolarizing currents provided by sodium channels and L-type calcium channels

As mentioned above, voltage-gated sodium channels initiate the cardiac action potential. They are expressed at high densities in the plasma membrane to ensure rapid conduction of electricity throughout the myocardium with over 100,000 channels for each ventricular myocyte (Makielski et al., 1987). While sodium channels initiate the action potential, calcium channels also assist in the depolarization of the cell and more importantly transduce the electrical signals into a series of biochemical and mechanical events. Sarcoplasmic calcium channels constitute the major pathway for calcium entry into the cell and are regulated by membrane potential (Bouchard et al., 1995), guanine nucleotide-binding (G proteins), intracellular calcium (McDonald et al., 1994), ATP and MgATP (O’Rourke et al., 1992). L-type calcium channels are activated by catecholamines and other stimuli that activate adenyl cyclase or cyclic adenosine monophosphate-dependent protein kinase (Reuter and Scholz, 1977; Tsien et al., 1972). Unlike sodium channels, there are approximately 30,000 calcium channels in a single ventricular myocyte. The L-type calcium channel (I_{Ca,L}) is well characterized with respect to its role in cardiac excitability and excitation-contraction coupling (please refer to section on excitation-contraction coupling). Everything else remaining equal, an increase in calcium current can prolong action potential duration and to some extent increase action potential amplitude.

1.3.5 Repolarizing currents provided by potassium channels

Potassium channels have received much attention from cardiac electrophysiologists as a result of their fundamental importance in setting the resting
potential near the K⁺ equilibrium potential and initiating and/or modulating repolarization of the action potential. More recently, potassium channels were found to be implicated in inherited and drug-induced cardiac disorders such as long Q-T syndrome (Keating and Sanguinetti, 1996), diabetes (Shimoni et al., 1995), hypertension (Lee et al., 1997), ischemia (Findlay 1994), cardiac hypertrophy and failure (Tomaselli and Marban, 1999). They are either regulated by voltage or by intracellular messengers and factors such as ATP, G proteins, magnesium, polyamines and outside the cell by a variety of hormones including angiotensin II, cathecholamines, acetylcholine (Wickman and Clapham 1995) and thyroid hormone (Shimoni et al., 1992; 1995). Approximately 60 K⁺ channel subunits (α and β) have been cloned to date in rat heart and brain (reviewed in Deal et al., 1996). The successful cloning and heterologous expression studies (the most common one being Xenopus oocytes) led to the identification of many subfamilies of endogenous currents in the mammalian heart and the identification of the pore, voltage sensor, gates and drug/toxin binding sites. The new tools of molecular biology using immunofluorescence have revealed the complex heterogeneity of expression of some of these channels (Brahmajothi et al., 1999). There are several types of potassium channels in the rat myocardium which include $I_{K1}$ the inwardly rectifying current, $I_{K}$ the delayed rectifier current, $I_{to}$ the transient outward current (or sometimes referred to as $I_{peak}$), $I_{sus}$ the sustained current and $I_{Kx}$ a novel delayed rectifier channel. There are a variety of other classes of potassium channels in mammalian hearts but this review will be limited to channels that exist in the rat species. A more general review of K⁺ channel expression in mammalian myocardium can be found in some excellent review articles (Snyders, 1999; Roden and George, 1997; Deal et al., 1996).
1.3.5A The delayed rectifier potassium channel

One group of potassium channels are referred to as "delayed rectifiers" because they do require a time delay before opening following membrane depolarization. For this reason, they are measured at the end of a long voltage clamp depolarization (i.e. > 400 ms). Three delayed rectifier currents have been described in the rat including the sustained current, and two other delayed rectifiers which can be discriminated based on their inactivation properties and pharmacologic blockade (Himmel et al., 1999). In the rat heart, the delayed rectifier is predominately encoded by Kv1.2, Kv1.5, and Kv2.1 (Himmel et al., 1999; Barry et al., 1995; Po et al., 1993). These gene products exhibit currents with Iₖ-like properties when expressed in mammalian cell lines (Paulmichl et al., 1991).

1.3.5B The inward rectifier potassium channel

The inward rectifier are potassium-selective channels that open at relatively negative membrane potentials (Sakmann and Trube, 1984). These channels activate very rapidly (near instantaneously) and do not inactivate in response to step changes in voltage. At potentials positive to the potassium reversal potential (Eₖ), they conduct outward (repolarizing) current whereas at potentials negative to Eₖ, they conduct inward (or depolarizing) current. Since the membrane potential is slightly more positive than Eₖ (which is approximately −90 mV), these channels provide a repolarizing current to effectively set the resting membrane potential and contribute to the terminal phase of repolarization. They are termed inward rectifier since the outward current is much smaller than the inward current. The mechanism underlying rectification in these channels is block of the outward component of the current by internal magnesium
(Matsuda et al., 1987) and polyamines (Lopatin et al., 1994). More recently, it was demonstrated that the transient rise in subsarcolemmal Ca\(^{2+}\) occurring during an action potential as a consequence of Ca\(^{2+}\) influx via the calcium channel and release from the sarcoplasmic reticulum also contribute to \(I_{K1}\) rectification (Zaza et al., 1998). These channels are encoded by members of the inward rectifier family, IRK1 and IRK2 which belong to the Kir2.x family (Wible et al., 1995).

1.3.5C The transient outward potassium channel

In cells derived from rat hearts, a distinct \(K^+\) current referred to as the transient outward current (\(I_{to}\)) has been identified. The term "transient" refers to the fact that in response to maintained depolarization elicited under voltage-clamp conditions, \(I_{to}\) displays rapid activation kinetics and slower, but still relatively rapid, inactivation kinetics. Despite the fact that \(I_{to}\) exists in a variety of mammalian hearts, the reported magnitudes and gating characteristics of \(I_{to}\) vary widely among species and cardiac tissue type. There are two types of transient outward currents with differing pharmacological and kinetic properties. These currents include two separate \(K^+\) current systems: a larger voltage-activated Ca\(^{2+}\)-independent \(I_{to1}\) and a smaller Ca\(^{2+}\) activated \(I_{to2}\) which is present in larger animal models (Josephson et al., 1984; Tseng et al., 1989) and human atria (Shibata et al., 1989) and conducts potassium ions in some species and chloride ions in others. Since \(I_{to1}\) but not \(I_{to2}\) exist in the rat ventricle, the focus will be placed on \(I_{to1}\) and will be referred to as \(I_{to}\) in the future. Briefly, \(I_{to}\) has been reported to be blocked by tetraethylammonium (TEA) (Kenyon and Gibbons, 1979), 4-aminopyridine (Thompson, 1982) and various inorganic cations (Escande et al., 1987) (i.e Cs and Ba). The channel is highly selective for \(K^+\) over Na\(^+\) and Cl\(^-\) (Nakayama and Irisawa, 1985). Its activation
is a relatively rapid voltage-dependent process. Reported times to peak are on the order of milliseconds to tens of milliseconds and adequate voltage control is important to properly measure the peak. Beyond this obvious qualitative observation, the activation thresholds range from approximately $-40$ to $0$ mV (Campbell et al., 1995) and is well described by a single Boltzmann distribution. The reported variables such as half-activation range from $-10$ mV to $+30$ mV (Campbell et al., 1995) in the rat ventricle. In our study the midpoint for activation was approximately $14$ mV. One possible basis for this variability in activation could be due to differences in experimental conditions such as the external concentration of divalent cations ($\text{Ca}^{2+}$, $\text{Mg}^{2+}$, and $\text{Cd}^{2+}$). These divalents can effectively screen for membrane surface charges thereby change the gating properties of the channel (Agus et al., 1991).

1.3.5D Nomenclature and molecular correlates of the transient outward potassium channel

In mammalian hearts, the $\text{Kv}x.y$ nomenclature is used to classify a specific $\alpha$ subunits. $\text{Kv}$ reflects the fact that they are voltage-gated $\text{K}^+$ channels, $x$ represents the subfamily and $y$ represents the number of the gene within the subfamily for a given $\alpha$ subunit. The first potassium channel cDNA was isolated using $\text{Drosophila}$ genetics in a mutant fruit fly, $\text{Shaker}$ (Papazian et al., 1987). Flies displaying the $\text{Shaker}$ phenomenon which caused abnormal leg shaking in response to ether exposure, were found to be missing a $\text{K}^+$ channel from their leg and flight muscles (Salkoff 1985). To date, seven different $\text{K}^+$ channel cDNAs have been cloned from rat heart: four of these belong to the $\text{Shaker}$ ($\text{Kv1}$) subfamily, one to the $\text{Shab}$ ($\text{Kv2}$) subfamily, and two to the $\text{Shal}$ ($\text{Kv4}$) subfamily (Paulmichl et al., 1991; Roberds and Tamkun, 1991). Given the $n^4$ model of
Hodgkin and Huxley postulated in 1952, it is believed that assembly of four $\alpha$ subunits is required to create a functional and conducting K$^+$ channel. More, recently it was reported that only compatible $\alpha$ subunit chosen from a subfamily are able to co-assemble (Li et al., 1992) and a conserved region in the amino terminus is involved in the tetramerization (Shen et al., 1993).

Historically, it was believed that Kv1.4 encoded cardiac $I_{\text{to}}$ in mammalian species including man. When Kv1.4 was first isolated in human cDNA, it became a natural candidate for $I_{\text{to}}$ (Po et al., 1992). When the channel was expressed in Xenopus oocytes, it displayed rapid inactivation, but the recovery from inactivation was much slower than native cardiac $I_{\text{to}}$. Co-expression of Kv1.4 with Kv1.2 resulted in an inactivating channel with much more rapid recovery kinetics suggesting Kv1.4 and Kv1.2 may form heterotetramers and underlie $I_{\text{to}}$ (Po et al., 1993). The candidacy of Kv1.4 for $I_{\text{to}}$ became less convincing when immunostaining and westerns blots failed to show the presence of Kv1.4 protein although the Kv1.4 mRNA was readily detected in rat ventricular myocytes (Gidh-Jain et al., 1996; Dixon and McKinnon 1994). However more recently, we showed evidence for robust Kv1.4 protein in rat ventricular myocytes (Wickenden et al., 1999). Other studies indicate that Kv4.2 might be the molecular correlate of $I_{\text{to}}$ since its expression paralleled the gradient of $I_{\text{to}}$ expression in the rat ventricular free wall whereas Kv1.4 gene expression did not (Wickenden et al., 1998; Dixon and McKinnon, 1994). Also, most functional and pharmacologic properties of Kv4.2 correspond to those of $I_{\text{to}}$ recorded in rat and human ventricular myocytes including the state-dependant blockade by 4-aminopyridine (4-AP) (Yeola and Snyders, 1997). More recently, a related isoform Kv4.3 has emerged as the leading candidate $\alpha$ subunit gene that underlies $I_{\text{to}}$ in
rats and larger mammals such as dogs and humans (Dixon et al., 1996). Dominant-negative suppression by truncated or non-conducting Kv4.2 subunits has confirmed the role of Kv4 gene family as the molecular basis of $I_{no}$ (Johns et al., 1997) Furthermore, the use of anti-sense oligonucleotides against Kv4 channel subunits have enabled us to better correlate $K^+$ channel clones to electrophysiological recordings (Fiset et al., 1997). Knockout strategies using dominant-negative constructs (by means of deletion and substitution of specific amino acids) have been exploited to probe the molecular basis of $K^+$ channels in rodent hearts (Wickenden et al., 1999; London et al., 1998; Barry et al., 1998). With these results, the emerging picture is that Kv4.2, Kv4.3 and Kv1.4 contribute to $I_{no}$ in the rat heart.

1.3.5E Biophysical characteristics of the transient outward potassium channel

Channel gating is a voltage and time-dependent process by which channels can control conductance. Since there are three genes that encode for $I_{no}$, there is no unifying channel gating scheme that represent every single $I_{no}$ channel in a given myocyte. There are several complex kinetic models which have been proposed (Campbell et al., 1995). According to the kinetic model and experimental data, $I_{no}$ channels are in the closed and rested state at hyperpolarized potentials. Upon depolarization, the channels undergo a voltage-dependent conformational change which lead to channel opening. During the depolarization the channels go from a conducting and open state to a non-conducting and inactivated state. This process is referred to as inactivation and is a widespread property of voltage-gated ion channels including $K^+$ channels. $I_{no}$ channels remain in this inactivated state and cannot re-open unless the membrane repolarizes. Recovery from inactivation is the time required for the channels to return to the closed state. For Kv1.4-
based channels, there are two different mechanisms for channel inactivation, which are referred to as N-type and C-type. Both types of inactivation require channel activation to proceed with completely different mechanisms (Rasmusson et al., 1998). C type inactivation involves a specific residue in the extracellular H5-S6 loop. Therefore it is currently believed that C type inactivation involves closure of the extracellular mouth of the pore (Lopez-Barneo, Hoshi and Aldrich, 1993). N-type inactivation generally occurs on the order of milliseconds and is mediated by a "tethered ball" mechanism in which a segment of ~20 amino acids in the N terminus of the channel binds to the intracellular mouth of the pore (Hoshi et al., 1990). After channel activation, the ball tethered to the cytoplasmic chain swings in to occlude the pore (or permeation pathway). The C-type inactivation is much slower and can occur on the order of tens of milliseconds to seconds. Several amino acids in the S6 transmembrane segment on the carboxyl terminal are involved in this type of inactivation.

Recovery from inactivation kinetics have been reported to be region-dependent which explain the different $I_{to}$ phenotypes in mammalian hearts. Differences in the abundance of Kv1.4 compared to Kv4.x within a given myocardial region correlate with the appearance of a specific recovery kinetic phenotype (Brahmajothi et al., 1999). Using patch clamp, fluorescent in situ hybridization and immunofluorescence techniques, Brahmajothi and colleagues (1999) elegantly showed two $I_{to}$ phenotypes in ferret ventricles. They reported that Kv1.4 was primarily localized in the septum and left ventricular endocardium which coincided with the appearance of slowed recovery kinetics. Kv4.2 and Kv4.3 were primarily localized in the right ventricular free wall and epicardial layers of the left ventricle and electrical recordings demonstrated a clear
majority of myocytes exhibiting fast recovery kinetics. These results strongly support the hypothesis that Kv4.2, Kv4.3 and Kv1.4 are the molecular substrates for $I_o$ in the epicardium and endocardium, respectively. In the rat, epicardial myocytes exhibit fast recovery kinetics on the order of 70-100 ms whereas endocardial myocytes exhibit slow recovery kinetics on the order of hundreds of milliseconds to seconds (Josephson et al., 1984). These differences in the recovery kinetics are most likely due to the molecular composition of $I_o$ in epicardium and endocardium. Previous studies have not correlated levels of expressed $I_o$ channel $\alpha$ subunit expression (mRNA and protein) in specific anatomical regions of the heart to patterns of functional $I_o$ current phenotypes measured from individual myocytes from the same anatomical regions in the rat. In Chapter 3, we show that Kv4.2 underlie the fast recovery kinetics observed in the right ventricular myocytes whereas Kv1.4 underlie the slowed recovery kinetics observed in septal myocytes.

1.3.5F The transient outward potassium channel in cardiac development and hypertrophy

Shortly after birth, the characteristics of $I_o$ and action potential duration are quite different compared to the fully mature rat heart. Action potentials in neonatal myocytes are prolonged which is associated with decreased $I_o$ density (Wickenden et al., 1997; Kilborn and Fedida, 1990). With normal cardiac development, the action potential duration is abbreviated which is associated with an increase in $I_o$ density. With respect to $K^+$ channel genes, the molecular composition of $I_o$ in neonatal myocytes is mostly Kv1.4 (Xu et al., 1996; Wickenden et al., 1997) while Kv4.2 and Kv4.3 predominately encode for $I_o$ in adult cardiac myocytes (Barry et al., 1995; Dixon and McKinnon 1994). In
cardiac hypertrophy, Kv4.2 and Kv4.3 expression is reduced (Kaprielian et al., 1999; Takimoto et al., 1997; Gidh-Jain et al., 1996) while Kv1.4 expression is increased (Matsubara et al., 1993). This isoform switch from Kv4.2/Kv4.3 to Kv1.4 is consistent with a recapitulation of a fetal gene expression. This characteristic isoform switch is not confined to K+ channels, since SERCA2a, Na+/Ca2+ exchanger and myosin heavy chain expression patterns also revert to the neonatal profile in adult hypertrophied cardiac myocytes (Litwin and Bridge, 1997; Arai et al., 1994; Feldman et al., 1993).

At the present time, the mechanism for the reduction in K+ channel expression in cardiac hypertrophy is unknown but neurohormonal factors such as the adrenergic system and the renin angiotensin system seem to be involved. In-vitro and in-vivo studies have shown that altered expression of the transient outward channel can be induced by a number of stimuli including α-adrenergic (Parker et al., 1999; Gaughan et al., 1998; Han et al. 1997; Braun et al., 1990), angiotensin II (Nagatomo et al., 1995), endothelin (Shimoda et al., 1998; Damron et al., 1993), and growth factors such as nerve growth factor (Heath et al., 1998; Liu et al., 1998) and other paracrine (yet unidentified) paracrine factors (Guo et al., 1998). Experiments on Xenopus oocytes and rat ventricular myocytes showed that inhibition of the transient outward current may be mediated by protein Kinase C (PKC) which reduces the current by decreasing the expression of Kv4 α subunits (Nakamura et al., 1997) and Kv1.4 α subunits (Murray et al., 1994). It remains to be investigated whether adrenergic stimulation can inhibit K channel expression via a PKC mechanism. Recently, Takimoto and colleagues (1997) showed that an increase in cardiac afterload causes specific reductions in Kv4 subfamily channel mRNA expression and that this effect is likely to be mediated by angiotensin II. The
effect of Angiotensin II is not specific to Kv channels since it was shown to increase the amplitude of the rapid component of the delayed rectifier (I_{Kr}) in guinea pig ventricular myocytes (Daleau and Turgeon, 1994).

1.3.5G Regional differences in the transient outward potassium channel function and expression in ventricular myocardium

At the electrical level, the duration of the cardiac action potential is not uniform throughout the ventricular myocardium. In the normal heart, action potential duration (APD) is prolonged in endocardial myocytes compared with epicardial myocytes in humans (Li et al., 1998; Bailly et al., 1997) and a variety of animal species including the dog (Litovsky et al., 1988), cat (Furukawa et al., 1990), rabbit (Fedida and Giles 1991), and rat (Clark et al., 1993). The difference in action potential duration across the left ventricular wall is due to a greater density of the calcium-independent transient outward current (I_{to}) in epicardial myocytes compared to endocardial myocytes (Litovsky et al., 1988; Fedida & Giles 1991; Antzelevitch et al., 1991; Benitah et al., 1993; Clark et al., 1993; Li et al., 1998; Wickenden et al., 1999; Gomez et al., 1997). Across the left ventricular wall, I_{to} density is greater in the epicardium compared to the endocardium (Benitah et al., 1993; Clark et al., 1993) and greater in base compared to the apex of the heart. Electrophysiological and molecular studies have reported multiple I_{to} phenotypes and or I_{to} current densities among distinct anatomical regions of the heart such as the left ventricle, the interventricular septum and the right ventricle (Aimond et al., 1999; Gomez et al., 1997). Similar findings are reported in man where regional heterogeneity of APD and I_{to} density are reported in specific myocardial regions (Li et al., 1998; Bailly et al., 1997). The regional heterogeneity is important in the normal orderly spread of
depolarization and subsequent repolarization and for preventing abnormal patterns of
electrical activity such as re-entrant arrhythmias (Nuss et al., 1999; Tomaselli and
Marbán, 1999). In endocardial regions which are activated early in each cardiac cycle, the
action potential duration (APD) is longer than in epicardial regions, which are activated
later in the cardiac cycle. This gradient in repolarization explains the appearance of a
positive T wave in the electrocardiogram (Franz, 1999). The longer APD also results in
the endocardial refractory period significantly outlasting that observed in the epicardium,
thereby preventing re-excitation of the endocardium by the epicardium (Burgess, 1979).

1.3.6 **Excitation-contraction coupling in the normal myocyte**

Intracellular calcium is an important cation that influences cellular excitability
and is tightly regulated at approximately 100 nM under physiologic conditions during rest
or cardiac diastole (Barry and Bridge, 1993). For intracellular Ca\(^{2+}\) homeostasis to occur,
Ca\(^{2+}\) influx during the action potential must be balanced by an equivalent Ca\(^{2+}\) efflux
during repolarization and diastole. The contractile cycle in every cardiac myocyte is
sequentially composed of an electrical signal, the action potential (as described above),
the calcium transient and the force development. Calcium homeostasis is required in
cardiac myocytes to ensure proper electromechanical coupling. Excessive accumulation
of cellular calcium can lead to "calcium overload", a condition characterized by abnormal
diastolic bursts of calcium release from the sarcoplasmic reticulum (Wier et al., 1983)
leading to premature beats. Figure IV shows the general scheme of calcium handling in a
normal cardiac myocyte. It is widely believed that activation of excitation-contraction (E-
C) coupling in heart occurs in response to an incoming action potential which causes Ca\(^{2+}\)
influx via voltage-dependent L-type Ca\(^{2+}\) channels which in turn activates Ca\(^{2+}\) release
Figure IV. General scheme of the calcium handling during a cardiac cycle. During systole, electrical excitation and an incoming action potential depolarizes the plasma membrane resulting in the activation (i.e. opening) of L-type calcium channels and influx of Ca$^{2+}$ into the myocyte. The resulting increase in [Ca$^{2+}$], (about 10-20%) activates SR Ca$^{2+}$ release channels (ryanodine receptors or RyRs) which in turn allows a larger amount of Ca$^{2+}$ (about 80-90%) to move from the sarcoplasmic reticulum (SR) into the cytosol to activate myofilaments (MF) and contraction.

During diastole, 80-90% of the calcium is resequestered into the SR via the SR pump, while 10-20% is removed from the cytosol via the Na$^+$/Ca$^{2+}$ exchanger. (The figure is modified from D. Bers. *Excitation-contraction coupling and cardiac contractile force*).
from stores in the sarcoplasmic reticulum (SR) through ryanodine receptors (RyR). Since RyR receptors are activated by incoming calcium from the opening of L-type calcium channels and are responsible in the release of calcium from the SR, the process was termed “Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release” or CICR by Dr. Alexandre Fabiato (Fabiato 1983). Ryanodine receptors are located in the dyads across from the L-type calcium channels, an arrangement that is key to the CICR mechanism. The available data suggest that 80% to 90% of the Ca\textsuperscript{2+} that activates the myofilaments originates from the SR, and the remaining 10-20% is derived from Ca\textsuperscript{2+} influx from calcium channels during an action potential. This view is supported by numerous studies showing activation of contraction and Ca\textsuperscript{2+} transients in response to activation of Ca\textsuperscript{2+} current (Barcenaz-Ruiz and Wier, 1987; Beuckelmann and Wier, 1988; Nabauer et al., 1989). These studies have shown a close association between calcium current amplitude and Ca\textsuperscript{2+} transient amplitude, which is characterized by a bell-shaped relationship for both phenomena. Recent developments in confocal microscopy have allowed the measurement of local non-propagating elevations of Ca\textsuperscript{2+} (or Ca\textsuperscript{2+} sparks) at the level of individual sarcomeres (Cheng et al., 1993). These studies have allowed for better resolution of the calcium handling events and the coupling of L-type Ca\textsuperscript{2+} channels to the ryanodine receptors. It appears that Ca\textsuperscript{2+} sparks are triggered by a local sarcolemmal accumulation of Ca\textsuperscript{2+} established first in the region of the ryanodine receptor by the opening of a single L-type calcium channel (Cannell et al., 1995). This phenomena is referred to as the local control theory of excitation-contraction coupling (for reviews Wier and Balke, 1999; Stern, 1992). The close relationship between L-type Ca\textsuperscript{2+} currents and whole cell Ca\textsuperscript{2+} transients can be explained by recruitment and summation of their respective independent local events.
(namely, single L-type Ca\(^{2+}\) currents and Ca\(^{2+}\) sparks). From these experiments performed in early to late 80s, it appeared that calcium current through L-type calcium channels was the sole trigger for the release of calcium from the sarcoplasmic reticulum. However, this notion has been challenged by several reports indicating that other mechanisms may also support the release of calcium from the sarcoplasmic reticulum independent of L-type Ca\(^{2+}\) channels. In this regard, Ferrier and Howlett (1995) have shown that contraction in cardiac muscle can be observed even when Ca\(^{2+}\) influx through L-type calcium channels has been eliminated. They explained their results to be due to the presence of a voltage-gated release mechanism (also observed in skeletal muscle) which can directly activate ryanodine receptors. It appears that this mechanism is present across a variety of species (such as rabbit, rat and guinea pig) and is modulated by intracellular cAMP concentration (Hobai et al. 1997). The sodium-calcium exchanger operating in reverse mode may also be involved in triggering CICR by bringing Ca\(^{2+}\) into the cell in a voltage-dependent manner as a result of the increase in intracellular sodium that results from the sodium influx via I\(_{\text{Na}}\) (Wasserstrom and Vites, 1996; Leblanc and Hume, 1990). Calcium entry through T-type Ca\(^{2+}\) channels can also participate in triggering Ca\(^{2+}\) release from the sarcoplasmic reticulum but their overall contribution is minimal (Sipido et al., 1998). More recently, Santana and colleagues (1998) were able to show that calcium ions can also permeate through sodium channels following activation of protein kinase A and could contribute to the activation of Ca\(^{2+}\) release from the sarcoplasmic reticulum. The authors reasoned that PKA can possibly bring about a change in the selectivity of the Na\(^{+}\) channel to permit Ca\(^{2+}\) permeation. The ability of the Na\(^{+}\) channel to be changed such that Ca\(^{2+}\) ions are allowed to permeate the channel was
referred to as "slip-mode conductance". In summary, L-type Ca\(^{2+}\) channels have a privileged role in excitation-contraction coupling and is by far the most important mechanism to support the elaboration of a calcium transient and subsequent contraction, while the other mechanisms (i.e. voltage coupling, entry of calcium through T-type Ca\(^{2+}\) channels, reverse mode Na\(^{+}\)/Ca\(^{2+}\) and PKA-modulated Na\(^{+}\) channels) are less important. However, their role may become important in hypertrophied myocytes where the expression of T-type calcium channels is increased (Martinez et al., 1999), β-adrenergic stimulation and intracellular protein kinase A activity is increased (Swynghedauw 1999).

Irrespective of the mechanism, the increase in intracellular Ca\(^{2+}\) causes the myofilaments to contract and relaxation occurs when Ca\(^{2+}\) is released from the myofilaments. The decline of [Ca\(^{2+}\)] and relaxation are due to the re-uptake of Ca\(^{2+}\) into the SR by the SR Ca\(^{2+}\) ATPase pump and extrusion of Ca\(^{2+}\) via the Na\(^{+}\)/Ca\(^{2+}\) exchanger. From the total pool of cytosolic Ca\(^{2+}\), the majority of the Ca\(^{2+}\) (between 60-90%) is taken up by the SR while the remaining (between 10-30%) is extruded outside the cell by the Na\(^{+}\)/Ca\(^{2+}\) exchanger. The Na\(^{+}\)/Ca\(^{2+}\) exchanger competes with SR Ca\(^{2+}\) ATPase pump for removal of Ca\(^{2+}\) during relaxation (Bridge et al., 1990) and there are species differences in the relative activity of the Na\(^{+}\)/Ca\(^{2+}\) exchanger compared to SR Ca\(^{2+}\) ATPase (Sham et al., 1995). During the decline of the [Ca\(^{2+}\)] transient, the Na\(^{+}\)/Ca\(^{2+}\) exchange is about 2- to 3-fold faster in rabbit than in rat, whereas the SR Ca\(^{2+}\)-ATPase is 2- to 3-fold faster in the rat (Bassani et al., 1994). The role of the Na\(^{+}\)/Ca\(^{2+}\) exchanger is very important in excitation-contraction coupling since it may function to move Ca\(^{2+}\) either into or out of cells, depending on the net electrochemical driving force on the exchanger. Interestingly, this driving force, and thus the net Ca\(^{2+}\) movement mediated by the exchanger may
change direction when the membrane potential changes during an action potential or when cytosolic sodium or calcium concentrations are altered. In its "normal" or forward mode, the Na⁺/Ca²⁺ exchanger extrudes Ca²⁺ by exchanging intracellular Ca²⁺ for extracellular Na⁺. This efflux of Ca²⁺ balances Ca²⁺ influx during each cardiac cycle and is the principal mode of operation of the exchanger. The Na⁺/Ca²⁺ exchanger operating in the forward mode provides an important mechanism for Ca²⁺ homeostasis since it extrudes all the Ca²⁺ that enters the cell via the L-type Ca²⁺ channels (Bridge et al., 1990). The Na⁺/Ca²⁺ exchanger can also work in a reverse mode, causing Ca²⁺ influx by exchanging extracellular Ca²⁺ for intracellular Na⁺. Since the stoichiometry of the Na⁺/Ca²⁺ exchanger is 3 Na⁺ ions for 1 Ca²⁺ ion, it generates an electrical current and can play a role in determining the action potential duration. Since the Na⁺/Ca²⁺ exchanger was not the central part of the Thesis, the reader is advised to read an excellent review on the Na⁺/Ca²⁺ exchange and its physiological implications in a review published by Blaustein and Lederer (1999).

1.3.7 Rat models of cardiac hypertrophy and failure

Congestive heart failure is manifested by a diminished developed left ventricular pressure, reduced rates of left ventricular pressure development and relaxation (measured as ±dP/dt in Chapter 6), and markedly increased lung weight-to-body weight ratios. There are several models of cardiac hypertrophy and failure which are either naturally occurring and experimentally induced models. These models exist in a variety of animal species including, mouse (Dorn et al., 1994), dog (Armstrong et al., 1986; Kääb et al., 1996), pig (Spinale et al., 1994), rabbit (Litwin and Bridge, 1997), guinea pig (Siri et al., 1989), hamster (Hatem et al., 1994). In the past five years, gene-targeted disruption in
mice have also yielded cardiomyopathies and heart failure (Wickenden et al., 1999; Arber et al., 1997). There are great differences between species and models of heart failure and the connection of any of these with human patients is uncertain. Nevertheless, heart disease in these models are characterized by reductions in cardiac contractility as well as in the expression and function of ion channels, contractile proteins, calcium handling proteins and membrane receptors. I do not intend to review the merits of the different models, but briefly mention the models and report their relevance to the model of myocardial infarction studied in this Thesis (for review, Hasenfuss, 1998).

There are currently five models of cardiac hypertrophy and failure in the rat, two of which are produced surgically (i.e. coronary artery ligation and aortic banding). There are also hypertensive models induced by salt intake such as the salt-sensitive Dahl rats and by selective breeding such as the spontaneous hypertensive rat and the spontaneous hypertensive rat which develop cardiac failure. It is believed that with the onset of the disease, a state of compensated cardiac hypertrophy occurs for several weeks to months followed by a gradual transition to heart failure. These temporal changes occur in the aortic banding model where cardiac hypertrophy occurs for about 15-20 weeks post-surgery and cardiac failure develops after 20 weeks (Feldman et al., 1993). Interestingly, a decrease in sarcoplasmic reticulum ATPase mRNA occurs only after 20 weeks, leading investigators to suggest that a decrease in SR Ca\(^{2+}\) ATPase message is a marker of the transition from cardiac hypertrophy to failure. Similarly, the Dahl sensitive rat develops systemic hypertension after receiving a high-salt diet which causes a concentric compensated hypertrophy at 8 weeks, which is followed by gross ventricular dilatation and heart failure at 15-20 weeks (Inoko et al., 1994). The spontaneous hypertensive rat
(SHR) is a well-established model in which cardiac hypertrophy occurs, but cardiac pump function is preserved at 1 year of age. Between 18 and 24 months, signs of cardiac failure develop along with a marked up-regulation of extracellular matrix was observed (Bing et al., 1991; Boluyt et al., 1994). Cardiac failure can be induced in SHR rats that have been selectively bred with rats carrying the facp gene which encodes a defective leptin receptor (Chua et al., 1996). Myocardial infarction following coronary artery ligation is a widely used rat model and was used in my studies. While cardiac hypertrophy is a common finding as early as 3 weeks following the surgery, it is difficult to assess the presence or absence of failure in these rats. Unlike SHR rats and banded rats, myocardial infarction in rats causes cardiac hypertrophy, but the failing period is rather unpredictable and may occur after 16 weeks (Swynghedauw 1999; Hasenfuss, 1998). It appears that the progression of the disease from a compensated state of cardiac hypertrophy to a decompensated state leading to cardiac failure depends on the infarct size (Pfeffer et al., 1991) and time following the induction of myocardial infarction (Pfeffer et al., 1990). For example, DeFelice et al., (1989) showed that cardiac failure did not occur 4 weeks following myocardial infarction. While left ventricular end-diastolic volume and weight increase with time, marked reduction in cardiac function and ascites became apparent at 3 months after infarction (DeFelice et al., 1989; Yoshiyama et al., 1997). At the cellular level, a decline of SR Ca²⁺-ATPase function and SERCA2a are important cellular markers associated with the presence of cardiac failure in humans and experimental animal models. SR-Ca²⁺ ATPase function and SERCA2a expression (mRNA and protein) is either unchanged or increased at 3, 4, 8, 12 and 16 weeks in the right ventricle and remote non-infarcted myocardium (Yoshiyama et al., 1997; Zarain-
Herzberg et al., 1996; Afzal and Dhalla, 1992). In sharp contrast, SR Ca$^{2+}$-ATPase function and expression is decreased at 3, 4, 8, 12 and 16 weeks in the left ventricle. Using SR Ca$^{2+}$-ATPase function and expression as a criterion for the presence or absence of cardiac failure, it appears that the transition from cardiac hypertrophy to failure is region and time dependent. The methods used for coronary artery ligation are described in Chapter 2 (Methods section).

1.3.8 Mechanisms of cardiac hypertrophy

Myocardial growth or hypertrophy occurs early during the clinical course of heart disease and is an important risk factor for subsequent cardiac morbidity and mortality. Since adult cardiac myocytes are terminally differentiated and cannot undergo cellular division, the growth of the heart occurs through an increase in size of the myocytes. Although initially an adaptive response, if the stimulus to hypertrophy persists, the heart often undergoes a transition to contractile failure. Aside from a disease condition, myocardial hypertrophy may also occur during normal cardiac development and following exercise training. The so-called physiologic hypertrophy is associated with proportional increases in the length and width of cardiac myocytes. There are two types of pathologic hypertrophy which are the concentric and eccentric types. Concentric hypertrophy occurs during pressure-overload where contractile-protein units are assembled in parallel resulting in a relative increase in the width of individual cardiac myocytes. Concentric hypertrophy occurs at the expense of a decreased ventricular cavity. Eccentric hypertrophy is characterized by a relatively greater increase in the length than in the width of myocytes which cause wall thinning and increased wall stress on the surviving myocytes. In the case of myocardial infarction, the noninfarcted
myocytes appear to undergo eccentric and concentric hypertrophy (Pfeffer et al., 1990, 1991). While hypertrophic response can initially compensate for the loss of myocytes, sustained hypertrophy may lead to dilated cardiomyopathy, heart failure and sudden death, a process referred to as decompensation or broken compensation.

The ability to culture primary cardiac myocytes has resulted in the availability of a well characterized *in vitro* system where the hypertrophic response may be studied. At the cellular level, cardiac myocytes respond to stresses resulting from simple stretch to hypoxia by increasing their size. Cell stretch not only directly activates the hypertrophic response, but also induces the release of neurohormonal factors that are potent stimulator of the hypertrophic response in cultured ventricular myocytes and likely play a role in vivo (Force et al., 1999; Hunter and Chien 1999). At the whole heart level, hypertension and various forms of myocardial injury such as activation of neurohormonal pathways can initiate the process of hypertrophy. In this regard, angiotensin II (Sadoshima and Izumo, 1993), cathecholamines and associated receptors (Gaughan et al., 1998; Milano et al., 1994a, 1994b), atrial natriuretic peptide, endothelin I (Choukroun et al., 1998; Shubeita et al., 1990), cytokines (Sheng et al., 1997) and insulin growth factor I (Duerr et al., 1995) have been associated with cardiac hypertrophy. The hormones and growth factors are transmitted to the nucleus through an intricate network (for review, Hunter and Chien, 1999). The molecular mechanisms which link these stimuli to intracellular responses is known as signal transduction. Components of this network mediate the hypertrophic response as a result of protein-protein interactions generally by phosphorylation, leading to the production of macromolecular signaling complexes (Force et al., 1999). There are a variety of pathways including the activation of MAP
kinase (Sugden and Clerk, 1998), calmodulin-dependent calcineurin (Molkentin et al., 1998), G<sub>Gaq</sub> (Adams et al., 1998), cytokines (cardiotropin 1 and members of the interleukin-6 family), and growth factors (p38β, ras, c-jun) which are believed to be involved in cardiac hypertrophy (Yamazaki et al., 1995; Yamazaki et al., 1993). Activation of these pathways, along with activation of calcium dependent second messenger systems such as protein kinase C (Ailo et al., 1992) and adenylate cyclase are believed to mediate cardiac hypertrophy. For instance, the signal transduction of α₁ adrenoceptor stimulation which has been investigated in greatest detail involves the activation of protein kinase C (PKC) (Kariya et al., 1994) and a re-expression of fetal genes, activation of protein synthesis, and cellular growth. Once activated, these pathways translocate to the nucleus, where they can phosphorylate transcription factors and regulate gene expression (Sugden and Clerk, 1998; Force et al., 1999). These kinases which can be activated by calcium provide important switches in the pathways between apoptosis and compensated hypertrophy (Hunter and Chien, 1999). While hormones are known to cause cardiac hypertrophy, simple mechanical stretch can also cause cardiac hypertrophy by an autocrine mechanism causing the release of angiotensin II and endothelin. These mechanisms have been demonstrated in neonatal cardiac but have yet to be shown in adult cardiomyocytes. While cardiac enlargement occurs, a transition can occur whereby irreversible decompensation in cardiac function occurs during the dilation of the heart and thinning of the walls of the ventricular chamber. It is believed that changes in gene expression may lead the myocardium into a state of decompensation and cell death which is referred to as apoptosis. Apoptotic signals are believed to be initiated by cytokines including the interleukin-6 family such as glycoprotein 130, tumor necrosis
factor α, p38α and p31ras stimuli. The signals that regulate apoptosis are not well elucidated. It is believed that a balance between apoptotic and anti-apoptotic signals occur in the normal cell, and cell death occurs in response to a shift in this balance (Hunter and Chien 1999).

1.3.9 Alterations in electrical phenotype: Action potential prolongation

Action potential prolongation is a consistent finding in human heart failure and animal models of cardiac hypertrophy and failure. In 1970, Coltart and Meldrum were the first to report action potential duration from patients with hypertrophic cardiomyopathy. Action potential duration was also shown to be prolonged in cardiac trabeculae and ventricular myocytes from the hearts of patients with end-stage heart failure caused by either dilated cardiomyopathy, hypertrophic cardiomyopathy and ischemic heart disease (Gwathmey et al., 1987; Beuckelmann et al., 1992; Beuckelmann et al., 1993). Prolongation of the cardiac action potential duration also occurs in a variety of animal models of cardiac hypertrophy and failure. Table 1 shows the presence of prolonged action potentials in various experimental models such as hypertension, left coronary artery ligation, pulmonary artery ligation, aortic banding, hormone treatment and tumors. More recently targeted potassium channel downregulation have been shown to prolong action potentials (Wickenden et al., 1999).

1.3.9A The mechanism of action potential prolongation.

Action potential duration depends on a delicate balance between depolarizing (L-type calcium current, \( I_{Ca,L} \), sodium current, \( I_{Na} \), sodium-calcium exchanger, \( I_{Na/Ca} \) and chloride current \( I_{Cl} \)) and repolarizing currents (the transient outward potassium current, \( I_{to} \), the delayed rectifier, \( I_K \), the inward rectifier, \( I_{K1} \), chloride current, \( I_{Cl} \) and the sodium-
potassium ATPase). There is conflicting data concerning the effects of cardiac hypertrophy and failure on ion channel expression encoding depolarizing and/or repolarizing currents. The discrepancy may be due to the differences in the models used which generally result in distinct phenotypes. There is conflicting data concerning the effects of cardiac hypertrophy and failure on L-type Ca^{2+} channel density. The effects on L-type calcium channel activity seem to depend on the experimental model and the time course of the disease (review: Tomaselli and Marban 1999; Wickenden et al., 1998). In the rat model of coronary artery ligation, L-type calcium channel density was unchanged at 3-4 weeks post-MI (Zhang et al., 1999; Holt et al., 1998; Zhang et al., 1996; Qin et al., 1996) and decreased at 1-2 months post-MI (Santos et al., 1995). Therefore, it appears that the expression of the number of functional calcium channels is unaltered and hence I_{Ca,L} may not account for the prolongation of action potential duration observed in rats following myocardial infarction. While the number of expressed calcium channels does not change, prolongation of the action potential itself following myocardial infarction can result in a time-dependent increase in calcium influx into the myocyte (Bouchard et al., 1995). The mechanisms controlling inactivation, deactivation, and reactivation of Ca^{2+} channels are complex, and involve an interaction between time- and voltage-dependent gating as well as Ca^{2+}-induced inactivation (McDonald et al., 1994). Therefore, a change in the shape and duration of the action potential alter Ca^{2+} influx. For example an increase in the duration of the plateau potential leads to a reduced electrochemical driving force for Ca^{2+}, thereby reducing the peak current. While the peak current is decreased, a prolonged action potential increases the total time that the calcium channels are open, thereby increases the net influx of calcium.
The late repolarization phase of the action potential can also be modulated by the
electrogenic sodium-calcium exchanger due to its electrogenicity (review: Blaustein and
Lederer, 1999). The sodium-calcium exchange currents were found to be decreased in
compensated cardiac hypertrophy induced by coronary artery ligation (Zhang et al.,
1996). On the other hand, the function and expression of the Na\(^+/Ca^{2+}\) exchanger is
increased in experimental animal models of heart failure such as long term pressure
overload induced by infarction (Litwin and Bridge, 1997), aortic banding (Ryder et al.,
1993) pacing (O’Rourke et al., 1999) and human heart failure (Flesch et al., 1997). The
increase in Na\(^+/Ca^{2+}\) exchange activity may occur in part to compensate for the decreased
expression of the SR Ca\(^{2+}\)-ATPase that occurs in pressure overload (Bailey and Houser,
1993) and pacing model of heart failure (O’Rourke et al., 1999) and human heart failure
(Mercadier et al., 1990). The Na\(^+/Ca^{2+}\) exchanger may therefore, contribute to action
potential prolongation in cardiac failure, but not cardiac hypertrophy. Altered background
hyperpolarizing currents could also impact on the action potential profile of
hypertrophied and failing hearts, although the extent to which these currents contribute to
action potential prolongation is unclear. Thus, in animal models of myocardial infarction,
the activity of the Na\(^+/K^+\)-ATPase is decreased (Dixon et al., 1992). One study to date
has shown evidence for expression of a background chloride current which would tend to
shorten action potential duration (Benitah et al., 1997).

Potassium currents are the major repolarizing currents active during the plateau
of the action potential in myocytes isolated from a variety of model species and human
hearts. Several groups have measured K\(^+\) currents in human heart failure and in animals
models of cardiac hypertrophy and failure. Of all the mechanisms that may contribute to
action prolongation, decreased K⁺ currents appear to be the most common. Thus, in the studies outlined in Table I, the density of the transient outward current (I_t) is reduced in hypertrophied hearts regardless of species, precipitating factors or the disease stage. Indeed, preliminary evidence suggests that reduced I_t density represents an early event in the response to decreased pump performance (Lue and Boyden, 1992). Decreased I_t therefore, would appear to be a significant contributor to action potential prolongation in cardiac hypertrophy and failure. In animal models of cardiac hypertrophy and human heart failure a reduction in steady-state level of Kv4 mRNA has been associated with down-regulation of I_t (Please refer to Table I for the changes in K⁺ currents and K⁺ channel genes between the different models) In the rat, reductions in the steady-state level of mRNA is associated with a commensurate decrease in the level of immunoreactive Kv4 protein (Gidh-Jain et al., 1996). While Kv4 mRNA and protein (Kv4.2 and Kv4.3) are decreased in cardiac hypertrophy secondary to myocardial infarction and renovascular hypertension, Kv1.4 was found to be decreased (Gidh Jain et
<table>
<thead>
<tr>
<th>Model</th>
<th>APD</th>
<th>$I_{lo}$</th>
<th>$I_{K1}$</th>
<th>$I_K$</th>
<th>$K^+$ Channel genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beuckelmann et al., 1993</td>
<td>..........</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>Wettwer et al., 1994</td>
<td>All derived from end-stage hearts</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>Nabauer et al., 1996</td>
<td>↑ 90%</td>
<td>↓ septum</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Bailly et al., 1997</td>
<td>↑</td>
<td>↓ septum</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Kaab et al., 1998</td>
<td>↑</td>
<td>↓ septum</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Pressure/volume overload</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kleiman &amp; Houser, 1989</td>
<td>RVH/Cat?</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Brooksby et al., 1993</td>
<td>SHR 16 wks</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>Benitah et al., 1993</td>
<td>AC/rat 6-7 wks</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>Furukawa et al., 1993</td>
<td>AC/cat?</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>Ryder et al., 1993</td>
<td>AC/gp 20 wks</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>Matsubara et al., 1993</td>
<td>Goldblatt/rat</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↑ Kv1.4 ↓</td>
</tr>
<tr>
<td>Cerbai et al., 1994</td>
<td>SHR 16 &amp; 18 mths</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>Li and Keung, 1994</td>
<td>Goldblatt/rat</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>Tomita et al., 1994</td>
<td>AC/rat 4-6 wks</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>Potreau et al., 1995</td>
<td>AC/ferret 4-6 wks</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Bryant et al., 1997</td>
<td>AC/gp?</td>
<td>↑ epi</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Gomez et al., 1997</td>
<td>AC/rat 6-7 wks</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>Takimoto et al., 1997</td>
<td>Goldblatt 7 wks</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>McIntosh et al., 1998</td>
<td>Renal hypert. 7-10 wks</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>Ischemia/infarction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lue and Boyden, 1992</td>
<td>Dog/CAL 5 days</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Qin et al., 1996</td>
<td>Rat/CAL3-4 wks</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Gidh-Jain et al., 1996</td>
<td>Rat/CAL3-4 wks</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Rozanski et al., 1998</td>
<td>Rat/CAL 16 wks</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Aimond et al., 1999</td>
<td>Rat/CAL4-6 mths</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Drug/hormone induced</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xu and Best, 1991</td>
<td>Wistar-FurthRat GH tumor</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Coulombe et al., 1994</td>
<td>DOCA 13 wks</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Bryant et al., 1999</td>
<td>Rat/Isoprenaline Daily-7 day</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Lee et al., 1997</td>
<td>Rat/monocrotaline Daily-14-28 day</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Pacing-induced tachycardia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kaab et al., 1996</td>
<td>Dog 3-4 wks</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Rozanski et al., 1997</td>
<td>Rabbit 2-3 wks</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Genetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thuringer et al., 1996, 1996</td>
<td>Hamster &gt;60 days</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Dominant-negative mutations in murine models</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barry et al., 1998</td>
<td>Kv4.2W362F</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>London et al., 1998</td>
<td>Kv1.1N206</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Xu et al., 1999</td>
<td>Kv2.1N216</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Wickenden et al., 1999</td>
<td>Kv4.2N</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>
al., 1996) or increased in hypertrophied myocytes secondary to myocardial infarction (Nishiyama et al., 1998). Matsubara et al. (1993) showed that Kv1.4 mRNA expression increased in spontaneously hypertensive rats (Matsubara et al., 1993). These data show that Kv1.4 is differentially regulated compared to Kv4.2 and Kv4.3. The role of the increase in Kv1.4 is presently unclear especially with the evidence that cardiac I_{to} in the rat is mostly Kv4.2 (Yeola and Snyders, 1997; Johns et al., 1997; Fiset et al., 1997).

The role of other repolarizing K^{+} currents such as the delayed rectifier is not as clear. In the majority of studies, the density of I_{sus} is not significantly altered in hypertrophied myocytes, although I_{sus} has been reported to be decreased in one report (see Table I). The inward rectifier (I_{K1}) contributes to the late phase of repolarization and, more importantly, to the setting of the resting membrane potential. The density of I_{K1} has been shown to be decreased in human heart failure and the results are quite variable in the animal models (see Table I).

1.3.10 Calcium handling in human cardiac failure

Aside from action potential prolongation, hearts from heart failure patients are characterized by depression of developed force, prolongation of relaxation and blunting of the frequency-dependent facilitation of contraction. Indeed it was demonstrated a decade ago that end-stage human hearts display a delayed relaxation (Gwathmey et al., 1987; Beuckelmann et al., 1992, 1995), an increase in diastolic calcium (Beuckelmann et al., 1992) and a decrease in peak (or systolic) calcium [Ca^{2+}]_{i} (Gwathmey et al., 1987; Beuckelmann et al., 1992, 1995). The abnormalities in Ca^{2+} handling coupled with changes in contractile proteins (Lowes et al., 1997) explain the characteristic decrease in ventricular function observed in heart failure. The changes in calcium handling pointed to
defect in the expression or function of the sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase which sequesters Ca\textsuperscript{2+} during each cardiac cycle. Indeed, quantification of the steady state levels of SERCA 2a mRNA levels has revealed that they are markedly reduced in these failing human hearts (Arai et al., 1993, 1994; Schwinger et al., 1995). A decrease in the SR Ca\textsuperscript{2+}-ATPase causes a decrease in peak [Ca\textsuperscript{2+}]\textsubscript{i} and delays relaxation. Interestingly, an increase in Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange activity (detected at the RNA and protein levels) occurs in heart failure which somewhat compensates but does not restore for the decrease in Ca\textsuperscript{2+} re-uptake by the SR (Studer et al., 1994; Flesch et al., 1996).

1.3.11 Calcium handling in animal models of cardiac hypertrophy and failure

As with human heart failure, animal models of heart failure also show a decrease in the peak amplitude and a prolongation of the [Ca\textsuperscript{2+}]\textsubscript{i} duration. Reductions in peak [Ca\textsuperscript{2+}]\textsubscript{i} has been documented in myocytes from aortic-banded rats that worsened as heart failure developed (Siri et al., 1991), aortic-banded cats (Bailey and Houser 1992), rats with renovascular hypertension (Moore et al., 1991), dogs with pacing-induced cardiomyopathies (O'Rourke et al., 1999; Maltsev et al., 1998), Dahl salt-sensitive rats (Gomez et al., 1997) and a population of spontaneously hypertensive rats that develop accelerated heart failure (Gomez et al., 1997). The decrease in peak [Ca\textsuperscript{2+}]\textsubscript{i} is most likely attributed to changes in the activity of the SR Ca\textsuperscript{2+}-ATPase (Arai et al., 1994). In most studies of failing human myocardium or animal failure models, SERCA2a mRNA levels are reduced, while protein levels are reduced or unchanged (reviewed in Phillips et al., 1998). However, at the functional level, the consensus of multiple studies supports the notion that SERCA2 ATPase activity is reduced in heart failure. In this regard, several groups demonstrated a decrease in the expression of the sarcoplasmic reticulum gene
SERCA2a) following aortic banding (Arai et al., 1996; Maier et al., 1998; McCall et al., 1998 Qi et al., 1997), renovascular hypertension (Flesch et al., 1996), pacing in the dog model (O'Rourke et al., 1999). In a recent report, Gomez and colleagues (1997b) showed that despite the presence of normal SR function, the inadequate coupling between sarcolemmal calcium channels and SR calcium-release channels explains the reduction in peak [Ca^{2+}]_i and the probability of evoking calcium sparks in a rat model of cardiac failure. They concluded that a defective excitation-contraction coupling may develop in cardiac failure which is presumably due to changes in the microarchitecture of the dyad such that the distance between the L-type Ca^{2+} channel and the ryanodine receptor is increased. Animal models of cardiac failure are also characterized by increases in the expression of Na^+/Ca^{2+} exchanger (Litwin and Bridge 1997; O'Rourke et al., 1999).

Measurement of the amplitude of the [Ca^{2+}]_i transients from several animal models of cardiac hypertrophy have yielded opposite results compared to animal models of heart failure. An increase in peak [Ca^{2+}]_i was observed in the 6-18 week spontaneously hypertensive rat (Bing et al., 1991; Brooksby et al. 1993, Shorofsky et al., 1999). Despite the presence of cardiac hypertrophy, the progression of the disease does not lead to failure in SHR rats since expression of SERCA2a, phospholamban and ryanodine receptors was unchanged in these rats (Ohta et al. 1995; Shorofsky et al., 1999; Keller et al., 1997). Interestingly, SERCA2a is known to be upregulated in mild cardiac hypertrophy and down-regulated in severe cases of hypertrophy and failure (Feldman et al., 1993; Arai et al. 1996; Yoshiyama et al., 1997).
1.3.12 Regional modulation of gene expression in hypertrophic myocytes

The normal heart has considerable anatomic and functional heterogeneity in myocytes and non-cardiac myocytes, yet working together into a durable and efficient pump. Accumulation of evidence has reshaped our view of the hypertrophic heart, as ventricular disease is heterogeneous at the molecular, biochemical and cellular level. There is no clear explanation for this finding but a non-uniform distribution of wall stress across the ventricular wall and other parts of the heart may explain the transmural effects on gene expression and cardiac function in hypertrophic hearts.

In 1997, Gomez and colleagues were the first to report that significant region dependant changes in \( I_{\alpha} \) density and the loss of action potential heterogeneity in a pressure overload model of cardiac hypertrophy. Prolongation of action potential duration and regional differences in the extent of prolongation may predispose the heart to both triggered arrhythmia by favoring the development of triggered arrhythmias and re-entrant type arrhythmias, respectively. More recently, Aiond and colleagues (1999) showed that \( I_{\alpha} \) is significantly downregulated in the right ventricle but not in the septum following long term myocardial infarction. Similarly, the greatest decreases in \( I_{\alpha} \) density were observed in sub-epicardial and mid-myocardial with minimal changes in sub-endocardial myocytes in rat with catecholamine-induced hypertrophy (Bryant et al., 1999). In this regard, sub-epicardial were also found to be more prone to the depressing effect of \( I_{\alpha} \) than sub-epicardial myocytes in humans (Nabauer et al., 1996). One explanation for these findings may be related to the fact that \( K_v4 \) encoded \( I_{\alpha} \) channels are predominately affected following disease and therefore right ventricular or epicardial myocytes are more prone compared to septal or endocardial myocytes. Alongside the regional changes in potassium
channel expression, there is a heterogeneous distribution of calcium cycling proteins across the left ventricular wall in human cardiac tissue (Prestle et al., 1999). While cellular hypertrophy was observed in myocytes following myocardial infarction, abnormalities in single cell function such as velocity of shortening and relengthening and the extent of shortening were more prominent in the left ventricle compared to the right ventricle (Meggs et al., 1993). In this regard, the cardiac actomyosin ATPase activity (Geenen et al., 1989) was more depressed in the left ventricle compared to the right ventricle following myocardial infarction. Furthermore, SR Ca\(^{2+}\) ATPase (Afzal and Dhall a 1992) and adenylyl cyclase (Sethi et al., 1997) activity are increased in the right ventricle, but markedly decreased in the left ventricle following myocardial infarction. At the biochemical level, angiotensin II-stimulated phosphoinositol turnover was more enhanced in left and right myocytes, respectively, after infarction (Meggs et al., 1993). Collectively, these studies suggest that cardiac remodeling is region dependent during the development of cardiac hypertrophy. Furthermore, these findings support the view that the right ventricle may be playing a compensatory role following myocardial infarction and that the left ventricle exhibit the phenotypic changes that characterize heart failure.

1.3.13 Connection between action potential prolongation, [Ca\(^{2+}\)]\(_i\) and cardiac hypertrophy

Several studies have shown that Ca\(^{2+}\) is a primary signal for cardiac hypertrophy. Agents such as angiotensin II, adrenergic agents and endothelin-1 (known to be upregulated in heart disease) have been shown to elevate intracellular calcium, activate [Ca\(^{2+}\)]\(_i\)–dependent signaling systems and cause cardiac hypertrophy (Gaughan et al., 1998; Sadoshima and Izumo, 1993; Leite et al., 1994). Similarly, myocyte stretch,
increased loads on working heart preparations, elevation of extracellular calcium, stimulation with Ca\(^{2+}\) channel agonists, treatment with calcium ionophores all elevate [Ca\(^{2+}\)]\(_i\) and induce cardiomyocyte hypertrophy (Perreault et al., 1994; Hongo et al., 1995; Ito et al., 1991).

At the onset of cardiac hypertrophy, the amplitude of the Ca\(^{2+}\) transient increases. However, as hypertrophy progresses to heart failure, the amplitude of the Ca\(^{2+}\) transient decreases (Shorofsky et al., 1999; Balke and Shorofsky, 1998; Gomez et al., 1997; Bailey et al., 1992; Moore et al., 1991). It is unclear at the present time which calcium (diastolic or systolic) is important for the induction of hypertrophy. It was recently proposed that a Ca\(^{2+}\)-sensitive signaling system for cardiac hypertrophy may exist in cardiac myocytes and may be involved in the regulation hypertrophy (Olson and Molkentin, 1999). Several groups have demonstrated that modulation of action potential duration may be an important determinant of calcium influx through L-type calcium channels (Bouchard et al., 1995; Volk et al., 1999; Puglisi et al., 1999). Furthermore, the duration (O’Rourke et al., 1999; Maltsev et al., 1998) and amplitude of the calcium transient (Bouchard et al., 1995) were shown to be strongly influenced by membrane potential (O’Rourke et al., 1999; Maltsev et al., 1998). Apkon and Nerbonne (1988) were the first to show that α\(_1\) adrenergic agonists which are known to be upregulated in cardiac hypertrophy (see above) can suppress K\(^+\) currents. More recently, it was reported that α\(_1\)-adrenergic stimulation can reduce K\(^+\) currents, prolong action potential duration (Fedida and Bouchard, 1992; Gaughan et al., 1998) and cause an increase in [Ca\(^{2+}\)]\(_i\) and contractions (Fedida and Bouchard, 1992). Therefore, it is possible that α\(_1\)-adrenoreceptor stimulation
following neurohumoral activation in cardiac hypertrophy may contribute to the initial action potential prolongation.

Prolonged membrane depolarization and calcium entry may cause alterations in gene expression in cardiac myocytes (Wickenden et al., 1998). Similarly, and consistent with the hypothesis, gene expression of trophic factors in cardiac myocytes and activation of stress pathways have been shown to be dependent on calcium. For example, norepinephrine treatment of cultured neonatal myocytes induces a decreased expression of SERCA2 and the decrease in SERCA2 can be abolished in contractile arrested and verapamil treated myocytes (Muller et al., 1997), implying that excitation-contraction coupling and calcium current through the L-type calcium channel are important in the phenotypic response to \( \alpha_1 \)-adrenergic agonists. Furthermore, the induction of the immediate early genes such as \( c-fos \) and \( c-myc \) are responsible for the onset of cardiac hypertrophy and are inhibited by calcium channel blockers (Grohe et al., 1994).

### 1.3.14 Thyroid hormones

The thyroid gland primarily produces two hormones, L-thyroxine (T4) and tri-iodothyronine (T3). It appears that thyroid hormones are essential for normal development, growth and metabolism in vertebrate species (Goglia et al. 1999). The heart has long been known to be a major target of these hormones (Morkin et al., 1983; Dillman 1990), and deviations from normal levels have profound effects on the cardiovascular system.

Hyperthyroidism induces an increase in both cardiomyocyte cross-sectional area and cell length, but, interestingly, \( \textit{in vivo} \) hypertrophy associated with hyperthyroidism requires concomitant hemodynamic loading (Campbell and Gerdes, 1988) or activation of the
renin-angiotensin system (Kobori et al., 1999). Thyroid hormones also display effects on cardiac function which include a positive chronotropic and inotropic effect. They increase the expression of SERCA (Chang et al., 1997), decrease the expression of Na⁺/Ca²⁺ exchange activity (Cemohorsky et al., 1998) and increase α-myosin heavy chain gene expression in rat (Hoh et al., 1984) in the developing rat heart. Although the mechanisms by which thyroid hormones regulates developmental processes are not fully understood, intracellular T3 levels are likely to be a critical aspect of the process (Bates et al., 1999). Availability of T3 in peripheral tissues such as the heart mostly depends on local deiodination of T4 by a series of deiodinases. Therefore, T4 deiodination is crucial in the local homeostasis of thyroid hormones. In the past few years, it has been demonstrated that T3 exerts profound effects on the expression of several genes. These effects are mediated by the nuclear T3 receptor (TR). TR are transcription factors that can modulate transcription mainly by binding to specific regulatory sequences on DNA known as ‘thyroid hormone receptor elements’ or (TRE) and thereby modify expression of a particular gene.

1.3.14 A. Effect of thyroid hormones on potassium channels

Thyroid hormones may also exert important effects on electrical function. In this regard, experimental hypothyroidism was shown to prolong the Q-T interval (Bosch et al., 1999) as well as action potential duration (Bosch et al., 1999; Sharp et al., 1985; Binah et al., 1987). Thyroid hormone has been found to shorten action potential duration in guinea pig, rabbit, and rat ventricular myocytes (Sharp et al., 1985; Binah et al, 1987; Meo et al., 1994). Hypothyroidism was shown to reduce Iₒ in rat ventricular myocytes (Shimoni and Severson 1995; Shimoni et al., 1995). In this regard, hypothyroidism was
shown to increase Kv1.4 and decrease Kv4.2 expression while hyperthyroidism was shown to decrease Kv1.4 and increase Kv4.2 expression (Nishiyama et al., 1998). Furthermore, thyroid status did not affect Kv4.3 expression (Nishiyama et al., 1998).

Interestingly, I_o in neonatal rat ventricular myocytes is predominately encoded by Kv1.4 compared to Kv4.2. In this regard, the recovery kinetics of I_o are typically slowed in neonatal ventricular myocytes since these channels are primarily encoded by Kv1.4 (Tseng-Crank et al., 1990). Following development the ratio of Kv4.2 to Kv1.4 expression is increased resulting in an acceleration of the recovery kinetics (Wickenden et al., 1997). In summary, it is believed that the rise in circulating thyroid hormones (Shimoni et al., 1997; Wickenden et al., 1997) is responsible for the changes in I_o density and kinetics observed following development. In animal models which lack I_o such as the guinea pig, hypothyroidism was shown to reduce the slow component of the delayed rectifier (I_K), with no changes in the rapid component (I_Kr) (Bosch et al., 1999).
CHAPTER 2. METHODS

The methods and techniques were similar for all studies and are consolidated in this section to avoid repetition. All cellular recordings were performed at room temperature (19-21 °C) except for monophasic action potential which were performed between 34-36 °C.

2.1 Left anterior descending coronary artery ligation procedure

Left anterior descending coronary artery ligations were performed on male LBN-F1 rats (Harlan, Indianapolis, Indiana) at 10-12 weeks of age using the methods outlined previously (Orenstein et al., 1995). Animals were anesthetized with ketamine hydrochloride (45 mg Kg⁻¹) and xylazine (5 mg Kg⁻¹) intraperitoneally. Once anesthetized, rats were intubated with a 14-gauge polyethylene catheter and ventilated with room air using a small animal ventilator (model 683; Harvard Apparatus, Boston MA.). A left thoracotomy was performed in the fifth intercostal space and the pericardium opened. The proximal left coronary artery was encircled and ligated using a 6-0 silk suture. The muscle and skin were closed in layers. Infarct size was assessed 8 weeks following MI by dissecting the left ventricular free wall and measuring the fraction of the left ventricular free wall which was replaced with fibrous tissue. Sham-operated animals were treated identically except the left coronary artery was not tied. All experimental protocols were approved by the Committee on Animal Research at the Toronto General Hospital and were performed in accordance with the "Position of the American Heart Association on Research Animal Use". After the surgical procedure, rats were housed in a climate controlled environment at ambient temperature of 21 °C with 12 hour light/dark cycles. Water and standard Purina rat chow were given ad libitum.
2.2 Treatment with 3,5-diiodothyropropionic acid (DITPA)

Five weeks following the LAD ligation procedure, animals were treated with either DITPA (3.75 mg Kg⁻¹ body weight and 10 mg Kg⁻¹ body weight) or 0.9 g 100 ml⁻¹ NaCl. Stock solutions of DITPA (Sigma Chemicals, St-Louis, Mo) containing 3.75 or 10 mg ml⁻¹ were prepared as previously described (Pennock et al., 1993) and diluted in 0.9 g 100 ml⁻¹ NaCl. The doses of DITPA used in the present study have previously been shown to exert beneficial effects on contractile performance in animal models of cardiac hypertrophy and failure (Pennock et al., 1993; Mahaffy et al., 1995). Animals received daily subcutaneous injections for 21 days. Eight weeks following sham or LAD ligation procedure, the animals were sacrificed and mRNA levels, protein levels, current densities and action potentials were measured using methods described below. In addition, the left ventricular free wall was carefully dissected from base to apex and along the border edge of the right ventricle. Infarct size was determined by measuring the infarcted area on the epicardial surface and is expressed as a percentage of the area of the left ventricular free wall.

Our recordings were confined to myocardium derived from the right ventricle eight weeks following myocardial infarction. Previous characterization of this model has established that this time point represents a phase of compensated hypertrophy, rather than failure (Gidh-Jain et al., 1996) and would encompass the period during which there is an increased incidence of arrhythmias (Qin et al., 1996). Therefore, eight weeks post-infarction is a relevant time point to study mechanisms with potential relevance to hypertrophy and arrhythmogenesis.
2.3 In vivo monophasic action potential and hemodynamic studies

Monophasic action potentials (MAPs) are extracellularly recorded waveforms that under optimal conditions, can reproduce the repolarization time course of transmembrane action potentials (TAPs) with high fidelity (Franz 1999). MAP recordings are suitable for studying the characteristics of local myocardial electrophysiology and global repolarization in vivo. After a mid-line thoracotomy, the heart was perfused retrogradely with a Ca^{2+}-free Tyrode’s solution containing (mM): NaCl (140), KCl (5.4), HEPES (10), MgCl₂ (1), D-Glucose (10), pH balanced to 7.4 with NaOH at 37 °C. A custom-made contact probe was used to record monophasic action potentials (MAPs) as previously described (12). The MAP probe consisted of a bipolar electrode 0.8 mm in diameter sintered Ag/AgCl pellet (E255, In Vivo Metric, Healdsburg, CA, USA) encased in polyethylene tubing (with a total outer diameter of 1.3 mm). The MAP probe was gently held against the centro-lateral surface of the right ventricular wall. The reference probe was in electrical continuity with the Tyrode’s solution flowing over the epicardial surface of the heart. A constant pressure was applied onto the surface of the heart to eliminate motion artifacts caused by slippage or uneven contact onto the myocardium. MAPs were recorded at 10 KHz and action potential durations were evaluated at 50% (APD₅₀) and 90% (APD₉₀) repolarization.

For hemodynamic assessment, the animals were anesthetized with ketamine hydrochloride (45 mg Kg⁻¹) and xylazine (5 mg Kg⁻¹) intraperitoneally. The right carotid artery was cannulated with a 200 PE tubing. The catheter was then advanced into the aorta and then into the left ventricle. Left Ventricular End-Diastolic Pressure (LVEDP) and Left Ventricular Systolic Pressure (LVSP) were measured from the ventricular pressure traces. The maximal rates of LV pressure development and decline (+dP/dt and
-dP/dt, respectively) were obtained by differentiating ventricular pressure traces. For each animal, averaged data was used from three measurements for all parameters.

2.4 Isolation of ventricular myocytes

All rats were anti-coagulated (Heparin- 4000 U Kg⁻¹ i.p.) and anesthetized (pentobarbital sodium- 60 mg Kg⁻¹ ip.) eight weeks following surgery. Once an adequate depth of anesthesia was reached, the hearts were quickly removed and placed in a standard Tyrode’s solution. The hearts were then cannulated as previously described and retrogradely perfused via the aorta for about 3 minutes with a calcium-containing standard Tyrode’s solution of the following composition (mM): NaCl (140), KCl (5.4), HEPES (10), MgCl₂ (1), CaCl₂ (1) d-Glucose (10), 37 °C, pH balanced to 7.4 with NaOH. The heart was then perfused with Ca²⁺-free standard Tyrode’s solution for 5 minutes before the hearts were digested with the same solution containing collagenase (type II, 0.55 mg ml⁻¹, Manheim-Boehringer) and protease (type XIV, 0.05 mg ml⁻¹, Sigma Chemicals) for 8-9 minutes. The enzyme solution was subsequently removed by perfusing with a KRAFTBRÜHE (high K⁺) solution containing (mM): K⁺-glutamate (120), KCl (20), HEPES (20), MgCl₂ (1), K-EGTA (0.3), and d-Glucose (10) for 5 minutes. Following the enzyme washout period, the atria and blood vessels were removed and the ventricles were separated. All solutions were pre-bubbled with 100% O₂ for 5 minutes. The right ventricular free wall and septum were dissected and minced in a high K⁺ solution containing bovine serum albumin (0.02% w/v). Myocytes were then filtered through a nylon mesh and re-suspended in a KRAFTBRÜHE solution with gentamicin, 50 mg ml⁻¹. Calcium-tolerant quiescent, rod-shaped cells with clear, regular cross-striations were selected for electrophysiological recordings. Cells were transferred into
a perfusion bath situated on the stage of an inverted microscope and perfused with recording solution at a rate of 1-2 ml min⁻¹.

2.5  Basic electrophysiological circuitry

There are two fundamental laws which are important in the field of electrophysiology. Ohm's law states that the potential difference between two points linked by a current path is given by: \( \Delta V = I \cdot R \) where \( I \) is the current and \( R \) is the resistance. A second important concept is Kirchoff's current law which states that the sum of currents in a closed loop is equal to zero. As shown in figure V (panel A), electrophysiological recordings consist of a ground (or bath) electrode, a measuring circuitry which basically consists of an amplifier (connected to a computer not shown) and a recording electrode. Any current from the amplifier must pass through the recording electrode which typically ranges between 2-4 MΩ before reaching the membrane which contains its own resistance by virtue of the presence of ion channels. Therefore, two voltage drops exist in series in the circuitry including a voltage drop across the recording electrode known as the series resistance and across the membrane. The equivalent circuit is shown in panel B.

The voltage drop \( (V_S) \) across the series resistance is given by: \( V_S = V_C \left[ \frac{R_S}{R_M + R_S} \right] \)

whereas the voltage drop \( (V_M) \) across the membrane is given by: \( V_M = V_C \left[ \frac{R_M}{R_M + R_S} \right] \).

\( R_M \) is the membrane resistance, \( R_S \) is the series resistance \( V_C \) is the command voltage coming from the amplifier. For every cellular recording, the voltage drop across the series resistance must be quantified and compensated electrically with the
Figure V. Circuitry in electrophysiological studies. A is a schematic representation of the recording circuitry. Using a micropipette, cells are dialyzed with an intracellular solution. The series resistance from the pipette causes a voltage drop in the circuitry which is estimated and compensated for, while a ground (or bath) electrode is used to complete the circuit. B shows the equivalent electrical circuit (Axon Instruments Handbook).
amplifier. The purpose of the electric compensation is to eliminate $R_S$ such that it reaches almost 0, thereby allowing the quotient $[R_M/R_M+R_S]$ to become unity. Upon elimination of the series resistance, the closed circuitry may be described by: $V_C = V_S + V_M$.

### 2.6 Whole-cell electrophysiological evaluation

Currents flowing through individual channel molecules were resolved for the first time through the use of a technique known as “patch clamp”, which earned its discoverers, Erwin Neher and Bert Sakmann, the Nobel Prize in 1991. With the use of the patch-clamp technique, it is possible to study whole cell currents. As the ions move through a given ion channel, a current may be recorded which is the flow of electrical charge (expressed in Coulombs) passing a point per unit of time (expressed in seconds). Whole-cell current is described by the equation $I = N(P_o)i$ where $I$ is the measured current, $N$ is the number of functional channels, $P_o$ is the probability of channel opening, and $i$ is the unitary channel current amplitude. At any given activating voltage, $P_o$ and $i$ are constant assuming that the parameters for steady state activation are unchanged. Therefore, any decrease in whole cell current is a direct reflection of the number of channels. Current ($I$) is reported in units of amperes which is equivalent to 1 coulomb per second.

**Normalization of current amplitude**

In these studies, it was important to normalize the measured current to membrane surface area to ensure that the changes in currents were independent of cellular hypertrophy. For example, irrespective of changes in channel expression, an increase in cell surface area alone, can also cause a reduction in the recorded current. Normalization of currents to cell
surface area was routinely performed for every cell to ensure that the changes in current amplitude was related to the changes in channel expression. The currents were normalized to cell capacitance which is routine practice in the field of electrophysiology. The capacitance is expressed in picofarads (pF) in this Thesis and can be measured by applying voltage clamp pulses to the myocyte to voltages where ion channels are not activated. The conduction of electricity through the cell membrane (i.e including the invaginations such as the t-tubular structures) elicits a capacity transient which can be integrated to calculate cellular capacitance. It is generally accepted that the high electrical capacitance of biological membranes is a direct consequence of their molecular dimensions. The relationship between cellular capacitance and cell surface area is given by: 

$$ C = \varepsilon \varepsilon_0 \frac{A}{d} $$

where $C$ is the cell capacitance, $\varepsilon$ is the dielectric constant of the hydrocarbon chain = 2.1, $\varepsilon_0$ is the polarizability of free space = $8.85 \times 10^{-12}$ C/V.m , $A$ is the cell surface area, $d$ is the thickness of the membrane = 2.3 nm. The dielectric constant, polarizability of free space and the thickness of cell membrane is constant in normal and hypertrophied cardiac myocytes. Hence the cellular capacitance is directly related to cell surface area. Therefore the formula above simplifies to $C = c \ast$ area where $c =$ specific capacity. The word “specific” refers to the electrical properties of a 1 cm$^2$ of area of membrane and is estimated to be 1 $\mu$F/cm$^2$ for all cell membranes. Therefore the measured changes in whole cell currents for any given ion channel reflects changes in the number of functional channels following normalization to cellular capacitance.

In my experiments, I used different recording methods to measure membrane potential and currents through specific ion channels. In a current-clamp experiment, I applied a small square wave (depolarizing) pulse for a brief time period and measured the
change in membrane potential. In a voltage clamp experiment, I controlled the membrane voltage and measured the transmembrane current passing through a specific ion channel. I also measured membrane currents using the action potential clamp technique. Briefly, the transmembrane potential is recorded in the current clamp configuration during steady state stimulation. The action potential waveform is stored in a computer. After switching to the voltage-clamp configuration, the acquired action potential may be used as the command signal to drive membrane potential in a given myocyte. This is particularly important for the voltage-sensitive calcium channel whose activation and inactivation may change depending on the contour and shape of the action potential. The third chapter shows a figure where the action potential clamp technique was used during the measurements of Ca$^{2+}$ transients and L-type calcium currents. Since there are many currents that may be activated during the action potential, it is important to isolate the particular current by modifying solutions or using specific ion channel blockers.

2.7  

tsa-201 cell culture, transfection and electrophysiological recordings

At the outset, I must mention that Dr. Wickenden carried out the transfection and recording on the tsa-201 cells to complement the manuscript. Briefly, tsa-201 cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum and gentamicin (50 µg ml$^{-1}$) in an incubator at 37 °C with a humidified atmosphere of 5% CO$_2$. Media was replaced every 48 - 72 h. 24 h before transfection, tsa-201 cells were harvested by brief trypsinization (0.5 mg ml$^{-1}$ in phosphate buffered saline) and re-plated at a density of $3 \times 10^5$ cells per 35 mm culture dish. Cells were transfected using Lipofectamine reagent (GIBCO BRL) according to the manufacturers instructions. Cells were incubated for a period of 5 h with a mixture of 10 µl lipofectamine reagent, 1 µg
GFP and either 0.5-1 μg pRcCMV<sub>Kv4.2</sub>, 1 μg pcDNA3<sub>Kv4.3</sub>, 0.03-1 μg pGW1HK<sub>V1.4</sub> or a similar amount of vector alone in OPTI-MEM serum-free media. 24 - 48 h post-transfection, cells were prepared for electrophysiological evaluation. Cells were removed from the culture dish by brief trypsinization (as described above), collected by centrifugation (1000 rpm, 5 min) and replated in growth medium at low density.

Electrophysiological recordings from tsa-201 cells were made with the cells adhered to the 35 mm culture dishes in which they were plated. Culture medium was replaced with extracellular solution immediately prior to recording. Pipette tips were heat polished to a resistance of 1-3 MΩ when filled with an intracellular solution of the following composition (mM): KCl (140), MgCl₂·6H₂O (1), EGTA (10), HEPES (10), MgATP (5), pH 7.2-7.3 with KOH. In some experiments with cultured cells a KF based pipette solution was used to improve seal stability. The composition (mM) of the KF solution was KF (100), KCl (40), NaCl (5), MgCl₂ (2), HEPES (10), EGTA (5), glucose (5), pH 7.2-7.3 with KOH. Recovery kinetics were similar with KCl and KF based intracellular solutions. Successfully transfected tsa-201 cells were identified by their green fluorescence under appropriate conditions.

2.8 Electrophysiological recordings in ventricular myocytes

Current densities and action potentials were recorded using the whole cell patch clamp technique (Hamill et al., 1981) with an Axopatch 200A amplifier (Axon Instruments, CA, USA). Microelectrodes were pulled from thin-walled borosilicate glass (1.5 mm diameter, World Precision Instruments, Sarasota, Fla.) using a Flaming-Brown micropipette puller (Sutter Instruments). The pipette tip was heat polished with a heating filament. When filled with intracellular solutions, tip resistances were typically 2-4 MΩ. Series resistance
compensation ranged between 60-90%. After membrane rupture, the cell capacitance was estimated by integrating the area of the capacitance transients following a 5 mV step from a holding potential of -70 mV.

In order to measure Ca\(^{2+}\) currents (I_{Ca,L}), voltage-clamp recordings were made in sham myocytes superfused with a solution containing (mM): NaCl (140), MgCl\(_2\) (1), HEPES (10), CsCl (4), CaCl\(_2\) (1), d-Glucose (10), pH adjusted to 7.4 with NaOH. I_{Ca-L} was also recorded using the action potential voltage-clamp technique in myocytes superfused with a solution containing (mM): CsCl (145), HEPES (10), MgCl\(_2\) (1), CaCl\(_2\) (2), d-Glucose (10), pH adjusted to 7.4 with CsOH. Intracellular solutions always contained (mM): CsCl (150), HEPES (10), MgCl\(_2\) (1), EGTA (5) MgATP (5), pH adjusted to 7.2 with CsOH. Na\(^+\) and K\(^+\) free extracellular solutions were used during action potential voltage-clamp recordings to minimize K\(^+\), Na\(^+\) and Na\(^+\)/Ca\(^{2+}\) exchange currents activated during an action potential. These solutions contained (mM): CsCl (150), MgCl\(_2\) (1), HEPES (10), CaCl\(_2\) (2), d-Glucose (10), pH adjusted to 7.4 with CsOH. Sham myocytes were stimulated with a short or long action potential waveforms which were derived from sham and post-MI hearts, respectively. Due to the heterogeneous nature of action potential waveform, we recorded 20 action potentials from 5 hearts in either sham and post-MI right ventricular myocytes before selecting action potentials as command waveforms for each respective group. Action potential waveforms that most closely approached the mean action potential duration at 50% and 90% repolarization were used to examine the Ca\(^{2+}\) currents under action potential voltage-clamp conditions. I_{Ca,L} was estimated as the current in the absence of CdCl\(_2\) minus the current remaining after the addition of 0.3 mM CdCl\(_2\). The difference currents were integrated to estimate the total influx of calcium (\(\int I_{Ca,L}\)). In action potential clamp
experiments, action potential waveforms from either sham or post-MI myocytes were used as command signals to measure \([Ca^{2+}]_i\). To allow equilibration between the cell and the pipette, recordings were made 5 minutes after rupturing the cell membrane.

For K⁺ currents and action potential recordings, myocytes were superfused with a standard Tyrode’s solution containing (mM): NaCl (140), HEPES (10), MgCl₂ (1), KCl (4), CaCl₂ (1), d-Glucose (10), pH adjusted to 7.4 with NaOH. CdCl₂ (0.3 mM) was routinely added for K⁺ current recordings to block \(I_{Ca,L}\). Intracellular solution for K⁺ currents and action potentials had the following composition (mM): K-aspartate (130), KCl (20), HEPES (10), MgCl₂ (1), NaCl (5), EGTA (5), MgATP (5), pH adjusted to 7.2 with Trizma base. Action potentials were corrected by -9 mV to compensate for liquid junction potentials that arose from the use of K-aspartate in microelectrodes. To measure Na⁺ currents, myocytes were superfused with a solution containing (mM): NaCl (5), TEA-Cl (125), MgCl₂ (1), HEPES (20), CsCl (5), MnCl₂ (1), D-Glucose (10), pH adjusted to 7.4 with CsOH. The intracellular solution was composed of CsCl (125), TEA-Cl (20), HEPES (10), EGTA (10), MgATP (5), pH adjusted to 7.2 with CsOH. To examine \(I_{Na}\) density, a series of 50 ms voltage steps from a holding potential of -100 mV were given stepwise from -80 mV to +10 mV in 5 mV increments. A P/4 protocol was used for leak and capacitance subtraction.

2.9 Fluorescence microscopy in contracting myocytes

Fluorescence is the phenomenon in which absorption of light of a given wavelength by a fluorescent molecule is followed by the emission of light at longer wavelengths. The development of light-emitting indicators such as Fura-2 has made it possible to record changes in intracellular calcium in contracting myocytes. The fura-2
pentapotassium salt is added to the pipette solution and is allowed to freely diffuse into the myocyte. The fura-2 molecule binds calcium tightly and is excited by exposure to light at 340 nm and 380 nm. When Fura-2 binds to calcium ion, its fluorescence property is altered allowing for the measurement of intracellular calcium. To quantitatively characterize the relative concentration of calcium bound to fura-2 and free calcium, and thus measure $[Ca^{2+}]_i$, I had to measure the ratio (R) of fluorescence intensity at the two excitation wavelengths. Since the fluorescence microscopy measures the total emitted fluorescence in the field of the objective lens (which include the myocyte under study, the tip of the micropipette and other neighboring myocytes), I had to account for this background fluorescence and subtract it. This was done at both wavelengths (340 nm and 380 nm) after obtaining a gigaohm seal and prior to rupturing the cell membrane. The ratio of the background subtracted fluorescent signal (340/380) was used to estimate $[Ca^{2+}]_i$ using the equation given by Grynkiewicz et al., 1985:

$$[Ca^{2+}]_i = K'_D \cdot (R - R_{min}) / (R_{max} - R)$$

where $K'_D$ is the apparent dissociation constant, R is the ratio of the background-subtracted fluorescence at 340 nm excitation to that at 380 nm excitation. The effective dissociation constant ($K_D'$) is defined as $K_D \times \beta$ where $K_D$ is the dissociation constant of fura-2 for $Ca^{2+}$ which is $\sim 0.22 \mu M$ in the presence of 1 mM MgCl$_2$ (Molecular Probes-Handbook of fluorescent probes and research chemicals, 1996). In our experiments $\beta$ which is defined as the ratio of fluorescence measured with 380 nm excitation light in absence of $Ca^{2+}$ to that measured at saturating $Ca^{2+}$ levels, $R_{max}$ was 6.43 and $R_{min}$ was 0.20.

Briefly, fluorescence measurements were performed using light from a 75 Watt Xenon lamp passed through band-pass filters centered at 340 or 380 nm via an epifluorescence port and a
40X Fluor objective. The emitted fluorescence was collected by the objective and passed through a 510 nm filter to a photomultiplier tube (PMT) housing unit. The unit is used to detect very low levels of light which is amplified and converted to a voltage signal. The output from the PMT is then recorded using an A/D data acquisition board (2801A, Data translation) and stored in the computer for analysis.

\( [\text{Ca}^{2+}]_i \) was measured in ventricular myocytes under current clamp, action potential voltage-clamp and voltage-clamp conditions. For action potential voltage-clamp experiments, the action potential waveform from either sham or post-MI myocytes were used as command signal to drive membrane potential of the same cell and \( [\text{Ca}^{2+}]_i \) was recorded. Following patch rupture, 5 minutes was allowed for fura-2 to diffuse into the myocyte before recordings begun. For voltage-clamp recordings, sham and post-MI myocytes were delivered with short pulses (100 ms duration) from a holding potential of -80 to +10 mV. All \( [\text{Ca}^{2+}]_i \) measurements were made under steady-state conditions by stimulating the myocyte at 0.25 Hz and recording fluorescence at both wavelengths between the 17th and 20th beat. Bath solution consisted of a standard Tyrode’s solution in the presence of 2 CaCl_2. The intracellular solution contained (mM): K-aspartate (130), KCl (20), HEPES (10) MgCl_2 (1), NaCl (6), fura-2 pentapotassium salt (0.075), MgATP (5), pH adjusted to 7.2 with Trizma base.

2.10 Sarcoplasmic reticulum calcium content in ventricular myocytes

SR Ca\(^{2+}\) content was estimated by applying a train of 10 conditioning pulses from -70 to +10 mV (100 ms clamp steps separated by 1 s intervals) to establish uniform SR loading conditions. Bath solutions consisted of a modified Tyrode’s solution containing (mM): NaCl
(140), HEPES (10), MgCl$_2$ (1), CsCl (4), CaCl$_2$ (2), pH adjusted to 7.4 using CsOH. Recording electrodes contained (mM): CsCl (150), HEPES (10), MgCl$_2$ (1), EGTA (0.1), MgATP (5), pH adjusted to 7.2 with CsOH. After loading the SR, myocytes were rapidly superfused with the modified Tyrode's solution containing 10 mM caffeine. The application of caffeine induced a large contraction accompanied by an inward current which was previously shown to be caused by the Na$^+$/Ca$^{2+}$ exchanger, which extrudes the Ca$^{2+}$ released by the SR (Varro et al., 1993). $I_{Na^+/Ca^{2+}}$ was estimated as the difference in inward current induced by caffeine exposure in the presence and absence of sodium chloride (equimolar substitution with tetraethylammonium chloride). During caffeine applications, holding potential was set at –70 mV to enhance $I_{Na^+/Ca^{2+}}$ and the SR Ca$^{2+}$ content was calculated by integrating $I_{Na^+/Ca^{2+}}$ as described by Varro et al. (1993). Cell volume was calculated from membrane capacitance and then converted to volume by assuming a surface to volume ratio of 0.5 $\mu$m$^{-1}$ (Page, 1978) and a specific membrane capacitance of 1 $\mu$F/cm$^2$.

2.11 Molecular assays to study ion channel function and expression

At the outset, I must mention that Ms. Tin Nguyen (Technician in the laboratory) conducted all the molecular assays. In the analysis of gene expression, the steady-state level of RNA transcripts is one of the most convenient parameters used to monitor the gene activity in tissues and cell lines. A variety of methods such as fluorescent or non-fluorescent in situ hybridization, RNase protection, Northern blotting may be used to measure RNA levels, and the choice of the assay depends on the information required, levels of sensitivity, and limitations of the particular in vivo system being examined. The Northern blot technique provides information on the size and abundance of RNAs.
derived from a gene, but the RNA isolated must be of high quality and not degraded. This is not a problem for in situ hybridization and RNAse protection methods, since these assays use enzymes to degrade RNA as part of the procedure. Northern analysis is also generally considered to be less sensitive for $K^+$ channel expression and requires larger amounts of RNA which may not be possible from hearts especially for the regional studies. The use of RNAse protection assay on potassium channels is another method by which one may confirm the presence of a particular $K^+$ channel clone and was used in previous studies (Takimoto et al., 1997; Gidh-Jain et al., 1996; Barry et al., 1995; Dixon and McKinnon, 1994) including my study. A brief description of the technique follows. The RNAse protection assay is based on the resistance of RNA:RNA hybrids to single-stand specific RNases, after annealing to a complementary $^{32}$P-labeled probe in solution. Briefly, a short (10-15 base pairs in length) sequence $^{32}$P-labelled riboprobes complementary to the cellular mRNA sequence is used. After hybridization occurs, specific RNAse are used which cut single-stranded, but not double-stranded RNA. The protected probe is detected and quantitated by polyacrylamide gel electrophoresis under denaturing conditons, followed by autoradiography (Appendix: The RNAse protection protocol in Molecular Cloning Sambrook, Fritsch and Maniatis, 1989). As an internal control, the cyclophilin cRNA probe is usually used to detect cyclophilin mRNA levels which is not altered during conditions of cardiac hypertrophy. Another housekeeping gene, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) may also be used to control for RNA loading conditions.

While the presence of the mRNA encoding a particular $K^+$ channel may be used as an index of expression, it does not prove its expression on cell membranes. Also
expression of channels may vary regionally and following a particular intervention such as induction of cardiac disease or treatment with drugs, it is important to be able to visualize or quantify changes in expression. Immunohistochemistry with isoform-specific antibodies made against specific cloned channels have enabled us to confirm the presence and or abundance of a particular channel protein and correlate it to cellular recordings (Brahmajothi et al., 1999). Another method used to quantify for the presence of a particular $K^+$ channel subunit is by extracting protein from myocardial samples and measuring the protein level with the use of specific antibodies. Western blotting allowed us to better correlate protein expression with electrophysiologic recordings since some channels such as the inward rectifier family (IRK) are known to undergo significant post-transcriptional modification.

2.11.1 RNA extraction and RNase protection assay

Hearts were removed rapidly, the right ventricle and/or septum were separated, rinsed briefly in 0.9% NaCl (w/v) and snap frozen in liquid nitrogen. Ventricular tissue was powdered and RNA extracted by the one-step acid guanidium phenol method (Chomczynski & Sacchi, 1987). The concentration of RNA was measured spectrophotometrically and confirmed by agarose gel electrophoresis. Gels were loaded with 10 $\mu$g of total RNA obtained from several hearts ($n=3$ to 7) for each group. RNase protection assays (RPA) were performed using an RPAII Ribonuclease Protection Assay Kit (Ambion, Austin, Texas, USA) as previously described by Dixon and McKinnon (1994). Antisense probes were labelled with $[\alpha^{-32}P]$-UTP (3000 Ci/mmol, Mandel Scientific, Guelph, Ontario, Canada) by in-vitro transcription using T7 RNA polymerase. Probes (2 ng, $2 \times 10^4$ counts per minute) were added separately to 10 $\mu$g of total RNA in 20 $\mu$L of 300 mM
sodium acetate, pH 6.4, containing 100 mM sodium citrate, 80 mM formamide and 1 mM EDTA. Probe and RNA were hybridized for 18 h at 45 °C and the unannealed RNA was digested with RNase A (5 units ml⁻¹) and RNase T1 (200 units ml⁻¹) at 37 °C for 30 min. RNase resistant hybrids were recovered, analyzed on 8 M urea- 4-6 % polyacrylamide sequencing gels and visualized by autoradiography.

The Kv and IRK probes were kindly provided by Dr. David McKinnon (State University of New York at Stony Brook) and have been described previously (Dixon & McKinnon, 1994). The cyclophilin probe was purchased from Ambion (Austin, Texas, USA). Abundance of mRNA transcripts was quantified by densitometry (BIORAD GS670 Imaging densitometer). Signals were normalized to a cyclophilin internal standard to ensure that findings were not influenced by minor variations in loading. Absolute cyclophilin levels (densitometric units) were not significantly different between right ventricle, septum, sham and post-MI samples indicating that cyclophilin expression was not influenced by anatomic region or presence of disease. All mRNA samples for each sample was normalized to cyclophilin mRNA levels in the same sample.

2.11.2 Protein extraction and Western blotting

Venricular tissue and brain were quickly rinsed in a standard Tyrode’s solution and snap frozen in liquid nitrogen and stored at −70 °C for western blot analysis. Frozen tissue was homogenized in 10 volumes of 0.3 M sucrose and 30 mM histidine. This homogenate was then centrifuged at 3000 g for 15 min. The collected supernatant was re-centrifuged at 4500 g for 1 hour to precipitate membrane proteins. The membrane pellet was re-suspended in 1% triton X-100 and 50 mM Tris (pH=6.8), left on ice for one hour and then centrifuged at 14 000 g for 15 minutes. The final supernatant was saved for the
protein assay using the Lowry method. All solutions in this procedure were chilled on ice and contained the protein inhibitors (0.1 mM PMSF, 5 μg ml⁻¹ of aprotinin, leupeptin, antipain, and pepstatin). For Western blot analysis, 50-100 μg of total heart protein and 10-20 μg of total brain protein were fractionated on a 10% polyacrylamide-SDS gel. After electrophoretic transfer to polyvinylidifluoride (Bio-Rad), the membranes were incubated with Kv1.4 and Kv4.2 antisera (generously provided by Dr. Owen T. Jones, Toronto Western Hospital, Toronto, Canada). Bound primary antibody was detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham) in the blocking buffer for one hour at room temperature. The membrane was washed again with Tris Buffer Saline (TBS) containing 0.05% tween-20 and 1% triton X-100. Immunoreactivity was detected with the enhanced chemiluminescence (ECL) reagent (Amersham) and quantified by densitometry of the developed film. Quantitative analysis was performed with the Molecular Analyst Program (Bio-Rad).
CHAPTER 3

RELATIONSHIP BETWEEN POTASSIUM CHANNEL DOWN-REGULATION AND [Ca^{2+}] IN RAT VENTRICULAR MYOCYTES FOLLOWING MYOCARDIAL INFARCTION

R. KAPRIELIAN, A.D. WICKENDEN, Z. KASSIRI, T.G. PARKER, P.P. LIU AND P.H. BACKX

ABSTRACT

1) Cardiac hypertrophy and prolongation of the cardiac action potential are hallmark features of heart disease. We examined the molecular mechanisms and the functional consequences of this action potential prolongation on calcium handling in right ventricular myocytes obtained from rats 8 weeks following ligation of the left anterior descending coronary artery (post-MI).

2) Compared to sham myocytes, post-MI myocytes showed significant reductions in transient outward K+ current densities ($I_{to}$) (sham $19.7 \pm 1.1 \text{ pA pF}^{-1}$ versus post-MI $11.0 \pm 1.3 \text{ pA pF}^{-1}$), inward rectifier K+ current densities (sham $-13.7 \pm 0.6 \text{ pA pF}^{-1}$ versus post-MI $-10.3 \pm 0.9 \text{ pA pF}^{-1}$) and resting membrane potentials (sham $-84.4\pm1.3 \text{ mV}$ versus post-MI $-74.1 \pm 2.6 \text{ mV}$). Depressed $I_{to}$ amplitude correlated with significant reductions in Kv4.2 and Kv4.3 mRNA and Kv4.2 protein levels. Kv1.4 mRNA and protein were increased which coincided with the appearance of a slow component of recovery from inactivation for $I_{to}$.

3) In current clamp recordings, post-MI myocytes showed significant elevations of $[\text{Ca}^{2+}]$, transient amplitudes compared to sham myocytes. Using voltage-clamp depolarizations, no intrinsic differences in Ca$^{2+}$ handling by the sarcoplasmic reticulum or in L-type Ca$^{2+}$ channel density ($I_{Ca,L}$) were detected between the groups.

4) Stimulation of post-MI myocytes with an action potential derived from a sham myocyte reduced the $[\text{Ca}^{2+}]$ transient amplitude to the sham level and visa versa.

5) The net Ca$^{2+}$ influx per beat via $I_{Ca,L}$ was increased about 2-fold in myocyte stimulated with post-MI action potentials compared to sham action potentials.
6) Our findings demonstrate that reductions in $K^+$ channel expression in post-MI myocytes prolong action potential duration resulting in elevated $Ca^{2+}$ influx and $[Ca^{2+}]_i$ transients.

**INTRODUCTION**

Ischemic heart disease as a result of myocardial infarction (MI) is the most prominent etiology in the majority of patients who develop congestive heart failure (Teerlink et al., 1991). The loss of myocytes following MI causes a drop in cardiac output which in turn leads to activation of neurohumoral systems which initially acts to compensates for the loss of myocardial mass, but in the long-term can become cardiomyotoxic leading to the progression of the disease (Rouleau, 1996). During the period of compensation, hearts generally undergo hypertrophy with significant cellular and molecular remodeling of both the left and right ventricles resulting in functional and biochemical alterations of the myocardium (Beuckelmann et al., 1991, 1992, 1993; Beuckelmann et al., 1995; Bailly et al., 1997).

One common animal paradigm used to study MI is the rat coronary artery ligation model. It has been shown that left ventricular myocardial infarction in rats is associated with an elevated right ventricular systolic pressure and hypertrophy of the free wall of the right ventricle (Pfeffer et al., 1979). Most previous studies in heart disease following MI have focused on remodeling changes in the left ventricle and septum (Orenstein et al., 1995; Anand et al., 1997). However, considerable remodeling and hypertrophy also occurs in the right ventricle (Sethi et al., 1997) with changes in the contractile properties (De Tombe et al., 1996) and SR $Ca^{2+}$-ATPase reported (Afzal & Dhall, 1992). Alterations in the right ventricle following left ventricular infarction are not unexpected.
since neurohumoral activation produces circulating factors that will affect both ventricles (Afzal and Dhall, 1992; Sethi et al., 1997). Furthermore, impairment of left ventricular output will affect right ventricular load by increasing its afterload via elevations in pulmonary arterial pressure (Pfeffer et al., 1979).

Prolongation of action potential durations have also been reported in rat left ventricular myocardium following infarction (Qin et al., 1996; Rozanski et al., 1998), as observed in patients with cardiomyopathies (Bailly et al., 1997) and terminal heart failure (Beuckelmann et al., 1993). The cause of action potential prolongation has been correlated with a decrease in repolarizing K⁺ currents in human (Beuckelmann et al., 1993) and animal models of heart disease (Qin et al., 1996; Rozanski et al., 1998). While the precise electrical alterations are likely to vary with the stage of the disease (Cerbai et al., 1994; Rozanski et al., 1998), it has been suggested that action potential prolongation can alter electrical heterogeneity (Gomez et al., 1997) and promote various types of arrhythmias leading to sudden death (Tomaselli et al., 1994). Another possible consequence of action potential prolongation is increased [Ca²⁺] transients amplitude (Brooksby et al., 1993; Bouchard et al., 1995; Wickenden et al., 1998) which, while expected to offset the reduced eject properties of the compromised infarcted heart, can lead to delayed after-depolarization arrhythmias (Tomaselli et al., 1994). With the level of expression of various K⁺ channels (Brahmajothi et al., 1996) and the magnitude of K⁺ currents (Clark et al., 1993) varying regionally, it is conceivable that distinct changes in right and left ventricle and Ca²⁺ handling properties also occur.

As in human heart failure, progressive changes in contractile function in isolated muscle preparations (De Tombe et al., 1996) have been observed in the remaining non-
infarcted myocardium in the rat coronary artery ligation model. Associated with these contractile alterations, changes in \([Ca^{2+}]_i\) transient amplitudes in isolated left ventricular myocytes have also been reported following MI (Cheung et al., 1994; Zhang et al., 1995). However, the \(Ca^{2+}\) uptake rates by the sarcoplasmic reticulum (SR) \(Ca^{2+}\)-ATPase were found to be depressed in the viable left ventricle while being enhanced or unchanged in the right ventricle (Afzal and Dhall, 1992) suggesting that regional differences might explain discrepancies observed in \(Ca^{2+}\) handling following infarction.

Accordingly, the aim of the present study was to study the electrical changes in rat right ventricular myocytes derived from hearts 8 weeks following infarction. Furthermore, our studies were designed to identify the molecular basis for these changes and to elucidate the consequences of these changes on \([Ca^{2+}]_i\). A preliminary account of some of the findings has appeared (Kaprielian, Kassiri, Tsopor, Dawood, Liu, Parker & Backx, 1996)

Data analysis

All data are presented as mean ± S.E.M. In brackets \((n=*/*)\) indicates number of cells/number of preparations. Comparisons of all recordings in cells from sham and post-MI hearts were performed using a two-tailed unpaired Student’s \(t\) test or 2 way analysis of variance (ANOVA), whenever necessary. Steady state activation \((g)\) and inactivation \((h_{\infty})\) curves were fit to the following Boltzmann functions:

\[
g = \frac{1}{1+\exp[(-V-V_{1/2})/k]} \]

\[
h_{\infty} = \frac{1}{1+\exp[V-V_{1/2}/k]} \]
where \( V \) is the step or conditioning potential, \( V_{1/2} \) is the midpoint of the function and \( k \) is the slope factor. Mono-exponential or bi-exponential functions were used to fit recovery from inactivation data. For mono-exponential fits: 

\[
\frac{I}{I_0} = 100 - (A_{\text{fast}} \cdot \exp(-x/\tau_{\text{fast}}))
\]

For bi-exponential fits: 

\[
\frac{I}{I_0} = 100 - (A_{\text{fast}} \cdot \exp(-x/\tau_{\text{fast}})) + (100-A_{\text{fast}}) \cdot \exp(-x/\tau_{\text{slow}})
\]

where \( A_{\text{fast}} \) and \( A_{\text{slow}} \) are the amplitudes of the fast and slow components for recovery and ‘\( t \)’ is the time spent at the recovery potential. We calculated the Chi\(^2\) value to determine whether bi-exponential fits to the data gave significantly superior fits compared to mono-exponential fits. An experimental alpha level of \( p<0.05 \) was considered statistically significant.

**METHODS**

Please refer to section 2.1, 2.4, 2.8, 2.9, 2.10, 2.11

**RESULTS**

Following left coronary artery ligation, the normal myocardium of the left ventricular free wall was replaced to varying degrees by connective tissue. The mean infarct sizes were 51.4 ± 2.4 % (range 39.4 - 64.0 %) in the epicardium and 37.9 ± 2.9 % (range 27.6 - 48.9 %) in the endocardium (n=18). Six rats in the post-MI group died shortly after surgery, while another five developed a small infarction (<20%) and excluded from the study based on previously defined criteria (Orenstein et al., 1995; Pfeffer et al., 1979). Table 1 summarizes some of the observed differences between the sham and post-MI hearts measured 8 weeks following surgery. Compared to sham, post-MI hearts showed a 2-fold increase in the right ventricle-to-body weight ratio, pulmonary congestion measured using
the lung wet-to-dry weight ratio and a significant increase in membrane capacitance (215 ± 12 pF versus 123 ± 4 pF, p<0.05).

**Membrane potential changes following infarction**

Initially we sought to examine the electrical changes in right ventricular myocytes. Right ventricular myocytes derived from sham-operated hearts will be referred to as “sham myocytes” whereas those derived from infarcted hearts will be referred to as “post-MI myocytes”. Figure 3.1 shows that action potentials were significantly prolonged in post-MI myocytes compared to sham myocytes when evaluated at either 50% repolarization (sham: 4.8 ± 0.7 ms (n=29/10) versus post-MI: 13.5 ± 3.6 ms (n=13/6) p<0.01) or 90% repolarization (sham: 29.3 ± 3.4 ms (n=29/10) versus post-MI: 75.8 ± 16.3 ms (n=13/6) p<0.01). In addition to alterations in action potential profile, the resting membrane potential in post-MI myocytes was significantly (p<0.001) depolarized by ~10 mV compared to sham with no effect on the peak of the action potential (Table 1).

**Inward rectifier currents and channels**

Depolarized resting membrane potentials in post-MI myocytes compared to sham myocytes suggests possible changes in inward rectifier (I_{K1}) currents. Figure 3.2A illustrates representative barium subtracted I_{K1} traces recorded during 500 ms step depolarizations from −130 mV to −10 mV from a holding potential of −80 mV from a typical sham (left panel) and post-MI (right panel) myocyte. Current traces have been normalized for cellular capacitance. Figure 3.2B plots the current-voltage (I-V) relationships demonstrating the barium-subtracted I_{K1} densities were significantly reduced (p<0.05) in post-MI myocytes.
compared to sham myocytes at all voltages below and including -100 mV. At voltages above -90 mV, I_{K1} was reduced in post-MI myocytes, but the reduction was not significant, probably because of its small magnitude. Despite differences in I_{K1} current, Figure 3.2C shows that mRNA expression levels of IRK1 and IRK2 which are thought to encode the cardiac I_{K1} (Kubo et al., 1993) were not significantly altered.

**Transient outward current (I_{to}) and sustained current (I_{sus})**

The voltage-dependent transient outward current, I_{to} is a major K\(^+\) current in rat contributing to the rapid membrane repolarization (Apkon and Nerbonne, 1991). Figure 3.3A shows representative I_{to} traces in a sham and a post-MI myocyte following 500 ms step depolarizations to a range of voltages from -30 mV to +70 mV while holding at -80 mV. In order to isolate K\(^+\) currents, sodium currents were inactivated by 70 ms prepulses to -40 mV while I_{Ca,L} was blocked by adding 0.3 mM CdCl\(_2\) extracellularly. I_{to} was defined as the difference between the peak outward current and the sustained component remaining at the end of the depolarizing pulse (I_{to} = I_{peak} - I_{sus}) (Apkon & Nerbonne, 1991). The mean current-voltage relationship for I_{to} is illustrated in figure 3.3B. I_{to} densities were reduced at +60 mV from 19.7 ± 1.1 pA pF\(^{-1}\) (n = 42/10) for sham cells compared to 11.0 ± 1.3 pA pF\(^{-1}\) (n = 21/6) for post MI myocytes.

In contrast to the changes in I_{to}, the current remaining at the end of a 500 ms depolarizing pulse (I_{sus}) was not statistically different (p=0.09) between groups as summarized in figure 3.3C. I_{sus} density measured at +60 mV was 8.1±0.5 pA pF\(^{-1}\) (n = 42/10) and 6.7±0.6 pA pF\(^{-1}\) (n = 21/6) in sham and post-MI myocytes, respectively. The molecular correlate of I_{sus} remains uncertain, but three K\(^+\) channel genes (Kv1.2, Kv1.5 and
Kv2.1) known to encode for delayed rectifier-type currents have been studied previously. Expression at the transcriptional level of Kv1.2, Kv1.5 and Kv2.1 were not different between sham and post-MI right ventricles (data not shown). RNase protection assays for Kv1.2, Kv1.5 and Kv2.1 was reduced by 16.0±9.2% (n=7/7), 20.1±8.9% (n=7/7) and 20.4±10% (n=7/7) in the post-MI group relative to the sham group (p>0.05).

Since cardiac hypertrophy and disease has been previously shown to switch gene expression from an adult to a fetal-like program with respect to contractile proteins (Orenstein et al., 1995). We investigated whether the I_o in post-MI myocytes showed features resembling those observed in neonatal myocytes. Previous studies have demonstrated that I_o in neonatal myocytes have a sizeable component that recovers slowly from inactivation (Kilborn and Fedida, 1991; Wickenden et al., 1997). Figure 4A shows typical recordings in a sham and a post-MI myocyte using a double pulse protocol (from –80 mV to +60 mV) designed to measure the rate at which I_o channels recover from inactivated to closed conformations. Figure 3.4B graphically depicts the mean recovery data for all the sham and post-MI myocytes. For each myocyte, the time course of recovery was fit using mono and bi-exponential function (see methods). For sham myocytes (n=36/10), \( \tau_{fast} \) was 30.1±1.7 ms and accounted for 99.7±0.2 % of the current. For post-MI myocytes (n=16/8), \( \tau_{fast} \) was 35.7±4.5 ms and accounted for 89.4±2.2% of the current while \( \tau_{slow} \) was 1686.0±265.3 ms and accounted for 10.6±2.2 % of the current. In spite of the changes in the recovery from inactivation, steady-state activation and inactivation relationships between the groups were not altered significantly (p > 0.2). The mid-points for steady-state activation (i.e. \( V_{1/2} \)) were 17.1±0.8 mV (n=42/10) and 16.3±1.5 mV (n=21/6) while the slopes for activation were 16.7±1.3 and 16.3±1.3 in sham and post-MI myocytes, respectively. The
mid-points for \( I_{\text{to}} \) inactivation were \(-31.4\pm0.7 \text{ mV (n=6/3)} \) and \(-33.5\pm1.3 \text{ mV (n=6/3)} \) with the slopes measuring \(3.3\pm0.1 \) and \(3.4\pm0.2 \) in sham and post-MI myocytes, respectively.

The changes in \( I_{\text{to}} \) were further investigated by measuring mRNA levels of three \( K^+ \) channel genes Kv4.2, Kv4.3 and Kv1.4 that are known to encode for \( I_{\text{to}} \)-like currents in the rat ventricle (Dixon and McKinnon, 1994; Dixon et al., 1996). Figure 3.5A shows representative gels of RNase protection assays along with cyclophilin which was used as the internal standard to account for variable loading and possible RNA degradation between experiments (Gidh-Jain et al., 1996). Figure 3.5A further demonstrates a reduction of Kv4.2 by \(30.3\pm10\% \) \((n=7/7)\), and of Kv4.3 by \(20.4\pm5.1\% \) \((n=7/7)\), while Kv1.4 mRNA levels were increased by \(23.4\pm3.2\% \) \((n=7/7)\) in post-MI hearts compared to sham \((p<0.05)\). The pattern of mRNA expression for post-MI hearts is similar to that observed in 1-2 day-old neonatal hearts (Wickenden et al., 1997).

To determine whether the changes in mRNA level were accompanied by changes at the protein level, we performed Western blot analysis. Figure 3.5B shows representative gels of Western blots for Kv4.2 and Kv1.4 from post-MI \((n=6/6)\) and sham \((n=6/6)\) controls. Westerns for Kv4.3 channels were not performed since no antibody currently exists for this channel. Densitometric measurements of Kv4.2 immunoreactive protein for post-MI membrane preparations revealed a \(46.0\pm8.5\% \) decrease relative to sham \((p<0.05)\). In contrast, Kv1.4 protein levels were increased by \(94.6\pm42.8\% \) in the post-MI group relative to the sham group \((p=0.05)\). The decrease of Kv4.2 correlated with the large decrease in \( I_{\text{to}} \) density (Figure 3.3B). While the increase of Kv1.4 was correlated with the appearance of a slow component of recovery from inactivation in post-MI myocytes.
Action potential duration and $[\text{Ca}^{2+}]_i$

Action potential prolongation have previously been associated with increased incidences of arrhythmias in rats following MI (Qin et al., 1996). In principle, prolongation of the action potential duration is also expected to elevate $[\text{Ca}^{2+}]_i$ transients in the myocyte by increasing $\text{Ca}^{2+}$ entry via the L-type calcium current (Bouchard et al., 1995). In the absence of 5 mM EGTA and in the presence of $[\text{Ca}^{2+}]_i$ transients, action potentials were also prolonged at 50% (sham: $17.6\pm2.8\ ms, n=13/5$ versus post-MI: $34.9\pm4.0\ ms, n=14/5$ p<0.01) and 90% repolarization (sham: $92.3\pm15.4\ ms, n=13/5$ versus post-MI: $633.9\pm130.1\ ms, n=14/5$, p<0.01) (Figure 3.6). The action potential duration in these experiments differed from those illustrated in Figure 1 because of the contribution of the sodium calcium exchange current ($I_{\text{Na}^+/\text{Ca}^{2+}}$) when $[\text{Ca}^{2+}]_i$ transients are present. Mean systolic $[\text{Ca}^{2+}]_i$ were significantly (p<0.001) elevated in post-MI myocytes (1085.3±112.3 nM, n= 13/7) compared to sham myocytes (401.1±115.0 nM, n=12/6) while diastolic $[\text{Ca}^{2+}]_i$ were not significantly (p=0.11) different between the groups (sham: 78.4±22.6 nM versus post-MI: 94.6±5.5 nM).

To assess whether changes in action potential profile were responsible for the elevated $[\text{Ca}^{2+}]_i$ transients, we performed action potential voltage-clamp experiments. Figure 3.7A shows a typical experiment where a sham myocyte was stimulated with its own action potential and a typical representative action potential recorded from a post-MI myocyte while figure 3.7B shows the converse experiment in a post-MI myocyte. Peak systolic $[\text{Ca}^{2+}]_i$ were significantly elevated when sham myocytes were stimulated with a post-MI action potential compared to its own intrinsic action potential ($V_{C_{\text{Sham}}}=283.3\pm44.1\ nM$ versus $V_{C_{\text{Post-MI}}}=813.3\pm18.6\ nM$, n=3/3, p<0.05) but diastolic $[\text{Ca}^{2+}]_i$ remained unchanged.
Similar experiments in post-MI myocytes revealed the opposite pattern. Systolic $[\text{Ca}^{2+}]_i$ was reduced when post-MI myocytes were stimulated with a sham action potential ($V_{C_{\text{Post-MI}}}=90.0.3\pm9.0\text{ nM, }n=3/3$, $p=0.33$). Again, there was no detectable change in diastolic $[\text{Ca}^{2+}]_i$ ($V_{C_{\text{Post-MI}}}=982.1\pm184.1\text{ nM versus }V_{C_{\text{Sham}}}=333.3\pm159.2\text{ nM, }n=3/3$, $p=0.05$). These results demonstrate that $[\text{Ca}^{2+}]_i$ recorded in sham myocytes with post-MI action potential command waveform were not different compared to post-MI myocytes stimulated with their intrinsic action potential.

The results above suggest that elevated $[\text{Ca}^{2+}]_i$ transients primarily arise from action potential prolongation. To assess this further, we measured $[\text{Ca}^{2+}]_i$ transients in the two groups under voltage-clamp conditions following a brief 100 ms depolarization steps to +10 mV from a holding potential of −80 mV applied at 0.2 Hz. Figure 3.8A shows representative $[\text{Ca}^{2+}]_i$ recordings in a sham and a post-MI myocyte with the summarized data in Figure 3.8B. The difference in the peak systolic $[\text{Ca}^{2+}]_i$ between sham and post-MI myocytes disappeared when myocytes were stimulated with identical square voltage waveforms (sham: $648.9\pm143.3\text{ nM, }n=10/6$ versus post-MI: $736.8\pm100.8\text{ nM }n=10/4$, $p>0.2$). The diastolic $[\text{Ca}^{2+}]_i$ were also unchanged between sham (91.7±3.0 nM) and post-MI (84.7±3.2 nM) myocytes ($p=0.14$). Furthermore, under our recording conditions, there was no apparent difference in the rate of relaxation of the $[\text{Ca}^{2+}]_i$ transient between the two groups (data not shown). These results establish that reductions in $I_{\text{lo}}$ density are associated with action potential prolongation which is directly responsible for elevated $[\text{Ca}^{2+}]_i$ transients in post-MI myocytes.
Depolarizing currents

The changes in APD observed following MI might also originate from an increase in L-type Ca$^{2+}$ currents ($I_{Ca,L}$) as observed in other models of heart disease (Keung 1989; Ryder et al., 1993). Figure 3.9A shows representative cadmium-subtracted $I_{Ca,L}$ traces recorded from a sham and a post-MI myocyte in response to 500 ms step depolarizations from $-60$ mV to $+70$ mV while holding at $-80$ mV. On average, Figure 3.9B establishes that a small, but non-significant, reduction in $I_{Ca,L}$ density ($p > 0.05$ at $+10$ mV) occurred in post-MI myocytes which goes in the wrong direction to contribute to action potential prolongation.

The results above demonstrate that $I_{Ca,L}$ density is unchanged in post-MI myocytes. However, increases in the net influx of Ca$^{2+}$ as a result of action potential prolongation could occur and thereby contribute to the observed elevations in [Ca$^{2+}$]; transient amplitude. Therefore, we examined $I_{Ca,L}$ in action potential voltage-clamp in conditions that eliminated Na$^+$, K$^+$ and Na$^+/Ca^{2+}$ exchange currents. Figure 3.10A shows typical cadmium-subtracted Ca$^{2+}$ currents recorded at steady-state in a sham myocyte stimulated with an action potential waveform derived from either sham (left panel) and post-MI (right panel) myocytes. The waveforms were applied randomly on the same cell followed by cadmium wash to block $I_{Ca,L}$. The post-MI waveform caused a decrease in the peak $I_{Ca,L}$ with a marked slowing of its decline (inactivation). The integral of $I_{Ca,L}$ which provides a direct measure of calcium entry as a function of time (Bouchard et al., 1995) was increased almost 2-fold in sham myocytes given a post-MI action potential compared to sham action potentials. Table 2 summarizes data for the steady-state Ca$^{2+}$ currents obtained from 7 sham myocytes and establishes that action potential prolongation in post-MI myocytes causes a large increase in Ca$^{2+}$ entry per beat.

95
Figure 3.10B shows a plot of $I_{Ca,L}$ as a function of voltage during the course of an action potential. The trajectory of the $I_{Ca,L}$-voltage relationship when a post-MI action potential is applied follows very closely the relationship shown in Figure 3.10B following step depolarizations with the peak occurring at about +10 mV. In contrast, the $I_{Ca,L}$-voltage relationship following application of the sham action potential is shifted leftward relative to the post-MI action potential with a peak at about −25 mV. Moreover, a substantial amount of current is observed at voltages below −30 mV which is well below the threshold for L-type Ca$^{2+}$ channel activation. These results strongly suggest that the rate of membrane depolarization in the sham myocyte occurs more rapidly than the rate of channel deactivation similar to that observed in tail current measurements (McDonald, Pelzer, Trautwein & Pelzer, 1994). These results suggest that the kinetic nature of Ca$^{2+}$ entry through the L-type Ca$^{2+}$ channels is distinctly changed by action potential prolongation as seen in post-MI myocytes.

**Sarcoplasmic reticulum Ca$^{2+}$ content**

While prolonged action potentials clearly elevate [Ca$^{2+}$]$_i$ and increase calcium entry via $I_{Ca,L}$, there could also be changes in the Ca$^{2+}$ handling by the sarcoplasmic reticulum in post-MI myocytes. In order to test for this, we measured SR Ca$^{2+}$ content by applying identical step depolarizations (100 ms) under voltage-clamp conditions and integrating Na$^+$/Ca$^{2+}$ tail currents following application of 10 mM caffeine. Figure 3.11A shows the inward currents recorded in response to application of 10 mM caffeine in a sham and a post-MI myocyte with the mean data shown in Figure 3.11B. SR Ca$^{2+}$ content in sham and post-MI myocytes
were 97.8±4.9 μM, (n=9/3) and 96.8±19.2 μM (n=6/3), respectively (p>0.05), establishing that no differences in SR Ca²⁺ handling exist between the two groups.

**DISCUSSION**

This study was designed to investigate the ionic and molecular basis of action potential prolongation in cardiac hypertrophy and to test if these electrical changes could be linked to alterations in Ca²⁺ handling. Our studies were performed in right ventricular myocardium derived from hearts 8 weeks following left anterior descending coronary artery ligation. As reported previously, both the right and left ventricles of hearts with left-sided infarctions undergo significant compensatory cardiac hypertrophy (Afzal & Dhalla, 1992). Numerous studies have previously documented the pathological, functional, electrical and ionic changes in the left ventricle (Orenstein et al., 1995; Zhang et al., 1995; Kääb et al., 1996; Qin et al., 1996; Rozanski et al., 1998). Few studies have examined changes in the right ventricle following coronary artery ligation which also undergoes significant hypertrophy and remodeling when the infarct size is greater than 35% of the left ventricular free wall (Table 1). Moreover, functional, biochemical and genetic changes have previously been reported in the right ventricle, which are distinct from changes in the left ventricle of left-infarcted rats hearts (Afzal and Dhalla, 1992; De Tombe et al., 1996; Sethi et al., 1997). In addition, the severity of changes in the right ventricle are strongly correlated with mortality in human patients (Yu et al., 1996).

**Ionic and molecular mechanisms of action potential duration prolongation**

Our data reveal that action potential prolongation occurs in right ventricular myocytes 8 weeks following infarction. The action potentials were significantly prolonged whether in the presence of EGTA (5 mM) in the pipette to minimize the Na⁺/Ca²⁺
exchange activity and under conditions where the Na\(^+\)/Ca\(^{2+}\) exchanger is fully operational in the presence of [Ca\(^{2+}\)]\(_i\) transients. These results establish that K\(^+\) channel down-regulation is the primary determinant of action potential prolongation.

Underlying these APD changes in post-MI myocytes, I\(_{\text{to}}\) density was reduced which correlated with reductions in Kv4.2 mRNA and protein levels and Kv4.3 mRNA levels; two genes known to encode for transient outward currents. Reductions in I\(_{\text{to}}\) density and Kv4.2 message and protein was also observed in rat left ventricle 3-4 weeks following infarction (Qin et al., 1996). Interestingly, the level of Kv1.4 message was increased despite reductions in I\(_{\text{to}}\). These results are similar to changes of left ventricular Kv1.4 mRNA expression in spontaneously hypertensive rats (Matsubara et al., 1993), but differ from the nearly 2-fold reduction in Kv1.4 message observed in the left ventricle 3-4 weeks following infarction (Gidh-Jain et al., 1996). These results may indicate temporal or regional differences in the regulation of K\(^+\) channel expression in diseased hearts. The increase in Kv1.4 message and protein coincided with the emergence of a slow component of I\(_{\text{to}}\) recovery kinetics similar to that reported in end-stage human heart failure (Näbauer et al., 1993). The time constant for the slow component is very similar to that recorded previously in *xenopus* oocytes expressing Kv1.4 channels (Tseng-Crenk et al., 1990).

The prolonged action potential duration and slowed recovery kinetics of I\(_{\text{to}}\) following infarction coincides with that reported in neonatal myocytes (Kilborn and Fedida, 1991; Wickenden et al., 1997). Moreover, the finding that Kv4.2 and Kv4.3 mRNA levels decreased while Kv1.4 increased, is consistent with the re-expression of a fetal electrophysiological phenotype in rat heart, since the reverse changes are known to occur with post-natal development in the rat heart (Kilborn and Fedida, 1991; Wickenden et al.,
1997). These results show that switching to a "fetal gene program" also occurs in the electrophysiological phenotype and K⁺ channel expression as observed for other cardiac genes in diseased hearts (Schwartz et al., 1986). The significance of the re-expression of an early phenotype is unclear. However, as is the case for other aspects of re-expression of the fetal phenotype (Orenstein et al., 1995), these changes might reflect an attempt to maintain contractile performance and hypertrophy (Wickenden et al., 1998).

I₄₆ was not significantly reduced in post-MI myocytes compared to sham myocytes. Expression at the transcriptional level of Kv2.1, Kv1.5 and Kv1.2 mRNA levels were slightly decreased (p>0.05) in post-MI myocytes. These three channel genes encode for currents with delayed rectifier properties (Swanson et al., 1990), but their functional correlation in the rat heart remains uncertain. These changes are similar to those observed in the left ventricle 3-4 weeks following MI.

While reductions in Iₒ density explain, in large part, the prolongation of the action potential duration, reductions in I_K₁ density were also observed. Since, this current contributes to the late phase of repolarization, reductions in I_K₁ could further exacerbate action potential prolongation. Indeed, the degree of prolongation at 90% repolarization exceeded from that observed at 50% repolarization. In addition, the decrease in I_K₁ might also explain the higher resting membrane potentials observed in post-MI myocytes compared to sham hearts although differences in other background currents cannot be ruled out. For example, a recent report in rat ventricular myocytes has demonstrated an increase in a volume-regulated chloride conductance in response to left ventricular hypertrophy induced by aortic banding (Benitah et al., 1997). Our results are consistent with previous studies showing depolarization of the resting membrane potential (Bouron...
et al., 1992) and reductions in $I_{K1}$ density in diseased hearts (Beuckelmann et al., 1993; Kääb et al., 1996). Despite the decrease in the density of $I_{K1}$, expression of $IRK1$ and $IRK2$ mRNA was unaltered. These findings raise the possibility that $I_{K1}$ expression is downregulated at the post-transcriptional or post-translational level during the development of cardiac hypertrophy.

The role of action potential duration prolongation on $[Ca^{2+}]_i$ transients.

Previous studies of cardiac disease have demonstrated increases (Brooksby et al., 1993), decreases (Beuckelmann et al., 1992; Cheung et al., 1994) or no change (Cheung et al., 1994) in the $[Ca^{2+}]_i$ transient amplitude. In general, the direction of $[Ca^{2+}]_i$ transient changes depends on the severity and stage of the disease (Wickenden et al., 1998). Our current-clamp recordings in right ventricular myocytes establish that infarction is associated with a prolonged action potential duration and elevations in peak systolic $[Ca^{2+}]_i$ without changes in the diastolic $[Ca^{2+}]_i$ between sham and post-MI right ventricular myocytes under our experimental conditions. In action potential voltage-clamp experiments, the mean peak $[Ca^{2+}]_i$ transient amplitude was increased by 3-fold when sham myocytes were stimulated with an AP waveform derived from a post-MI rat as compared to stimulation with its own action potential. Conversely, peak $[Ca^{2+}]_i$ transients were reduced when post-MI myocytes were stimulated with sham action potentials compared to its intrinsic long action potential. Importantly, the amplitude of the $[Ca^{2+}]_i$ transients observed in sham myocytes were identical to that observed in the post-MI myocytes stimulated with sham action potentials. On the other hand, $[Ca^{2+}]_i$ transient amplitude in post-MI myocytes were identical to those observed in sham myocytes stimulated with post-MI action potentials. Differences in peak systolic $[Ca^{2+}]_i$ were abolished when identical voltage-clamp pulses were delivered to sham
and post-MI myocytes, as observed previously in the spontaneously hypertensive rat (Brooksby et al., 1993). These results establish that prolongation of action potential duration is directly responsible for the elevations in $[\text{Ca}^{2+}]_i$ transient amplitudes observed in the post-MI myocytes. Consistent with this conclusion, the sarcoplasmic reticulum (SR) calcium content was unchanged between the two groups when estimated using $I_{\text{Na}^+}/\text{Ca}^{2+}$ recordings following application of caffeine. The absence of differences in SR $\text{Ca}^{2+}$ content was also previously observed in rats subjected to aortic banding (McCall et al., 1998) and are consistent with previous SR vesicle studies in the rat infarction model (Afzal and Dhalla, 1992). Our results suggest that early in cardiac disease and hypertrophy, action potential duration is prolonged and $[\text{Ca}^{2+}]_i$ transient amplitude are elevated. In more advanced stages of heart disease and failure, there is consistently a decrease in $[\text{Ca}^{2+}]_i$ transient magnitude in spite of marked action potential prolongation which is associated with a severe reduction in sarcoplasmic reticular $\text{Ca}^{2+}$-ATPase and/or $\text{Na}^+/\text{Ca}^{2+}$ exchange activity (Beuckelmann et al., 1995). Thus it appears that the changes in systolic $[\text{Ca}^{2+}]_i$ in the right ventricle in our model are more representative of the early events occurring during compensated cardiac hypertrophy. The finding that intracellular calcium is elevated has a number of important implications for the pathogenesis and progression of heart failure. The increase in intracellular calcium may initially serve as a compensatory mechanism to increase myocardial contractility of the compromised heart. However, chronic elevations in $[\text{Ca}^{2+}]_i$ transient amplitude could also contribute to cardiac growth and disease progression by modulating gene expression, since $\text{Ca}^{2+}$ is an important cofactor for growth factor signal transduction (Molkentin et al., 1998; Wickenden et al., 1998).
Calcium influx through L-type calcium channels

Unlike $I_{o}$ and $I_{K1}$, L-type Ca$^{2+}$ current ($I_{Ca,L}$) density were not altered in post-MI myocytes compared to sham controls (Figure 3.9). These results are consistent with earlier electrophysiological recordings in left ventricle of rat hearts following infarction (Qin et al., 1996), of dog hearts following pacing-induced heart failure (Kääb et al., 1996) and in human heart failure (Beuckelmann et al., 1991). Action potential voltage-clamp recordings did, however, reveal an increase in calcium entry through the L-type Ca$^{2+}$ channels when sham myocytes are stimulated with post-MI action potentials as opposed to sham action potentials (Table 2). This net increase in Ca$^{2+}$ entry in each cardiac cycle are likely to contribute significantly to the increase in [Ca$^{2+}$]i observed in the post-MI myocytes. Our results establish for the first time that while channel density may be unaffected in cardiac hypertrophy, calcium entry through the channel can increase secondarily through the modulation of hyperpolarizing K$^+$ channels and the subsequent action potential prolongation.

Interestingly, short action potentials evoked a peak $I_{Ca,L}$ which were 2-fold larger with a much faster decline than with prolonged action potentials. This was found to result from a lack of equilibration between channel deactivation and voltage during the rapid repolarization in sham action potentials causing a large increase in driving force for Ca$^{2+}$ entry similar to that seen in typical tail experiments (McDonald et al., 1994). These kinetic differences in the nature of Ca$^{2+}$ entry through the L-type Ca$^{2+}$ channels in sham compared to post-MI myocytes may have important implications on the nature of excitation-contraction coupling in cardiac hypertrophy.
In Summary, we report that the right ventricle undergoes hypertrophy, $I_{\text{to}}$ down-regulation and action potential prolongation similar to earlier findings in the left ventricle (Qin et al., 1996). We also found a marked slowing of the kinetics of $I_{\text{to}}$ channels which was correlated with elevation in Kv1.4 message and protein levels. Action potential prolongation following infarction is tightly connected to elevations in peak $[\text{Ca}^{2+}]_i$ via effects on $I_{\text{Ca-L}}$. Reduction of $K^+$ channel expression while arrhythmogenic, also elevates $[\text{Ca}^{2+}]_i$, which may be important regulators of cardiac hypertrophy and disease progression.
TABLE 1. Changes associated with myocardial infarction.

BW indicates body weight; WH/BW indicates whole heart weight/body weight; RVW/BW indicates right ventricular weight/body weight; Lung W/D indicates lung wet-to-dry weight ratio; RMP indicates resting membrane potential; Vpeak indicates the peak of the action potential. * indicates p<0.05 between sham and post-MI groups.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Post-MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>376.2±13.7 (25)</td>
<td>394.1±10.8 (20)</td>
</tr>
<tr>
<td>Lung W/D, g/g</td>
<td>3.8±0.7 (25)</td>
<td>4.0±0.4 (20)</td>
</tr>
<tr>
<td>WH/BW, %</td>
<td>0.27±0.01 (25)</td>
<td>0.37±0.03 (20)*</td>
</tr>
<tr>
<td>RVW/BW, %</td>
<td>0.036±0.002 (25)</td>
<td>0.070±0.010 (20)*</td>
</tr>
<tr>
<td>Capacitance, pF</td>
<td>123±4 (58/13)</td>
<td>215±12 (43/10)*</td>
</tr>
<tr>
<td>RMP, mV</td>
<td>-84.4±1.3 (29/10)</td>
<td>-74.1±2.6 (13/8)*</td>
</tr>
<tr>
<td>Vpeak, mV</td>
<td>52.4±3.8 (29/10)</td>
<td>47.6.5±9.4 (13/8 )</td>
</tr>
</tbody>
</table>

TABLE 2. Effect of action potential duration on the L-type calcium current.

Sham myocytes (n=7/7) stimulated with either a sham action potential or a post-MI action potential at 0.25 Hz. VC indicates action potential voltage-clamp command waveform. ICa,L indicates peak Ca^{2+} difference current derived by cadmium subtraction (0.3 mM); QCa indicates the charge movement derived by area integration under ICa,L; t1/2 indicates half-time of current relaxation to baseline. * indicates p<0.05 between sham action potential and post-MI action potential command waveforms.

<table>
<thead>
<tr>
<th></th>
<th>ICa,L, nA</th>
<th>QCa, pC</th>
<th>t1/2, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham AP</td>
<td>1.0±0.1</td>
<td>12.4±1.7</td>
<td>7.5±0.9</td>
</tr>
<tr>
<td>Post-MI AP</td>
<td>0.5±0.07*</td>
<td>22.4±4.7*</td>
<td>29.6±0.9*</td>
</tr>
</tbody>
</table>
FIGURE 3.1. Action potential characteristics in right ventricular myocytes following myocardial infarction. A shows representative action potential traces from a sham and a post-MI myocyte. Action potentials were elicited by a brief (5 ms) suprathreshold (2 x threshold) applied at 0.2 Hz. Intracellular solutions contained 5 mM EGTA. B shows mean action potential duration (APD) evaluated at 50% and 90% repolarization for sham (n=29/10) and post-MI (n=20/6) myocytes. Action potentials were significantly prolonged in the post-MI myocytes (APD50= 29.3±3.4 ms- APD90=75.8±16.3 ms) compared to sham myocytes (APD50= 4.8±0.7 ms- APD90=13.5±3.6 ms. 100% is complete repolarization to the resting membrane potential. — indicates 0 mV. * indicates p<0.05 for APD50% and APD90% between sham and post-MI myocytes.
**FIGURE 3.2.** The inward rectifier (I\textsubscript{K1}) in right ventricular myocytes following myocardial infarction. A shows normalized traces of the inward rectifier current (I\textsubscript{K1}) in a sham and a post-MI myocyte elicited by 500 ms voltage steps over the range -130 to -10 mV in +10 mV increments from a holding potential of -80 mV. CdCl\textsubscript{2} (0.3 mM) was added to avoid contamination by the calcium current. B shows I\textsubscript{K1} current density which was normalized to membrane capacitance and plotted against the test potential for sham (n=29/10) and post-MI (n=13/6) myocytes. The steady state current measured at the end of the test pulse in the presence of barium was subtracted from the current evoked at the same voltage step in the absence of barium. Myocytes were depolarized every 5 s. → indicates 0 pA/pF. * indicates p<0.05 between sham and post-MI myocytes at -120 mV. C shows mRNA levels for IRK1 and IRK2 in sham (left panel) and post-MI (right panel) right ventricles were measured using an RNase protection assay (RPA). The top and bottom bands represent IRK and cyclophilin protected mRNA fragments, respectively. The bar graph shows mean changes in IRK mRNA levels in the post-MI (solid bars) relative to sham (open bars).
FIGURE 3.3. The transient outward (I_o) and sustained currents (I_{sust}) in right ventricular myocytes following myocardial infarction. A shows normalized traces of the transient outward current (I_o) and sustained current (I_{sust}) in a sham and a post-MI myocyte elicited by 500 ms voltage steps over the range -30 to +70 mV in +10-mV increments from a holding potential of -80 mV (shown in the inset). I_o (B) and I_{sust} (C) were normalized to membrane capacitance and plotted against the test potential for sham (n=42/10) and post-MI (n=21/6) hearts. Myocytes were depolarized every 5 s. * indicates $p<0.05$ between sham and post-MI myocytes at +60 mV.
FIGURE 3.4. Biophysical properties of $I_{\text{to}}$ in right ventricular myocytes following myocardial infarction.

A shows normalized recovery from inactivation traces in a sham and a post-MI myocyte. B is a plot of recovery kinetics for sham ($n=36$) and post-MI ($n=14$) myocytes using a two pulse protocol (inset to B) with two identical depolarizing pulses from -80 to +60 mV applied every 10 s at selected intervals from 10 to 10 000 ms. The recovery kinetics in the sham myocyte was best described by a single exponential function, whereas the recovery kinetics in post-MI myocytes was best fitted by a double exponential function. $\rightarrow$ indicates 0 pA pF$^{-1}$. 

$\text{Fraction recovered (\%)}$

$\text{Interpulse interval (ms)}$
FIGURE 3.5. Representative comparison of candidate genes encoding the transient outward current in the right ventricle following myocardial infarction. A shows a representative comparison of mRNA levels for Kv4.2, Kv4.3 and Kv1.4 from sham (left panel) and post-MI (right panel) right ventricles. The top and bottom bands represent Kvx and cyclophilin protected mRNA fragments, respectively. The bar graph shows mean changes in Kvx mRNA in post-MI (solid bars, n=7/7) levels relative to sham (open bars n=7/7). B. Western blot analysis showing Kv channel subunit immunoreactive proteins for Kv4.2 and Kv1.4 from sham (left panel) and post-MI (right panel) right ventricles. The bar graph shows mean changes in Kvx mRNA in post-MI (solid bars, n=6/6) levels relative to sham (open bars, n=6/6).
FIGURE 3.6. Effect of action potential prolongation on [Ca^{2+}]_i in right ventricular myocytes. Records show action potential voltage-clamp measurements in a sham and a post-MI myocyte. In A, the left panel shows a sham myocyte stimulated with its action potential. The right panel shows the same myocyte stimulated with an action potential derived from a post-MI myocyte (shown in B, left panel). In B, the left panel shows a post-MI myocyte stimulated with its action potential. The right panel shows the same myocyte stimulated with an action potential derived from a sham myocyte (shown in A, right panel). → indicates 0 nM [Ca^{2+}]_i and — indicates 0 mV.
FIGURE 3.7. Effect of action potential prolongation on [Ca$^{2+}$]$_i$ in right ventricular myocytes. Records show action potential voltage-clamp measurements in a sham and a post-MI myocyte. In A, the left panel shows a sham myocyte stimulated with its action potential. The right panel shows the same myocyte stimulated with an action potential derived from a post-MI myocyte (shown in B, left panel). In B, the left panel shows a post-MI myocyte stimulated with its action potential. The right panel shows the same myocyte stimulated with an action potential derived from a sham myocyte (shown in A, right panel). $\rightarrow$ indicates 0 nM [Ca$^{2+}$]$_i$ and $\leftarrow$ indicates 0 mV.
**FIGURE 3.8.** Effect of short voltage-clamp pulses on the \([Ca^{2+}]_i\) transient in sham and post-MI right ventricular myocytes.

A shows representative \([Ca^{2+}]_i\) transients in a sham and a post-MI myocyte stimulated with short voltage-clamp pulses (100 ms) from a holding potential of -80 mV to +10 mV at 0.25 Hz. B shows mean values for systolic and diastolic \([Ca^{2+}]_i\) in sham (open bar, \(n=10/5\)) and post-MI (solid bar, \(n=10/4\)) myocytes. No significant difference was observed between sham and post-MI myocytes. \(\rightarrow\) indicates 0 nM \([Ca^{2+}]_i\) and \(\longrightarrow\) indicates 0 mV.
FIGURE 3.9. The L-type calcium current ($I_{Ca,L}$) in right ventricular myocytes following myocardial infarction. A shows representative current traces showing cadmium-sensitive difference currents (0.3 mM CdCl$_2$) from a sham and a post-MI myocyte elicited by 500 ms voltage steps to -40, 0, +10 and +30 mV from a holding potential of -80 mV. A prepulse to -40 mV (100 ms) was used to eliminate any contaminating sodium current. The $I_{Ca,L}$ was normalized to membrane capacitance and plotted against the test potential for sham (n=15/4) and post-MI (n=9/3) myocytes (B). Myocytes were depolarized every 5 s. No significant difference was observed between sham and post-MI myocytes. $\rightarrow$ indicates 0 pA pF$^{-1}$. 
FIGURE 3.10. Effect of action potential duration on the L-type calcium current ($I_{Ca, L}$). Records and graph show $I_{Ca, L}$ produced under action potential voltage-clamp condition. A shows a right ventricular sham myocyte stimulated with a representative sham (O) and post-MI (●) action potential with corresponding cadmium-subtracted $I_{Ca, L}$ traces. → indicates 0 pA — indicates 0 mV. B Current-voltage plot shows $I_{Ca, L}$ current during the action potential voltage-clamp for the sham and the post-MI action potential waveform. Arrows indicate increasing time.
FIGURE 3.11. Ca\textsuperscript{2+} content in the sarcoplasmic reticulum in right ventricular myocytes following myocardial infarction. A shows original records representing \( I_{\text{Na+Ca}^{2+}} \) tails following application of 10 mM caffeine in a sham and a post-MI myocyte. All myocytes were held at -70 mV throughout. Broken line marks non-exchange (leakage) current flowing during the sodium-free portion of caffeine application. Bar indicates timing of caffeine application. Sarcoplasmic reticulum calcium content was calculated by integrating the currents and the mean data is shown in panel B. No significant difference in sarcoplasmic reticulum Ca\textsuperscript{2+} content was observed between sham and post-MI myocytes.
CHAPTER 4

REGIONAL DIFFERENCES IN Kv1.4, Kv4.2 AND Kv4.3 TO THE TRANSIENT OUTWARD K⁺ CURRENT IN THE NORMAL AND INFARCTED RAT HEART

ROGER KAPRIELIAN, RAJAN SAH, ALAN D. WICKENDEN AND PETER H. BACKX

To be submitted to: American Journal of Physiology
ABSTRACT

Previous studies reported regional differences in the density of transient outward current (I_{to}) in the normal rat myocardium. In this study, we measured changes in action potentials, density of the transient outward K^+ channel (I_{to}) and [Ca^{2+}]_i transients recorded from rat ventricular myocytes and interventricular septum (S). Results demonstrated the following: Kv4.2 protein levels in sham-operated hearts were 28.4 ± 2.0 % higher (P<0.01) in the RVW compared to the S and correlated with significant differences in I_{to} density (RVW: 14.7±0.8 pA/pF versus S: 7.0±1.1 pA/pF P<0.01). I_{to} density was significantly reduced following MI in right ventricular myocytes (Sham: 14.7±0.8 pA/pF versus Post-MI: 8.6±1.1 pA/pF at +40 mV P < 0.01) with only modest reduction in the septum (Sham: 7.0 ± 1.1 pA/pF versus Post-MI: 5.1 ± 0.6 pA/pF at +40 mV P = 0.15). In keeping with these results, Kv4.2 protein were reduced by 54.3 ± 11.1 % (P<0.01) in the RVW compared to 16.0 ± 7.0 % (P=0.04) in the S. 3. In contrast to Kv4.2, Kv4.3 levels were not significantly different between the RVW and S and did not change following myocardial infarction, suggesting that this gene is not significantly regulated.

Kv1.4 protein expression was significantly high in the RVW compared to the S and increased significantly in both tissues following myocardial infarction. Action potential durations at 50% repolarization were significantly longer in the S compared to the RVW in sham myocytes. Following MI, marked prolongation of APD occurred in the RVW (4.8 ± 0.7 ms and 13.3 ±1.7 ms P < 0.01) with a modest prolongation in the septum (9.7± 1.1 ms and 12.2±1.3 ms P= 0.09) resulting in a loss of heterogeneity of APD between these regions. At steady state, systolic Ca^{2+} were significantly different between right ventricular and septal myocytes derived from sham-operated animals (RVW: 381.8 ± 44.7
nM versus S: 687.4 ± 84.9 nM P = 0.05). Following MI, systolic Ca$^{2+}$ were significantly elevated in the RVW myocytes (Sham: 381.8 ± 44.7 nM versus Post-MI: 1056.4 ± 92.9 nM P < 0.05) and S myocytes (Sham: 687.4 ± 84.9 versus Post-MI: 1177.5 ± 127.4 P < 0.05).

Our results demonstrate that significant differences in APD between RVW that are mirrored by changes in [Ca$^{2+}$]$_i$, I$_{o}$ density and Kv4.2 expression and these differences largely disappear following myocardial infarction. The regulation of Kv4.2 expression may explain the electrical and mechanical heterogeneity that occurs in the rodent heart and the loss of heterogeneity following infarction.

INTRODUCTION

The duration of the cardiac action potential in the mammalian heart is of longer duration in endocardial myocytes compared to epicardial myocytes. These differences in action potential duration help explain the electrical heterogeneity that exists in the normal myocardium which has been attributed to differences in the calcium-independent transient outward current density (I$_{o}$) (Litovsky et al., 1988; Fedida & Giles 1991; Antzelevitch et al., 1991; Benitah et al., 1993; Clark et al., 1993; Li et al., 1998). Several studies have further shown that the differences in I$_{o}$ density are correlated with regional variations in the expression of K$^+$ channel genes encoding for I$_{o}$ among distinct anatomical regions of the heart and between the epicardial and endocardial layers of the left ventricular wall (Wickenden et al., 1999; Brhamajothi et al., 1999; Dixon and McKinnon, 1994).

Reduction in I$_{o}$ density and concomitant action potential prolongation are commonly observed in rats following left anterior descending coronary artery ligation.
(Aimond et al., 1999; Kaprielian et al., 1999; Qin et al., 1996), aortic banding (Gomez et al.; 1997), treatment with catecholamines (Bryant et al. 1999) and treatment with monocrotaline (Lee et al. 1997; Lee et al., 1999). Action potential prolongation can increase calcium influx through L-type calcium channels resulting in elevations in $[Ca^{2+}]_i$ as observed in normal (Bouchard et al., 1995; Puglisi et al., 1999) and hypertrophied cardiac myocytes (Kaprielian et al., 1999). It appears that the extent of $I_{io}$ reduction and action potential prolongation is regionally modulated in the hypertrophied myocardium resulting in a loss in the gradient of $I_{io}$ density and action potential heterogeneity (Bryant et al., 1999; Aimond et al., 1999; Gomez et al., 1997) which can be associated with an increased propensity for the development of arrhythmias (Qin et al., 1996). In this study, we examined the regional differences in action potential duration, $I_{io}$ density and $K^+$ channel genes (i.e. Kv1.4, Kv4.2 and Kv4.3) and to assess their relative changes following MI.

**Statistical analysis and curve fitting**

All data are expressed as mean ± standard error of the mean (S.E.M). Steady state activation ($g$) and inactivation ($h_{\infty}$) curves were fit to the following Boltzmann functions:

$$g = g_{\text{max}} / 1 + \exp[-V-V_{1/2}/k]$$

$$h_{\infty} = 1 / \{1 + \exp[V-V_{1/2}/k]\}$$

where $V$ is the step or conditioning potential, $V_{1/2}$ is the mid-point of the function and $k$ is the slope factor. Mono-exponential or bi-exponential functions were used to fit recovery from inactivation data. For bi-exponential fits:

$$I/I_o = 100 - (A_{fast} \cdot \exp(-x/\tau_{fast}) + (100-A_{fast}) \cdot \exp(-x/\tau_{slow}))$$

where $A_{fast}$ and $A_{slow}$ (100- $A_{fast}$) are the amplitudes of the fast and slow components for recovery and "t" is the time spent at the recovery potential. When the recovery data was fit
to a mono-exponential, $A_{\text{fast}} = A_{\text{slow}} = A$ and $\tau_{\text{fast}} = \tau$. Correlation between action potential duration and $[\text{Ca}^{2+}]_i$ was performed by linear regression. For Western blots, the densitometric units were always normalized to those obtained in right ventricular tissue obtained in sham samples. Statistical comparisons were made with the one way analysis of variance using the SPSS program (Version 7.0 for Windows, SPSS Inc.). When ANOVA showed statistical significance by F test, inter-group comparisons were made by the Student-Newman-Keuls procedure. A $P < 0.05$ was considered significant.

RESULTS

Effect of left anterior descending coronary artery ligation

Significant hemodynamic alterations have previously been shown to occur in the rat model of coronary artery ligation (Kapриelian et al., 1999; Sethi et al., 1997). The infarct size of post-MI hearts used in our studies were $50.6 \pm 2.8 \%$ (range 34.4 - 64.0 %). Hearts with infarct sizes involving less than 30% of the left ventricular free wall were not used in our studies. Table 3 summarizes some of the observed differences between sham and post-MI hearts at 8 weeks post-surgery. Both the right ventricle and the septum had elevated tissue weight to body weight ratios, consistent with global cardiac hypertrophy. Associated with these changes in heart weight, membrane capacitance of post-MI myocytes from both regions (as a measure of sarcolemmal area) was significantly larger than myocytes isolated from sham-operated animals (sham: $132 \pm 3 \text{ pF}$, $n=65$ and post-MI: $225 \pm 13 \text{ pF}$ $n=40$) for the right ventricle and (sham: $157\pm8 \text{ pF}$, $n=42$ and post-MI: $241\pm13 \text{ pF}$, $n=40$) for the septum.
Regional changes in membrane potential following infarction

Figure 4.1 shows representative action potentials measured in right ventricular myocytes and septal myocytes derived from sham (Panel A) and post-MI (Panel B) hearts. Action potentials recorded from RVW sham myocytes were significantly shorter in duration than S sham myocytes (APD50 = 4.8 ±0.7 ms, n = 29 and 9.7 ± 1.1 ms, n = 26 P<0.01 and APD90= 29.3±3.4 ms, n=29 and 49.4±4.5 ms, n =26 P<0.01 in right ventricular and septal myocytes, respectively). Following MI, action potentials durations in right ventricular myocytes from post-MI hearts were prolonged compared to right ventricular myocytes from sham-operated hearts at 50% (Sham: 4.8 ± 0.7 ms, n=29 Post-MI: 13.3 ± 1.7 ms, n=24 P<0.05) and 90% (Sham: 29.3 ± 3.4 ms, n=29 Post-MI: 75.1 ±7.7, n=24, P<0.05) repolarization. By contrast, action potential duration in post-MI septal myocytes was only significantly prolonged at 90% repolarization (APD50=12.2 ± 1.3 ms, n=25 P=0.1 and APD90=71.2 ± 7.0 ms, n=25 compared to sham septal myocytes, P<0.05). Figure 4.1 also shows a frequency histogram of APD50 and APD90 for right ventricular and septal myocytes derived from sham-operated (Panel C) and post-MI hearts (panel D). These results show that 65.5% of right ventricular myocytes compared to less than 23.1% of septal myocytes exhibited an APD50 ranging from 1 ms to 5 ms in sham-operated hearts. Following myocardial infarction, 16.7 % of right ventricular myocytes compared to 23.1% of septal myocytes exhibited an APD50 ranging from 1 ms to 5 ms. Similar trends were observed for APD90 where 75.8% of right ventricular myocytes compared to less than 42.3% of septal myocytes ranged from 5 ms to 35 ms in sham-operated hearts. Following myocardial infarction, 20.8 % of right ventricular myocytes compared to 16.0 % of septal myocytes exhibited an APD90 ranging from 5 ms to 35 ms. These data establish that both the
average action potential properties and the distribution of action potential values become very similar between the RVW and S following MI. There was no regional difference in resting membrane potential, but myocardial infarction caused a significant depolarization of right ventricular myocytes (Sham: -84.9 ± 1.4 mV; Post-MI: -75.9 ±0.8 mV n=24 P<0.01) but not septal myocytes (Sham: -81.6 ± 1.1 mV n=26 Post-MI: -77.7 ± 1.4 mV n=25 P=0.12) (Table 3).

The transient outward current (I_{to})

In order to investigate the ionic basis for the differences in action potential duration between the RVW and S myocytes, we measured I_{to}, a major repolarizing K^+ current in rat heart. Figure 4.2 shows representative normalized I_{to} densities traces and current-voltage data, while Table 4 summarizes the biophysical properties of I_{to} recorded in right ventricular and septal myocytes derived from sham and post-MI hearts. I_{to} was defined in our studies as the difference between the peak outward current and the sustained current remaining at the end of depolarizing pulse (I_{to} = I_{peak} - I_{sust}) as previously described (Apkon and Nerbonne, 1991). The I-V relationship in Figure 4.2 reveal that I_{to} activate over the same range despite I_{to} density being significantly higher in the RVW compared to the S (RVW: 21.3 ±0.9 pA/pF versus S: 13.9±1.1 pA/pF) as shown previously (Wickenden et al., 1999) (Panel A). I_{to} current-voltage relationships show that I_{to} activated over the same range of voltages for the two groups (positive to −10 mV), but the average current densities were significantly different. Comparison of the current recordings in Figure 4.2 also shows that I_{to} was profoundly reduced in post-MI right ventricular myocytes compared to sham right ventricular myocytes. I_{to} density evaluated following depolarization to +40 mV was
significantly reduced (P<0.01) in RVW myocytes following MI (Sham: 14.7 ± 0.8 pA/pF n=42 Post-MI: 8.6 ± 1.1 pA/pF n=24 (P<0.05) while no significant reductions were recorded in S myocytes (Sham: 7.0±0.9 pA/pF n=19 Post-MI: 5.1 ± 0.6 pA/pF n=20). These changes in I\textsubscript{o} following MI mirrored the changes in APD\textsubscript{50}. We also recorded I\textsubscript{o} density (16.2±1.9 pA/pF at +40 mV, data not shown) in left ventricular epicardial cells from sham-operated animals which were not different (P = 0.5) than results obtained in sham right ventricular myocytes. A frequency histogram of I\textsubscript{o} evaluated at +40 mV shows that 15.7% sham right ventricular myocytes, compared to 83.3 % of sham septal myocytes exhibited current densities ranging from 1 pA/pF to 9 pA/pF (Panel G). 68.9 % of right ventricular myocytes and 100 % of septal myocytes had I\textsubscript{o} densities ranging between 1 pA/pF and 9 pA/pF showing a loss of heterogeneity of I\textsubscript{o} between these regions following MI (Panel H).

Since conductance provides a better measure of channel number, the maximal transient outward channel conductance (G\textsubscript{max}) was also calculated (Table 4). G\textsubscript{max} was significantly reduced from 136.5 ± 7.5 pS/pF (n = 42) in sham right ventricular myocytes to 78.6 ± 8.1 pS/pF (n = 24 P <0.05) in post-MI right ventricular myocytes. In contrast, G\textsubscript{max} was not significantly changed in the septum (Sham: 67.8 ± 3.6 pS/pF n=18 and Post-MI: 48.6 ± 5.9 pS/pF n=20 P < 0.05). There were no significant differences in the activation and inactivation parameters (i.e.\textit{V}_{1/2} and \textit{k}) in all of the groups as shown in Table 4. The changes in G\textsubscript{max} mirrored the changes in I\textsubscript{o} density. Figure 4.3 shows representative normalized recovery from inactivation traces in RVW (panel A) and S myocytes (panel B). Peak current amplitude was plotted as a function of the function of the interpulse duration and fitted with an exponential function. As summarized in Table 2, no slow component for recovery could be observed in sham myocytes from the RVW while about 10% of the I\textsubscript{o}
recovered with a slow time course. Following MI, both the RVW and S myocytes had a sizable component of the current that recovered with a slow time course. These results establish that the biophysical properties of I<sub>io</sub> in the RVW change following MI as shown previously (Kaprielian et al., 1999), but not in the S which may probably reflect the changes in expression profile of Kv4.x to Kv1.x channels in these regions.

To investigate the molecular basis for the changes in I<sub>io</sub> density and its time course for recovery kinetics, we examined the expression of Kv4.2, Kv4.3 and Kv1.4 in the right ventricular wall and septum derived from sham and post-MI hearts (figure 4.4). We find that Kv4.2 α subunit expression is significantly higher in the RVW compared to the S in sham hearts (right ventricle: unity n=4 versus septum: 0.76±0.05 n=4 P<0.01) while Kv4.3 protein expression was not significantly different between the right ventricular free wall and the septum (right ventricle: unity n=4 versus septum: 1.03 ± 0.2 n=4 P=0.51). These differences appear to occur at the transcriptional level (Wickenden et al., 1999). Following myocardial infarction, Kv4.2 protein levels were significantly decreased in the right ventricle reaching 45.7±11.1% of levels observed in right ventricle derived from sham-operated hearts (sham: unity n=4 versus post-MI: 0.46±0.11 n=4 P<0.05). In contrast, Kv4.2 protein was also decreased in the septum following myocardial infarction, but to a lesser degree compared to the right ventricle (sham: 0.76±0.05 n=4 versus post-MI: 0.56±0.05 n=4). Unlike Kv4.2, Kv4.3 protein expression was neither different in right ventricular myocytes and septal myocytes nor modulated by myocardial infarction. Another candidate for I<sub>io</sub>, Kv1.4 protein expression was not different between the RVW and S, but was modulated by infarction. Kv1.4 protein increased significantly in the RVW (2.09±0.4 n=6 P<0.01), but not in the S.
1.29±0.39 n=6 P=0.25) following MI. The pattern of expression of Kv1.4 channels mirrors the pattern of the slowly recovering component of $I_{o}$, consistent with previous suggestions that Kv1.4 encodes for the slowly recovering component of $I_{o}$ (Wickenden et al., 1999)

The non-inactivating sustained outward K$^+$ current ($I_{\text{sus}}$) which is defined as the current remaining at the end of a 500 ms depolarizing voltage step (Figure 4.2). The current-voltage relationship for $I_{\text{sus}}$ is shown for right ventricular myocytes and septal myocytes derived from sham (Panel E) and post-MI myocytes (panel F). Unlike the findings on $I_{o}$, there was no regional differences in $I_{\text{sus}}$ density. $I_{\text{sus}}$ density was not different between right ventricular myocytes isolated from sham and post-MI hearts (Sham: 6.6 ± 0.4 pA/pF n=42 and Post-MI: 5.3 ± 0.5 pA/pF n=24 at +40 mV P > 0.05). In comparison to RVW myocytes, $I_{\text{sus}}$ was significantly reduced in the S (Sham: 6.6 ± 0.4 pA/pF n=19 Post-MI: 4.7 ± 0.4 pA/pF n=20 at +40 mV P <0.05). Consistent with our results, $I_{\text{sus}}$ was found to be significantly reduced in the left ventricular endocardium, but not in right ventricle following short-term infarction (Yao et al., 1999). The molecular correlate of $I_{\text{sus}}$ remains uncertain, but three K$^+$ channel genes (Kv1.2, Kv1.5 and Kv2.1) known to encode for delayed rectifier-type currents have been studied previously (Swanson et al., 1990; Tseng-Crank et al.,1990). We performed RNase protection assays (data not shown) for Kv1.2, Kv1.5 and Kv2.1 α-sub-units on tissues derived from the right ventricle and septum. In the right ventricle, mRNA levels for Kv1.2, Kv1.5 and Kv2.1 were reduced by 23.7±15.3 % (n=5; P=0.19), 22.4±11.4% (n=5; P=0.11) and 27.0±9.8% (n=5; P=0.06) in the post-MI group relative to the sham group. In the septum, Kv1.2 and Kv2.1 were significantly decreased by 39.7± 4.5% (n=5 P=0.001) and 22.2± 7.5% (n=5 P=0.04), respectively whereas, Kv1.5 mRNA level were not changed (n=5 18.7 ±11.8 P=0.17).
The inward rectifier current

Figure 4.5 shows representative barium subtracted inward rectifier (I_{Kr}) traces in right ventricular and septal myocytes derived from sham (panel A) and post-MI hearts (panel B). Current-voltage relationships for Ba^{2+}-sensitive currents are depicted in panel C for the sham group and panel D for the post-MI group. Unlike the differences observed in I_o density, I_{Kr} density between right ventricular and septal myocytes were not significantly different. Following myocardial infarction, I_{Kr} density evaluated at -130 mV was decreased significantly in RVW myocytes (Sham: -16.0± 0.6 pA/pF, n = 28; Post-MI: -12.2 ±1.0 pA/pF, n = 20 P<0.05), but not in myocytes (Sham: -14.3±1.6 pA/pF, n=10; Post-MI: -12.8±1.0 pA/pF, n=18 P=0.11). At potentials greater than or equal to -110 mV and in the physiological range of test potentials (from -90 mV to -40 mV), I_{Kr} density was not statistically significant for right ventricular and septal myocytes. In contrast to right ventricular myocytes, I_{Kr} density was unaffected (P > 0.05) in septal myocytes following myocardial infarction at all test potentials.

The L-type calcium current

Since the regional change in APD observed following MI could potentially originate from differences in L-type Ca^{2+} current (I_{Ca,L}), we recorded calcium currents in the right ventricular and septal myocytes. Figure 4.6 shows representative cadmium subtracted I_{Ca,L} traces in right ventricular and septal myocytes derived from sham (panel A) and post-MI (panel B) hearts. There are no differences in the expression of calcium channels in the different myocardial regions and following myocardial infarction. As shown in panel C, I_{Ca,L} density was not significantly different between sham right ventricular myocytes and post-MI right ventricular myocytes (at +10 mV: sham: -6.0±0.4 pA/pF, n=15 and Post-MI: --
5.5±0.5 pA/pF, n=14 P=0.59). Similarly, I_{Ca, L} density were not significantly different between sham septal myocytes and post-MI septal myocytes as shown in Panel D (at +10 mV- sham: -5.8±0.4 pA/pF n=16 and Post-MI: -5.2±0.3 pA/pF n=16 P=0.59).

**Action potential duration and [Ca^{2+}]_{i}**

Since the expression and function of I_{o} channels is region-dependent, we examined whether differences in I_{o} expression may be associated with regional changes in calcium handling. Figure 4.7 shows simultaneous records of action potentials and [Ca^{2+}]_{i} (in current clamp) in right ventricular and septal myocytes derived from sham (Panel A) and post-MI (Panel B) hearts under conditions where the only Ca^{2+} buffer in the myocyte was 75 µM Fura-2 pentapotassium salt. Following myocardial infarction, action potentials were prolonged at 50% repolarization in right ventricular myocytes (sham: 6.5±1.4 ms, n=17 and post-MI: 37.2±5.8 ms, n=16 p<0.01), but not in septal myocytes (sham: 24.2±3.4 ms, n=15 and post-MI: 33.3±3.7 ms, n=20 P=0.09). At 90% repolarization, action potentials were significantly prolonged in both right ventricular (sham: 72.3±13.8 ms and post-MI: 673.6±89.9 ms, p<0.05) and septal myocytes (sham: 154.2±23.4 ms and post-MI: 592.3±74.3 ms, p<0.05). Associated with the longer action potentials, mean systolic [Ca^{2+}]_{i} was significantly higher in the septum compared to the right ventricle derived from sham hearts (RVW: 381.8±44.7 nmol/L, n=17 versus S: 687.4±84.9 nmol/L, n= 15 P<0.01). Systolic [Ca^{2+}]_{i} in post-MI right ventricular myocytes was 1056.4±92.9 nmol/L, n=16) as compared to sham myocytes (381.8±44.7 nmol/L, n=17 P<0.05). Similarly, in septal myocytes systolic [Ca^{2+}]_{i} was increased from (687.4±84.9 nmol/L, n= 15) in sham myocytes as compared to post-MI myocytes (1177.5±127.5 nmol/L, n=15). Diastolic [Ca^{2+}]_{i} were unaffected by region or MI. In right ventricular myocytes, diastolic [Ca^{2+}]_{i} were 81.3±5.4 nmol/L n=17 and 93.5 ±4.5
nmol/L n=16 in sham and post-MI myocytes, respectively. In septal myocytes, diastolic [Ca\(^{2+}\)]\(_i\) were 100.4±8.8 nmol/L n=15 and 93.5 ±10.5 nmol/L n=15 in sham and post-MI myocytes, respectively. We also examined whether factors other than membrane potential may explain the elevated systolic [Ca\(^{2+}\)]\(_i\). Myocytes were voltage clamped at a holding potential of −80 mV and brief 100 ms step depolarizations to +10 mV were applied at a rate of 0.2 Hz. Figure 4.8 shows representative [Ca\(^{2+}\)]\(_i\) in right ventricular (panel A) and septal (panel B) myocytes with the summarized data in panel C and D, respectively. In right ventricular myocytes, systolic[Ca\(^{2+}\)]\(_i\) were 648.9±143.3 nmol/L n=10 and 736.8±100.8 nmol/L n=10 P=0.69) while in septal myocytes, systolic[Ca\(^{2+}\)]\(_i\) were 841±127.7 nmol/L n=10 and 727.8±124.3 nmol/L n=9 P=0.69) in sham and post-MI myocytes, respectively. Diastolic [Ca\(^{2+}\)]\(_i\) were unaffected by region or MI. In right ventricular myocytes, diastolic [Ca\(^{2+}\)]\(_i\) were 91.7±3.0 nmol/L n=10 and 84.7±3.2 nmol/L n=10 in sham and post-MI myocytes, respectively. In septal myocytes, diastolic [Ca\(^{2+}\)]\(_i\) were 101.9±9.3 nmol/L n=10 and 82.0 ±5.7 nmol/L n=9 in sham and post-MI myocytes, respectively.
DISCUSSION

We previously showed that \( I_{\text{to}} \) density and Kv4.2 expression is high (Wickenden et al., 1999) and is significantly reduced following infarction in right ventricular myocytes (Kaprielian et al., 1999) and can be restored following thyroid hormone administration (Wickenden et al., 2000).

In the present study, we show that \( I_{\text{to}} \) density is significantly higher in the right ventricular myocytes compared to the septal myocytes and that myocardial infarction causes a significant reduction in \( I_{\text{to}} \) density in myocytes derived from the right ventricle, but not in myocytes derived from the interventricular septum. These regional changes in \( I_{\text{to}} \) density in normal and hypertrophied myocardium is primarily attributed to differences in the expression of Kv4.2 protein. Associated with these changes in \( I_{\text{to}} \) expression and function, action potential duration are significantly longer in septal myocytes compared to right ventricular myocytes resulting in a significant prolongation of action potential duration in right ventricular myocytes, but not septal myocytes. These regional changes in action potential duration are associated with significant elevations in \([\text{Ca}^{2+}]_{\text{i}}\) in right ventricular myocytes but not in septal myocytes. To our knowledge this is the first study documenting the ionic and molecular changes in the expression and function of \( I_{\text{to}} \) channels, action potential duration and \([\text{Ca}^{2+}]_{\text{i}}\) in specific myocardial regions in the presence and absence of myocardial infarction.

**Heterogeneity of action potential duration and \( I_{\text{to}} \) density**

Our findings clearly demonstrate that regional dissimilarities in action potential duration exist in the normal rat myocardium which is consistent with previous findings (Clark et al., 1993; Gomez et al., 1997; Wickenden et al., 1999). Differences in action potential
configuration are not only detected between specific anatomical regions of the heart, but also within the layers of the left ventricular wall itself in a variety of species (Fedida and Giles, 1991; Antzelevitch et al., 1991) and humans (Nabauer et al., 1996). These differences in action potential duration are attributed to differences in the repolarizing current provided by the transient outward $K^+$ channel (Clark et al., 1993; Gomez et al., 1997). Under our recording conditions, other repolarizing (i.e. $I_{sus}$ and $I_{K1}$) and depolarizing (i.e. $I_{Ca,L}$) currents that exist in the rat may also explain regional differences in action potential duration. In this study, we did not detect any difference in the density of these currents between right ventricular and septal myocytes which is consistent with previous findings (Aimond et al., 1999; Casis et al., 1998). Furthermore, depolarizing currents provided by the L-type calcium channel were unaffected by myocardial infarction which consistent with previous results (Kaprielian et al., 1999; Qin et al., 1996). We also report no regional differences in $I_{Ca,L}$ density. Therefore it seems likely that the regional variations in the shape of early repolarization in right ventricular and septal action potentials are most likely due to the differences in $I_{to}$ density in these regions.

**Regional changes in Kv4.2, Kv4.3 and Kv1.4 expression and $I_{to}$ density in normal and hypertrophied myocytes**

We previously reported that Kv4.2 expression at the mRNA and protein level predominates in the right ventricular wall compared to the septum and coincides with higher $I_{to}$ densities and fast recovery kinetics (Wickenden et al., 1999). Studies on the right ventricle and septum revealed similar findings in humans (Nabauer et al., 1993; 1996) and animal models (Aimond et al., 1999; Brahmajothi et al., 1999). The expression of $K^+$ channel genes encoding for $I_{to}$ have been shown to be different in various
anatomical regions of the normal mammalian heart (Wickenden et al., 1999; Brahmajothi et al., 1999; Dixon and McKinnon, 1994). In this study, we show that the downregulation of Kv4.2, reduction of I_to density and maximal conductance of I_to is more severe in the right ventricular wall compared to the septum which is consistent with previous data following long term MI (Aimond et al., 1999) and aortic banding (Gomez et al. 1997). Similarly, large reductions in I_to density were observed in sub-epicardial and mid-myocardial with minimal changes in sub-endocardial myocytes in catecholamine-induced hypertrophy (Bryant et al., 1999).

A likely explanation for our findings may be related to the large gradient of Kv4.2 expression that exists in the right ventricle compared to the septum in the normal heart (Wickenden et al., 1999; Dixon and McKinnon, 1994). Since myocardial infarction primarily affects the expression of Kv4.2 and Kv4.3 (Kaprielian et al., 1999; Gidh-Jain et al., 1996), it is then expected that I_to to be more depressed in the right ventricle compared to the septum. Following myocardial infarction, Kv4.2 protein but not Kv4.3 protein is reduced in the right ventricle compared to the septum. Alongside the regional differences in I_to density following MI, I_to recovery kinetics in septal myocytes are markedly slowed compared to right ventricular myocytes. The existence of a higher proportion of myocytes with slowed recovery kinetics may be related to the heterogeneous distribution of Kv4.2, Kv4.3 and Kv1.4 between the right ventricle and septum in control and hypertrophied myocytes. At the present time, the contribution of Kv1.4 relative to Kv4.2 and Kv4.3 in adult rat ventricular myocytes remains unclear (McKinnon, 1999). We previously showed that Kv4.2 and Kv4.3 is less predominant whereas Kv1.4 is more important in the septum compared to the right ventricle (Wickenden et al., 1999). In the majority of right ventricular
sham myocytes, we find that the recovery was fast, but a small proportion of the septal sham myocytes showed slowed recovery kinetics. The slowed recovery kinetics is most likely due to the low expression of Kv4.2 α subunits in the septum compared to the right ventricle. On the other hand, the marked down-regulation of Kv4.2 and Kv4.3 coupled with a significant increase in Kv1.4 protein in the right ventricle but not septum probably explain the emergence of slowed recovery kinetics in hypertrophied right ventricular myocytes compared to normal right ventricular myocytes.

**Action potential duration and \([\text{Ca}^2+]_i\)**

We previously showed that prolongation of the action potential duration increases calcium influx via the L-type calcium channels and is associated with a significant elevation in peak \([\text{Ca}^{2+}]_i\) transient amplitude in hypertrophic myocytes (Kaprielian et al., 1999). At the onset of cardiac hypertrophy, prolongation of action potential duration occurs and the amplitude of the \([\text{Ca}^{2+}]_i\) transient increases as previously observed in this and other animal models of cardiac hypertrophy (Kaprielian et al., 1999; Shorofsky et al., 1999; Brooksby et al., 1993a; Brooksby et al., 1993b; Bing et al., 1991). However as the hypertrophy progresses to heart failure and despite the prolongation of action potential duration, the amplitude of the \([\text{Ca}^{2+}]_i\) decreases and is prolonged probably due to a decrease in SR Ca^{2+}-ATPase activity and an uncoupling between L-type calcium channels and the ryanodine receptors (Gwathmey et al., 1987; Gomez et al., 1997; Balke and Shorofsky, 1998; O’Rourke et al., 1999). Eight weeks following myocardial infarction, we found no differences in calcium current, \([\text{Ca}^{2+}]_i\) in voltage-clamp experiments which indicate that there are no intrinsic differences in intrinsic calcium handling properties. Furthermore, SR Ca^{2+} content is not changed (Kaprielian et al., 1999).
Alongside the electrical heterogeneity, regional differences in metabolic, functional and mechanical function have been reported (Kainulainen et al., 1990; Robitaille et al., 1990; Burashnikov and Antzelevitch, 1999). In this study, we show that peak \([\text{Ca}^{2+}]_i\) were different in the right ventricle and septum which is consistent with a previous report demonstrating peak \([\text{Ca}^{2+}]_i\) were higher in the endocardium than at the epicardium in a perfused rat heart model (Figueroedo et al., 1993). These data confirm the presence of significant regional modulation of APD which can be an important determinant of peak \([\text{Ca}^{2+}]_i\).

**Limitations of the study**

Previous studies on animals models of cardiac hypertrophy have also demonstrated a reduction in action potential heterogeneity (Aimond et al., 1999; Gomez et al., 1997; Bryant et al., 1997; Bryant et al., 1999; Qin et al., 1996). The loss of the electrical heterogeneity would probably disturb the normal repolarization process and favor the onset of re-entry circuits within the wall and at the right or left ventricular wall-septum interface. We did not study the cellular mechanisms of arrhythmias in this study, but an earlier report showed evidence for ventricular tachycardia and triggered activity in recordings from strips of the left ventricular wall (Qin et al., 1996).

The transmural gradient of \(I_{\text{to}}\) expression across the different myocardial layers and between the different anatomical regions is universal to all mammalian hearts including humans (Aimond et al., 1999; Gomez et al., 1997; Nabauer et al., 1996; Wettwer et al., 1994; Clark et al., 1993; Fedida and Giles, 1991). In the present study, we did not intend to compare \(I_{\text{to}}\) expression in epicardial and endocardial myocytes. Also, we cannot generalize our findings in the right ventricular free wall and interventricular septum to be representative
of epicardial and endocardial myocytes, respectively. There is an advantage to studying
different anatomical regions of the heart rather than between the myocardial layers (i.e.
epicardium and endocardium) since endocardial cells drawn from different anatomical
regions of the same heart may still show clear differences in electrical properties (Watanabe
et al., 1983). Furthermore, with the presence of large infarcts in the left ventricle and the thin
cross-section of the right ventricular wall, it was difficult to separate the myocardial layers
and obtain enough tissue to perform the molecular studies. It has been shown that $I_{\text{to}}$
density, kinetics and the molecular profile of $K_{\text{v}x}$-based channels in left ventricular
epicardial myocytes and right ventricular myocytes are quite similar in the rat (Dixon and
McKinnon, 1994) and ferret (Brahmajothi et al., 1999). Furthermore, our own data did not
reveal any differences in $I_{\text{to}}$ density between left ventricular epicardial myocytes and right
ventricular myocytes. To this end, we can assume that myocytes derived from the right
ventricle are epicardial whereas myocytes derived from the septum are mostly endocardial
(Clark et al., 1993; Brahmajothi et al., 1999; Bryant et al., 1997). We are aware that such
assumptions cannot hold true for humans where a transmural heterogeneity and a gradient of
$I_{\text{to}}$ density have been detected in the right ventricular wall (Li et al., 1998) and septum
(Bailly et al., 1997).

**Conclusion:** In summary, these combined patch clamp and molecular studies provide
important information on the mechanism for the regional alteration in action potential
waveform following myocardial infarction. The level of channel expression between right
ventricular myocytes and septal myocytes reflect the observed differences in $I_{\text{to}}$ and APD
between the different regions in control and hypertrophied myocytes. These findings also
show the pronounced effects of changes in action potential duration on \([\text{Ca}^{2+}]_i\), and draw attention to the regional differences in action potential duration and \([\text{Ca}^{2+}]_i\) in the rat heart. A more quantitative study (perhaps by confocal microscopy) must be done before a more realistic understanding of the role of epicardial and endocardial myocytes on repolarization and calcium handling can be achieved.
TABLE 3. Changes associated with myocardial infarction in right ventricular and septal myocytes. BW indicates body weight; W/BW indicates tissue weight to body weight; APD\textsubscript{50} and APD\textsubscript{90} indicate action potential duration at 50% and 90% repolarization; \( \text{V}_{\text{peak}} \) indicates the peak of the action potential; \( \text{V}_{\text{m, rest}} \) indicates resting membrane potential. † Right ventricular sham myocytes versus right ventricular post-MI myocytes ‡ septal sham myocytes versus septal post-MI myocytes.

<table>
<thead>
<tr>
<th></th>
<th>Right Ventricle</th>
<th></th>
<th>Septum</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Post-MI</td>
<td>Sham</td>
<td>Post-MI</td>
</tr>
<tr>
<td>W/BW (%)</td>
<td>0.038±0.001(18)</td>
<td>0.066±0.001(6)†</td>
<td>0.049±0.001(7)</td>
<td>0.065±0.001(8)‡</td>
</tr>
<tr>
<td>Capacitance (pF)</td>
<td>132±3 (65)</td>
<td>225±13 (40)†</td>
<td>157±8 (42)</td>
<td>241±13 (40)‡</td>
</tr>
<tr>
<td>APD\textsubscript{50} (ms)</td>
<td>4.8±0.7 (29)§</td>
<td>13.3±1.7 (24)†</td>
<td>9.7±1.1 (26)</td>
<td>12.2±1.3 (25)</td>
</tr>
<tr>
<td>APD\textsubscript{90} (ms)</td>
<td>29.3±3.4 (29)§</td>
<td>75.1±7.7 (24)†</td>
<td>49.4±4.5 (26)</td>
<td>71.2±7.0 (25)‡</td>
</tr>
<tr>
<td>( \text{V}_{\text{peak}} ) (mV)</td>
<td>45.3±4.0 (29)</td>
<td>41.8±4.0 (24)</td>
<td>41.1±2.2 (26)</td>
<td>47.8±2.9 (25)</td>
</tr>
<tr>
<td>( \text{V}_{\text{m, rest}} ) (mV)</td>
<td>-84.8±1.4 (29)</td>
<td>-75.9±0.8 (24)†</td>
<td>-81.5±0.8 (26)</td>
<td>-77.7±1.4 (25)</td>
</tr>
</tbody>
</table>
TABLE 4. Regional differences in the characteristics of the transient outward current in right ventricular and septal myocytes following myocardial infarction. 

I_{peak} indicates the peak outward current; I_{sust} indicates the sustained current measured at the end of the 500 ms pulse; I_{to} indicates the transient outward current and is defined as I_{peak} - I_{sust}; G_{max} indicates the maximal conductance for the transient outward channel; V_{1/2} indicates the mid-point for activation; k indicates the slope factor; A_{fast} and A_{slow} indicate the fast and slow fractions of recovery; \tau_{fast} and \tau_{slow} represent the fast and slow time constant of recovery. † Right ventricular sham myocytes versus right ventricular post-MI myocytes; ‡ septal sham myocytes versus septal post-MI myocytes; § right ventricular sham myocytes versus septal sham myocytes; * right ventricular post-MI myocytes versus septal post-MI myocytes

<table>
<thead>
<tr>
<th></th>
<th>Right Ventricle</th>
<th>Septum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Post-MI</td>
</tr>
<tr>
<td>at +40 mV</td>
<td>42</td>
<td>24</td>
</tr>
<tr>
<td>I_{peak} (pA/pF)</td>
<td>21.3±0.9§</td>
<td>13.9±1.1†</td>
</tr>
<tr>
<td>I_{sust} (pA/pF)</td>
<td>6.6±0.4</td>
<td>5.3±0.5</td>
</tr>
<tr>
<td>I_{to} (pA/pF)</td>
<td>14.7±0.8§</td>
<td>8.6±1.1†</td>
</tr>
<tr>
<td>Steady state activation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>42</td>
<td>24</td>
</tr>
<tr>
<td>G_{max} (pS/pF)</td>
<td>136.5±7.5§</td>
<td>78.6±8.1†</td>
</tr>
<tr>
<td>V_{1/2} (mV)</td>
<td>13.8±0.9</td>
<td>11.7±2.1</td>
</tr>
<tr>
<td>k (mV)</td>
<td>14.9±0.5</td>
<td>14.3±0.5</td>
</tr>
<tr>
<td>Steady state inactivation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>V_{1/2} (mV)</td>
<td>-31.4±0.7</td>
<td>-33.5±1.3</td>
</tr>
<tr>
<td>k (mV)</td>
<td>3.3±0.1</td>
<td>3.4±0.2</td>
</tr>
<tr>
<td>Recovery from inactivation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>36</td>
<td>16</td>
</tr>
<tr>
<td>A_{fast} (%)</td>
<td>99.7±0.2§</td>
<td>89.4±2.2†</td>
</tr>
<tr>
<td>\tau_{fast} (ms)</td>
<td>30.1±1.7</td>
<td>35.7±4.5</td>
</tr>
<tr>
<td>A_{slow} (%)</td>
<td>0.3±0.2§</td>
<td>10.6±2.2†</td>
</tr>
<tr>
<td>\tau_{slow} (ms)</td>
<td>223.1±84.2§</td>
<td>1686.0±265.3†</td>
</tr>
</tbody>
</table>
FIGURE 4.1. Action potential characteristics in right ventricular and septal myocytes following myocardial infarction. Panel A shows representative traces for right ventricular (left) and septal (right) myocytes derived from sham hearts. Panel B shows representative traces for right ventricular (left) and septal (right) myocytes derived from post-MI hearts. Action potentials were elicited by a brief (5 ms) suprathreshold (2 × threshold) applied at 0.2 Hz. Recordings were made with 5 mM EGTA in the pipette. Panel C and Panel D show a frequency distribution of APD₅₀ and APD₉₀, respectively. Open bars represent right ventricular myocytes and solid bars represent septal myocytes. — indicates 0 mV.
FIGURE 4.2. The transient outward (I_{to}) and sustained current (I_{su}) in right ventricular and septal myocytes following myocardial infarction. Panels A and B show representative normalized traces of the transient outward current (I_{to}) and sustained current (I_{su}) in right ventricular myocytes and septal derived from sham and post-MI hearts elicited by 500 ms voltage steps over the range of -30 to +70 mV in +10-mV increments from a holding potential of -80 mV (shown in the inset). I_{to} (Panel C; right ventricular myocytes and Panel D; septal myocytes) and I_{su} (Panel E; right ventricular myocytes and Panel F; septal myocytes) were normalized to membrane capacitance and plotted against the test potential for sham and post-MI groups. Panel G and Panel H show a frequency distribution of I_{to} density evaluated at +40 mV for right ventricular myocytes and septal myocytes, respectively. Myocytes were depolarized every 5 seconds. \( \rightarrow \) indicates 0 pA/pF.
FIGURE 4.3. Biophysical properties of the transient outward current ($I_{to}$) in right ventricular myocytes and septal myocytes following myocardial infarction. Representative normalized recovery from inactivation traces from right ventricular and septal myocytes derived from sham (panel A) and post-MI (panel B) hearts. C and D is a plot of recovery kinetics for the respective groups. A two pulse protocol with two identical depolarizing pulses from -80 to +60 mV was applied every 10 seconds at selected intervals from 10 to 10 000 ms. Recovery curves were constructed by taking the ratio of the current available in the second pulse to that observed in the first pulse as a function of the interpulse interval. The recovery kinetics were fitted with a double exponential function $\rightarrow$ indicates 0 pA/pF.
FIGURE 4.4. Representative comparison of candidate K⁺ channel α-subunits encoding the transient outward current in the right ventricle and septum following myocardial infarction. Each blot shows side-by-side immunoblots for Kv4.2 (panel A), Kv4.3 (panel B) and Kv1.4 (panel C) taken from the right ventricle and septum in sham and post-MI hearts. The bar graph shows mean changes in Kvx protein in tissues derived from sham right ventricle (solid bars), sham septum (open bars), post-MI right ventricle (gray bars) and post-MI septal (broken bars) normalized to sham right ventricle. † sham, right ventricle vs. post-MI, right ventricle; § sham, right ventricle vs. sham, septum; ‡ sham, septum vs. post-MI, septum. P < 0.05
FIGURE 4.5. The inward rectifier current in right ventricular and septal myocytes following myocardial infarction. A shows normalized barium-subtracted traces of the inward rectifier current \( (I_{K1}) \) for right ventricular and septal myocytes derived from sham (panel A) and post-MI (panel B) hearts elicited by 500 ms voltage steps over the range -130 to -10 mV in +10-mV increments from a holding potential of -80 mV. CdCl₂ (0.3 mmol/L) was added to avoid contamination by the calcium current and a prepulse to -40 mV was used to inactivate the sodium channels. \( I_{K1} \) current were normalized to membrane capacitance and plotted against the test potential for sham (Panel C) and post-MI (Panel D) myocytes. The steady state current measured at the end of the test pulse in the presence of barium was subtracted from the current evoked at the same voltage step in the absence of barium. Myocytes were depolarized every 5 seconds. → indicates 0 pA/pF.
FIGURE 4.6. The L-type calcium current ($I_{Ca,L}$) in right ventricular and septal myocytes following myocardial infarction. Panels A and B show representative normalized cadmium-subtracted (0.3 mM CdCl₂) traces of the L-type calcium current in right ventricular myocytes and septal derived from sham and post-MI hearts elicited by 500 ms voltage steps over the range of -60 to +70 mV in +10-mV increments from a holding potential of -80 mV (shown in the inset). $I_{Ca,L}$ (Panel C; sham and Panel D; post-MI) were normalized to membrane capacitance and plotted against the test potential for right ventricular and septal myocytes. A prepulse to -40 mV was used to eliminate any contaminating sodium current ($I_{Na}$). Myocytes were depolarized every 5 seconds. → indicates 0 pA/pF.
FIGURE 4.7. Action potentials and [Ca^{2+}] in right ventricular and septal myocytes following myocardial infarction. Action potentials (top) and its associated Ca^{2+} (bottom) transient in right ventricular and septal myocytes derived from sham (Panel A) and post-MI (Panel B) hearts. Panel C and Panel D show mean values for systolic and diastolic [Ca^{2+}] in the respective groups. Panel E shows a scatter plot of the systolic [Ca^{2+}] plotted against action potential duration at 50% repolarization in right ventricular and septal myocytes derived from sham-operated animals. → indicates 0 nM [Ca^{2+}]; — indicates 0 mV.
FIGURE 4.8. Voltage-clamp stimulated [Ca$^{2+}$] in right ventricular and septal myocytes following myocardial infarction. Representative [Ca$^{2+}$]i derived from right ventricular and septal myocytes derived from sham (panel A) and post-MI (panel B) hearts elicited with short voltage-clamp pulses to +10 mV (100 ms) from a holding potential of -80 mV to +10 mV. Panel C and Panel D show mean values for systolic and diastolic [Ca$^{2+}$]i derived from the respective groups. No significant difference were observed between sham and post-MI myocytes. → indicates 0 nM [Ca$^{2+}$], and — indicates 0 mV.
THE THYROID HORMONE ANALOG, 3,5-DIODOTHYROPROPIONIC ACID (DITPA) RESTORES THE TRANSIENT OUTWARD POTASSIUM CHANNEL IN RATS FOLLOWING MYOCARDIAL INFARCTION

ROGER KAPRIELIAN*, ALAN D. WICKENDEN*, XIA-MANG YOU AND PETER H. BACKX


* These authors contributed equally to this work.
ABSTRACT

Previous studies have established that reductions in repolarizing currents contribute to life-threatening arrhythmias in myocardium. In this study, we investigated whether the thyroid hormone analog, 3,5 diiodothyropropionic acid (DITPA), could increase K⁺ channel expression and restore repolarization in a rat model of hypertrophy secondary to myocardial infarction (MI). The density of the transient outward K⁺ current (Iₒ) was reduced following MI (Sham: 14.0 ± 1.0 pA/pF versus Post-MI: 10.2 ± 0.9 pA/pF at +40 mV). MI caused reductions in Kv4.2 and Kv4.3, but increases in Kv1.4 mRNA levels. Corresponding changes in Kv4.2 and Kv1.4 protein were also observed. Chronic treatment of post-MI rats with 10 mg Kg⁻¹ DITPA restored Iₒ density (15.2 ± 1.1 pA/pF), Kv4.2 and Kv1.4 expression to levels observed in sham-operated controls, but did not reverse the changes in Kv4.3. Other membrane currents (Iₙa, Iₖ, Iₛus, Iₖ₁) were unaffected by DITPA treatment. Associated with the changes in Iₒ expression, action potential duration recorded in single right ventricular myocytes or right ventricular wall monophasic action potentials were prolonged following MI and were restored following DITPA treatment. Our results demonstrate that DITPA restores Iₒ density in the setting of MI which may be useful in preventing complications associated with Iₒ down-regulation.
INTRODUCTION

Ventricular remodeling is a well recognized response to myocyte loss, such as might occur following a myocardial infarction (Pfeffer et al., 1979). The remodeling process involves concentric myocyte hypertrophy and altered gene expression in surviving myocytes in both left and right ventricles (Young et al., 1998; Sethi et al., 1997; Afzal et al., 1992). An important feature of the remodeling process is action potential prolongation secondary to reductions in the transient outward K⁺ current (Iₒ) density which elevates [Ca²⁺]t transient amplitude (Kaprielian et al., 1999). It seems plausible that reductions in the expression of Iₒ and action potential prolongation represent early compensatory responses of the heart to increased load (Wickenden et al., 1998). On the other hand, chronic elevation in intracellular calcium associated with action potential prolongation may potentiate mitogenic signaling cascades, leading to maladaptive gene expression, thereby over-riding any short-term benefits and perhaps contributing to left ventricular dysfunction and failure (Wickenden et al., 1998). Action potential prolongation can also be arrhythmogenic and, as such, this compensatory mechanism may predispose the heart to lethal arrhythmias and sudden cardiac death (Tomaselli et al., 1994).

Given the potential contribution of Iₒ down-regulation and action potential prolongation to the pathogenesis of heart failure, normalization of Iₒ in the hypertrophied heart might be useful in the treatment of this condition. Interestingly in this regard, thyroid hormone can increase the expression of potassium channel genes encoding for Iₒ (Guo et al., 1998; Shimoni et al., 1997; Wickenden et al., 1997), alter the biophysical
properties of $I_o$ (Shimoni et al., 1997; Wickenden et al., 1997; Shimoni et al., 1992) and abbreviate action potential duration (Binah et al., 1987).

Although thyroid hormone treatment can increase cardiac performance in the post-infarction rat heart (Gay et al., 1988), the effects of thyroid hormone are complicated by a plethora of non-cardiac effects which may limit the beneficial effects of this agent (Gay et al., 1988). In this study, we examined the effects of the thyroid hormone analog-3,5-diiodothyropropionic acid (DITPA) on $I_o$ expression in the post-infarcted rat heart since this analogue binds to nuclear thyroid hormone receptors and alters the transcription of Tri-iodothyronine-responsive genes (Pennock et al., 1992). In animal models of cardiac hypertrophy, DITPA also increases cardiac contractility with apparently only minor effects on heart rate and metabolism compared to thyroid hormone itself (Mahaffy et al., 1995; Pennock et al., 1993). Our results show that chronic treatment of infarcted hearts with DITPA restored $I_o$ expression and hastened repolarization to levels observed in non-infarcted hearts. A preliminary report of our data has appeared (Wickenden et al., 1998).

**METHODS**

Please refer section 2.1, 2.2, 2.3, 2.4, 2.8, 2.11

**Statistical analysis**

All data are expressed as mean ± standard error of the mean (S.E.M). Data were collected from right ventricles and single right ventricular myocytes isolated from 3-8 hearts per group. For RNase protection assays, the arbitrary densitometric units were normalized to the value of the cyclophilin gene. Statistical analysis was done using the one-way ANOVA from SPSS program (Version 7.0 for Windows, SPSS Inc.). When ANOVA showed
statistical significance by F test, inter-group comparisons were made by the Student-Newman-Keuls procedure. A $P < 0.05$ was considered significant.
RESULTS

Effect of DITPA following myocardial infarction

In our study, we analyzed rats with infarct sizes greater than 30% (range: 30%-60%). Table 5 shows some of the general characteristics and hemodynamic parameters following MI and DITPA treatment. Infarct sizes were comparable between vehicle-treated post-MI and DITPA-treated post-MI animals (49.9 ± 4.1 %, n = 13, 52.7 ± 3.9 %, n = 4, and 53.2 ± 3.8 %, n = 13, for vehicle-treated, 3.75 mg Kg⁻¹ DITPA, and 10 mg Kg⁻¹ DITPA groups, respectively). Daily subcutaneous injections with DITPA had no effect on body weight. After DITPA treatment for 21 days, body weight increased by 5.6 ± 0.8 % (n = 14) in vehicle-treated post-MI animals, by 7.1 ± 1.6 % (n = 8) in 3.75 mg Kg⁻¹ DITPA-treated post-MI animals and by 4.2 ± 1.5 % (n = 14) in 10 mg Kg⁻¹ DITPA-treated post-MI animals. Lung-wet-weight to dry-weight ratios were slightly increased in the vehicle-treated post-MI animals compared to sham-operated animals but this did not reach statistical significance (4.4 ± 0.2, n = 11 versus 3.9 ± 0.2, n = 7 P = 0.1), and were unchanged in the DITPA-treated post-MI animals (3.75 mg Kg⁻¹ DITPA: 3.8 ±0.4, n = 4 10 mg Kg⁻¹ DITPA: 4.2 ± 0.2, n = 7, P > 0.05).

The effects of MI and DITPA treatment on hemodynamic properties of the hearts are summarized in Table 5. The most notable change was in left ventricular end-diastolic pressure (LVEDP) which was elevated following MI with or without DITPA treatment. Changes in the rate of contraction (+dP/dt) were unaffected following MI or DITPA treatment. The rate of relaxation (-dP/dt) was significantly (P < 0.05) decreased following
MI and was modestly restored following DITPA treatment. The lack of significance in these parameters might be explained by the variations in infarct sizes (Pfeffer et al., 1979).

Effect of DITPA on the transient outward current

From hereafter, right ventricular myocytes derived from sham-operated animals, vehicle-treated post-MI animals, 3.75 mg Kg\(^{-1}\) DITPA-treated post-MI animals and 10 mg Kg\(^{-1}\) DIPTA treated post-MI animals will be referred to as sham myocytes, post-MI myocytes, 3.75 mg Kg\(^{-1}\) DITPA post-MI myocytes and 10 mg Kg\(^{-1}\) DIPTA post-MI myocytes, respectively. Figure 6.1 shows representative normalized \(I_{\text{o}}\) traces derived from sham (A), post-MI (B), 3.75 mg Kg\(^{-1}\) DITPA post-MI (C) and 10 mg Kg\(^{-1}\) DIPTA post-MI myocytes (D). Comparison of Figures 5.1A and 5.1B illustrate that peak current density (\(I_{\text{peak}}\)) was reduced in post-MI myocytes compared to sham myocytes. Treatment with 3.75 mg Kg\(^{-1}\) DIPTA modestly increased \(I_{\text{peak}}\) while 10 mg Kg\(^{-1}\) DITPA restored \(I_{\text{peak}}\) to levels observed in sham-operated controls (Figure 5.1C). The contribution of individual K\(^{+}\) currents to the peak outward currents was determined by recording \(I_{\text{o}}, I_{\text{SS}}, I_{\text{K1}}\) separately (See Methods). \(I_{\text{o}}\) densities-voltage relationships are summarized in figure 5.1E. \(I_{\text{o}}\) density at +40 mV was significantly reduced from 14.0 ± 1.0 pA/pF (\(n = 21\)) to 10.2 ± 0.9 pA/pF (\(n = 41, P = 0.001\)) in sham myocytes compared to post-MI myocytes. Maximal whole cell transient outward channel conductance (\(G_{\text{max}}\)) was significantly reduced from 127.9 ± 9.8 pS/pF (\(n = 21\)) in sham myocytes to 84.6 ± 7.8 pS/pF (\(n = 41, P = 0.002\)) in post-MI myocytes. The voltage dependence of \(I_{\text{o}}\) activation in post-MI myocytes was slightly shifted in the hyperpolarizing direction compared to sham myocytes. The mid-point for steady-state activation (i.e. \(V_{1/2\text{act}}\)) was significantly reduced
while the slopes for activation (i.e. k) were unchanged in sham and post-MI myocytes, respectively.

Treatment of post-MI animals with 3.75 mg Kg⁻¹ DITPA increased Iₒ density to 11.2 ± 0.8 pA/pF, (@ +40 mV, n = 45) (P = 0.56) while 10 mg Kg⁻¹ DITPA treatment produced a marked increase in Iₒ density to 15.2 ± 1.1 pA/pF, (@ +40 mV, n = 45) (P = 0.001) compared to post-MI myocytes. Consistent with changes in Iₒ density, treatment of post-MI animals with 3.75 mg Kg⁻¹ DITPA did not alter Gₘₐₓ (107.2 ± 8.3 pS/pF, n = 45) while 10 mg Kg⁻¹ DITPA treatment in post-MI animals produced a significant increase in Gₘₐₓ (133.0 ± 10.7 pS/pF, n = 45). In spite of the changes in Gₘₐₓ, DITPA treatment had no effect on V₁/₂act. or k compared to vehicle treatment. Steady-state inactivation properties were not affected following MI or DITPA treatment as summarized in Table 6.

**Effect of DITPA on Kv4.2, Kv4.3 and Kv1.4 expression**

In order to investigate the molecular basis for the effects of DITPA on Iₒ, we employed RNase protection assays to measure mRNA levels of the “transient-outward like” K⁺ channel genes, Kv1.4, Kv4.2 and Kv4.3 (Dixon and McKinnon 1994). Since Iₒ was only marginally affected with 3.75 mg Kg⁻¹ DITPA, we only examined the effect with 10 mg Kg⁻¹ DITPA. To confirm that 10 mg Kg⁻¹ DITPA had the expected effects on gene expression under our experimental conditions, the mRNA levels of α-MHC and β-MHC were examined since these are strongly regulated by hypertrophy and thyroid hormone (Izumo et al., 1987). As shown in figure 5.2A, the ratio of β-MHC/α-MHC increased significantly following MI (Sham: 0.48 ± 0.01; n = 3 versus Post-MI: 1.79 ± 0.07; n = 3 P < 0.01). Consistent with previous results (Izumo et al., 1987), DITPA treatment normalized the ratio of β-MHC/α-MHC (0.59 ± 0.02, n = 3) to levels observed in sham-operated hearts.
Figure 5.2 also shows the results of RNase protection assays for Kv4.2 (B), Kv4.3 (C) and Kv1.4 (D) mRNA levels in right ventricle. Kvx mRNA levels were normalized for cyclophilin levels in each sample to account for possible variations in the loading conditions (Kaprielian et al., 1999; Wickenden et al., 1999). Mean Kv4.2/cyclophilin mRNA ratios were reduced from $0.73 \pm 0.03$ (n = 3) in sham samples to $0.51 \pm 0.02$ (n = 3) in vehicle-treated post-MI samples ($P < 0.01$). Following DITPA treatment Kv4.2 mRNA levels were significantly ($P < 0.01$) greater than those in untreated hearts ($0.63 \pm 0.01$, n = 3) indicating that DITPA partially restored Kv4.2 mRNA levels. Kv4.3/cyclophilin mRNA ratios were also significantly decreased for vehicle-treated post-MI hearts ($1.00 \pm 0.06$, n = 3) compared to sham-operated controls ($1.19 \pm 0.05$, n = 3, $P < 0.05$) but DITPA treatment did not reverse these changes ($0.889 \pm 0.002$, n = 3, $P > 0.05$).

As shown in Fig. 5.2C, Kv1.4 mRNA ratios were significantly ($P < 0.01$) increased in vehicle-treated post-MI ($0.55 \pm 0.03$, n = 3) compared to sham right ventricles ($0.36 \pm 0.04$, n = 3). DITPA treatment normalized Kv1.4 mRNA ratios to levels observed in sham-operated hearts ($0.37 \pm 0.02$, n = 3, $P > 0.05$).

Since the level of RNA expression may not always be indicative of protein expression levels, we also measured protein levels against Kv1.4 and Kv4.2 subunits. Kv4.3 protein was also measured but not reported due to rapid degradation of this protein under our experimental conditions. Figure 5.3A. shows a representative Western blot of protein from adult rat brain (Br, 10 µg) and right ventricles probed with anti-Kv4.2 antibody. The antibody labeled a single band in all samples with an approximate molecular weight of 77 kDa. As summarized in Figure 5.3B, the level of Kv4.2 protein was markedly reduced ($P = 0.03$) in the post-MI hearts compared to sham-operated hearts (Sham: $0.53 \pm 0.09$).
arbitrary units (n = 3) versus Post-MI: 0.21 ± 0.04 arbitrary units (n = 3). Fig 3A also shows that DITPA treatment of post-MI rats restores Kv4.2 protein (0.50 ± 0.04 arbitrary units, n = 3 P = 0.67) to levels observed in sham-operated animals correlating well with electrophysiological and Kv4.2 mRNA studies. Fig. 5.3C depicts a Western blot of the same protein samples probed with anti-Kv1.4 antibody and shows two distinct bands, in both brain and myocyte protein as shown previously in adult and cultured neonatal rat ventricular myocytes (Litovski and Antzelevitch, 1988). Consistent with the Kv1.4 mRNA measurements, Kv1.4 immunoreactivity increased from 0.43 ± 0.04 arbitrary units (P = 0.10) in sham-operated hearts (n = 3) to 0.68 ± 0.15 arbitrary units in post-MI hearts (n = 3). Following DITPA treatment, Kv1.4 protein levels were normalized to levels observed in sham-operated samples (0.224 ± 0.007 arbitrary units n = 3, P = 0.43).

**Effect of DITPA on other ion channels**

Given the effect of DITPA on $I_{K1}$, we wanted to examine whether similar findings can be applied to other ion channels. Figure 5.1F shows the current-voltage relationships for $I_{sus}$ evaluated at the end of a 500 msec depolarization period. At +40 mV, $I_{sus}$ density was not significantly different in post-MI myocytes (5.6 ± 0.3 pA/pF, n = 41) compared to sham myocytes (6.4 ± 0.4 pA/pF, n = 21) (P = 0.07). Treatment with 3.75 mg Kg$^{-1}$ DITPA (6.0 ± 0.3 pA/pF, n = 45 P = 0.06) and 10 mg Kg$^{-1}$ DITPA (7.0 ± 0.4 pA/pF, n = 45 P = 0.06) produced no changes in $I_{sus}$ density compared to vehicle-treated controls.

Figure 5.4 shows representative normalized $I_{K1}$ currents recorded from sham (A), post-MI (B), 3.75 mg Kg$^{-1}$ DITPA post-MI (C) and 10 mg Kg$^{-1}$ DITPA post-MI myocytes (D). Comparison of these figures demonstrates that $I_{K1}$ was lower in all post-MI myocytes compared to sham myocytes. $I_{K1}$ density evaluated at −130 mV was decreased significantly
(P < 0.006) in post-MI myocytes (-12.1 ± 0.8 pA/pF, n = 25) compared to sham myocytes (-15.7 ± 0.9 pA/pF, n = 25). Treatment with DITPA did not restore (P > 0.05) I_{K1} density to that observed in sham myocytes (-11.3 ± 1.0 pA/pF, n = 14 for 3.75 mg Kg⁻¹ DITPA and -10.8 ± 1.0 pA/pF, n = 10 for 10 mg Kg⁻¹ DITPA evaluated at -130 mV).

Next, we recorded L-type calcium currents (I_{Ca,L}). Fig. 5.5 shows representative normalized cadmium-subtracted I_{Ca,L} traces recorded in sham (A), post-MI (B), and 10 mg Kg⁻¹ DITPA post-MI (C) myocytes with the current-voltage relationship depicted in Fig. 5D. I_{Ca,L} density was unchanged following MI (I_{Ca,L} densities evaluated at 0 mV were -6.1 ± 0.5 pA/pF, n = 17, and -5.4 ± 0.3 pA/pF, n = 26 in myocytes from sham and post-MI myocytes, respectively; P > 0.05). DITPA treatment did not change I_{Ca,L} compared to vehicle-treated controls (I_{Ca,L} density evaluated at 0 mV was -6.6 ± 0.4 pA/pF, n = 20; P > 0.05).

I_{Na} density (data not shown) were also unchanged following MI and 10 mg Kg⁻¹ DITPA treatment (I_{Na} density evaluated at -35 mV was -17.6 ± 1.1 pA/pF n = 21, -16.5 ± 1.5 pA/pF n = 15 and -15.0 ± 1.0 pA/pF n = 11 in sham, post-MI and 10 mg Kg⁻¹ DITPA post-MI myocytes, respectively P > 0.05). Furthermore, mid-points for steady state inactivation (i.e. V_{1/2inact.} were -94.7 ± 3.2 mV (n = 4), -93.9 ± 1.1 mV (n = 4), and -91.8 ± 4.8 mV (n = 3) (P = 0.85) with the slopes measuring 4.6 ± 0.4 (n = 4), 5.5 ± 0.1 (n = 4), and 6.4 ± 0.6 (n = 3) (P < 0.05 only for sham versus post-MI DITPA) in sham, post-MI and 10 mg Kg⁻¹ DITPA post-MI myocytes, respectively.

**Effect of DITPA on action potential**

Since DITPA treatment was able to normalize I_{Io} density without affecting other current densities, we examined whether the increase in I_{Io} density in post-MI myocytes was
associated with action potential shortening. As shown in Figure 5.6, action potentials in sham myocytes typically had a spike-like appearance while the rate of repolarization in post-MI myocytes was slowed, resulting in a significant prolongation of action potential duration at both the 50% (APD$_{50}$) and 90% (APD$_{90}$) repolarization (APD$_{50}$ was 5.0 ± 0.8 ms, n = 21 and 12.6 ± 1.3, n = 15 P < 0.05 while APD$_{90}$ was 30.8 ± 3.5 ms, n = 21 and 55.7 ± 5.7 ms, n = 15 P < 0.05 for sham and post-MI myocytes respectively). Both 3.75 mg Kg$^{-1}$ DITPA and 10 mg Kg$^{-1}$ DITPA treatment accelerated repolarization and shortened action potential durations compared to vehicle-treated controls. The effect of 3.75 mg Kg$^{-1}$ DITPA was most apparent for APD$_{50}$ (8.2 ± 0.7 ms, n = 16 P < 0.01) whereas 10 mg Kg$^{-1}$ DITPA significantly (P < 0.01) shortened the APD$_{50}$ (8.0 ± 0.6 ms, n = 18) and APD$_{90}$ (35.3 ± 3.2 ms, n = 16) compared to the vehicle-treated controls. Resting membrane potential in post-MI myocytes was significantly (P < 0.001) depolarized (-71.1 ± 1.0 mV, n = 18) compared to sham myocytes (-76.0 ± 1.3 mV, n = 20). DITPA treatment did not affect the resting membrane potential (-69.2 ± 1.2 mV, n = 16 for 3.75 mg Kg$^{-1}$ DITPA post-MI and -71.1 ± 1.0 mV, n = 18 for 10 mg Kg$^{-1}$ DITPA post-MI, P > 0.05). It is conceivable that the changes in APD following DITPA treatment could result from changes in a number of currents during an action potential. However, as shown above, the changes in APD with DITPA treatment are not due to alterations in $I_{Na}$, $I_{K}$, $I_{Ca,L}$ and $I_{Na}$. The APD measurements (Figure 6.6) were performed under conditions where [Ca$^{2+}$]$_i$ transients might not be entirely eliminated. Therefore, the changes in APD with DITPA could have resulted from differences in sodium-calcium exchange currents ($I_{Na/Ca}$) secondary to changes in [Ca$^{2+}$]$_i$ transients. To test for this possibility, we also measured action potentials in the presence of 30 mM BAPTA in the pipette and 0.1 mM external Ca$^{2+}$ to practically eliminate [Ca$^{2+}$]$_i$
transients and $I_{Na/Ca}$. Under these conditions, the $APD_{50}$ (4.4 ± 0.8 ms, $n = 6$ and 10.5 ± 3.6 ms, $n = 5$ for sham and post-MI myocytes, respectively $P < 0.01$) and $APD_{90}$ (27.7 ± 4.6 ms and 49.1 ± 7.8 ms for sham and post-MI myocytes, respectively $P < 0.01$) were prolonged following MI. DITPA treatment (at 10 mg Kg$^{-1}$) restored $APD_{50}$ (4.9 ± 0.7 ms, $n = 6$) and $APD_{90}$ (31.2 ± 3.5 ms, $n = 6$) to levels observed in sham myocytes. To determine whether the changes in action potential duration recorded in single cells (under non-physiological conditions with respect to temperature, frequency and cellular dialysis) could be extended on a multicellular level and under physiological conditions, we recorded monophasic action potentials from the right ventricular wall in Langendorff hearts and are shown in Figure 5.7. Consistent with the single cell electrophysiology, $APD_{50}$ (17.9 ± 1.2 ms, $n = 14$ and 23.4 ± 1.4, $n = 10$, $P < 0.01$) and $APD_{90}$ (43.6 ± 2.0 ms, $n = 14$, and 55.0 ± 3.0 ms, $n = 10$, $P < 0.01$) were significantly prolonged following MI. DITPA treatment (at10 mg Kg$^{-1}$) normalized the APD in hearts derived from post-MI animals to values observed in hearts derived from sham-operated animals ($APD_{50} = 14.2 ± 0.8$ ms, $n = 9$ and $APD_{90} = 37.6 ± 1.1$ ms, $n = 9$).
DISCUSSION

Most previous studies in heart disease following left ventricular infarction have reported cellular, electrical and mechanical changes in the right and left ventricles (Kaprielian et al., 1999; Litwin and Bridge, 1997; Sethi et al., 1997; Qin et al., 1996; Orenstein et al., 1995; Afzal and Dhalla, 1992; Anversa et al., 1985). Alterations in the right ventricle following loss of left ventricular myocytes as a result of infarction are not unexpected for a number of reasons. For example, reductions in left ventricular contractility will directly increase the load on the right ventricle. Left ventricular changes will also affect right ventricular properties as a consequence of direct mechanical coupling between the two ventricular chambers. Furthermore, neurohumoral activation will produce circulating local factors that may affect both ventricles (Shimoni et al., 1997; Afzal and Dhalla, 1992). With respect to electrical alterations, down-regulation of the transient outward current ($I_{to}$) is consistently observed in human patients (Näbauer and Kääb, 1998; Näbauer et al., 1996) and in a variety of animal models of heart disease (Kaprielian et al., 1999; Litwin and Bridge, 1997; Kääb et al., 1996; Qin et al., 1996). $I_{to}$ down-regulation and action potential prolongation may lead to the onset of arrhythmias and may also contribute to elevations in intracellular calcium (Kaprielian et al., 1999) which can play a role in the pathogenesis of heart failure (Näbauer and Kääb, 1998; Wickenden et al., 1998). Normalization of $I_{to}$ may therefore be desirable in the diseased myocardium. Our study demonstrates that thyroid hormone can reverse the changes in cardiac $I_{to}$ expression following MI.

Effect of DITPA on the transient outward current Consistent with previous
observations (Kaprielian et al., 1999; Qin et al., 1996) $I_{in}$ density and conductance were significantly decreased following MI. The decrease in maximal whole cell conductance for the transient outward channel were mirrored by reductions in Kv4.2 and Kv4.3 expression. Interestingly, expression of the "fetal" $K^+$ channel gene, Kv1.4 (Wickenden et al., 1997; Xu et al., 1996) was increased following MI similar to that observed in hypertensive rats (Matsubara et al., 1993). The functional significance of an increased Kv1.4 expression despite a reduction in $I_{in}$ density is unclear. DITPA treatment restored $I_{in}$ density in post-MI myocytes to levels observed in sham myocytes. Increased $I_{in}$ density following DITPA treatment was accompanied with increases in Kv4.2 expression and decreases in Kv1.4 expression, reversing the pattern of changes observed following MI. The effects of DITPA on $K^+$ channel expression appear to be mediated at the transcriptional level, consistent with the known effects of thyroid hormone on Kv4.2 and Kv1.4 transcription in cardiac myocytes (Guo et al., 1998; Nishiyama et al., 1998; Shimoni et al., 1997; Wickenden et al., 1997). Interestingly, Kv4.3 RNA levels were unchanged following DITPA treatment which is consistent with previous findings reporting that Kv4.3 was not altered by thyroid status (Nishiyama et al., 1998).

Effect of DITPA on action potential duration

Consistent with previous observations, action potential prolongation occurs in non-infarcted right ventricular myocytes following MI (Kaprielian et al., 1999; Qin et al., 1996). Our study shows that DITPA treatment in post-MI hearts accelerated action potential repolarization. These results were further supported with whole heart recordings of monophasic action potentials which reflect more global changes in repolarizing currents. The mechanism regulating the rat action potential duration could conceivably
involve alterations in depolarizing (the L-type calcium current, \( I_{Ca,L} \), the sodium current, \( I_{Na} \), the sodium-calcium exchange current, \( I_{Na/Ca} \) and/or hyperpolarizing (the transient outward potassium current, \( I_{o} \), the delayed rectifier current, \( I_{K} \) or \( I_{sus} \), the inward rectifier current, \( I_{K1} \), the chloride current, \( I_{Cl} \) and the sodium-potassium ATPase) currents. Previous studies reported that sodium-calcium exchange currents were either increased (Litwin and Bridge, 1997) or decreased (Zhang et al., 1995) following MI and that the activity of the exchanger was decreased (Boerth and Artman, 1996) or unchanged (Cernohorsky et al., 1998) following daily thyroid hormone treatment. Any contribution by the \( \text{Na}^+/\text{Ca}^{2+} \) exchange current to the action potential were minimized in the present study by including 5 mM EGTA in the pipette or by reducing extracellular calcium to 0.1 mM and including 30 mM BAPTA in the pipette. Therefore, the changes in APD under our experimental conditions, at least in the single cell recordings, are unlikely to result from differences in \( I_{Na/Ca} \). A direct correlation between the changes in \( I_{o} \) and APD could not be definitively established in our studies. However, we found that \( I_{sus} \), \( I_{Ca,L} \) and \( I_{Na} \) were not affected by MI or DITPA treatment while changes in \( I_{K1} \) following MI were unaffected by DITPA treatment. \( I_{K1} \) was significantly decreased at the hyperpolarized potentials, but no statistical differences were detected at depolarized potentials (positive to \(-90\) mV), most likely due to the small magnitude of the current (Pinto and Boyden, 1999). The fact that resting membrane potentials were depolarized following MI is consistent with a decrease in \( I_{K1} \) density, although changes in other background currents cannot be ruled out.

**Limitations of the present study** A direct correlation between the changes in \( I_{o} \) expression and APD was not performed in this study, since it would require the
investigation of all the currents activated during a rat action potential and the use of a model to predict the action potential waveforms following MI and DITPA treatment. Therefore, we cannot directly correlate increase in $I_{io}$ expression to action potential shortening following DITPA treatment. Given our results, it seems nevertheless reasonable to conclude that changes in $I_{io}$ density contribute to the observed changes in APD following MI and DITPA treatment. In this study, we did not attempt to compare single cell action potentials derived with monophasic action potentials since the conditions (i.e. solutions and temperature) were quite different. The fact that the results are in agreement points to the relevance of our observations on global repolarization in the working heart.

The duration of the cardiac action potential is not uniform throughout the mammalian heart. Regional heterogeneity of action potential duration has been described in the normal myocardium (Näbauer et al., 1996; Clark et al., 1993; Litovski and Antzelevitch, 1988) and its modulation in the hypertrophied myocardium (Gomez et al., 1997). In the present study we made no attempt to determine whether the effects of MI and DITPA treatment varied regionally within the heart. Therefore, the anti-arrhythmic effects resulting from restoration of action potential duration and $I_{io}$ density remains to be determined. In Conclusion. We show that DITPA treatment in post-MI rats can restore $I_{io}$ density, and increase $Kv4.2$ expression to levels observed in sham-operated controls. DITPA treatment did not affect $I_{sus}$, $I_{K1}$, $I_{Ca,L}$ and $I_{Na}$. Consistent with the increase in the expression of $I_{io}$, a shortening of the action potential duration was observed following DITPA treatment. These data suggest that thyroid hormones and analogues might be useful in the reversal of electrical remodeling observed following MI.
TABLE 5. General characteristics and hemodynamic parameters following myocardial infarction and DITPA treatment. Lung W/D indicates lung wet-to-dry weight ratio. LVEDP indicates left ventricular diastolic pressure; LVSP indicates left ventricular systolic pressure; +dP/dT indicates the peak rate of pressure rise; -dP/dT indicates the peak rate of pressure decline. na: not applicable. § sham-operated versus post-MI vehicle; † sham-operated versus 10 mg Kg⁻¹ DITPA treatment; ‡ post-MI vehicle versus 10 mg Kg⁻¹ DITPA treatment; P < 0.05

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Post-MI Vehicle</th>
<th>Post-MI 10 mg Kg⁻¹ DITPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung W/D, g/g</td>
<td>3.9 ± 0.2</td>
<td>4.4 ± 0.2</td>
<td>4.2 ± 0.2</td>
</tr>
<tr>
<td>Infarct size, %</td>
<td>Na</td>
<td>49.9 ± 4.1</td>
<td>53.2 ± 3.8</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>2.3 ± 0.4§†</td>
<td>5.3 ± 0.98</td>
<td>7.8 ± 1.3</td>
</tr>
<tr>
<td>LVSP, mm Hg</td>
<td>100.1 ± 2.3</td>
<td>96.2 ± 1.5‡</td>
<td>102.8 ± 5.0</td>
</tr>
<tr>
<td>+dP/dt, mm Hg/s</td>
<td>7644 ± 356</td>
<td>5760.6 ± 641.3</td>
<td>7061 ± 358</td>
</tr>
<tr>
<td>-dP/dt, mm Hg/s</td>
<td>-5337.3 ± 250§</td>
<td>-4011.2 ± 362.2</td>
<td>-4674.5 ± 297.4</td>
</tr>
</tbody>
</table>
TABLE 6. Characteristics of the transient outward current following myocardial infarction and DITPA treatment. n indicates the number of myocytes. $G_{\text{max}}$ indicates the maximal conductance for the transient outward channel; $V_{1/2}$ indicates the mid-point for activation. $k$ indicates the slope factor. $\dagger$ sham-operated versus post-MI vehicle $\ddagger$ post-MI vehicle versus 10 mg Kg$^{-1}$ DITPA treatment $P < 0.05$.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Post-MI Vehicle</th>
<th>Post-MI 10 mg Kg$^{-1}$ DITPA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Steady state activation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>21</td>
<td>41</td>
<td>45</td>
</tr>
<tr>
<td>$G_{\text{max}}$ (pS/pF)</td>
<td>127.9 ± 9.8 $\dagger$</td>
<td>84.6 ± 7.8 $\ddagger$</td>
<td>133.0 ± 10.7</td>
</tr>
<tr>
<td>$V_{1/2}$ (mV)</td>
<td>14.3 ± 1.1 $\dagger$</td>
<td>9.4 ± 1.2</td>
<td>11.2 ± 1.1</td>
</tr>
<tr>
<td>$k$ (mV)</td>
<td>14.5 ± 0.4</td>
<td>12.8 ± 0.4</td>
<td>13.3 ± 0.4</td>
</tr>
<tr>
<td><strong>Steady state inactivation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>$V_{1/2}$ (mV)</td>
<td>-36.9 ± 1.1</td>
<td>-33.3 ± 1.1</td>
<td>-37.1 ± 2.1</td>
</tr>
<tr>
<td>$k$ (mV)</td>
<td>4.1 ± 0.1</td>
<td>3.9 ± 0.2</td>
<td>4.4 ± 0.2</td>
</tr>
</tbody>
</table>
FIGURE 5.1. Effect of DITPA on the transient outward (I<sub>to</sub>) and sustained currents (I<sub>sus</sub>) in right ventricular myocytes following myocardial infarction. Panels A-D show normalized traces of the transient outward current (I<sub>to</sub>) and sustained current (I<sub>sus</sub>) elicited by 500 ms voltage steps from -40 mV to +60 mV (in 20 mV steps) from a holding potential of -80 mV derived from sham, post-MI, 3.75 mg Kg<sup>-1</sup> DITPA post-MI, and 10 mg Kg<sup>-1</sup> DITPA post-MI myocytes. The I-V plot for I<sub>to</sub> (panel E) and I<sub>sus</sub> (panel F) for sham (- - -), post-MI (- - -) 3.75 mg Kg<sup>-1</sup> DITPA post-MI (- △ -) and 10 mg Kg<sup>-1</sup> DITPA post-MI (- ▽ -) myocytes. Myocytes were depolarized every 10 s. — indicates 0 pA pF<sup>-1</sup>. § sham versus post-MI; ‡ post-MI versus 10 mg Kg<sup>-1</sup> DITPA post-MI; P < 0.05.
![Image of a diagram showing mRNA levels for Kv4.2, Kv4.3, and Kv1.4 with sham, post-MI, and post-MI with DITPA treatments.](image)

**FIGURE 5.2.** Effect of DITPA on genes encoding the transient outward current and myosin heavy chain in the right ventricle. Representative comparison of mRNA levels for Kv4.2 (panel A), Kv4.3 (panel B) and Kv1.4 (panel C) from sham (S1, S2, S3), post-MI (V1, V2, V3) and 10 mg Kg\(^{-1}\) DITPA post-MI (D1, D2, D3) hearts. The top and bottom bands represent Kv and cyclophilin protected mRNA fragments, respectively. Panel D shows the expression of \(\alpha\) and \(\beta\) myosin heavy chain (MHC) isoforms. The bar graphs on the right side show mean changes in Kv mRNA and \(\alpha\) MHC (expressed as a ratio of \(\beta\)-MHC/\(\alpha\)-MHC normalized to cyclophilin levels) in sham (open bars, n=3), post-MI, (broken bars, n=3), and 10 mg Kg\(^{-1}\) DITPA post-MI (solid bars n=3) hearts, respectively. \(\dagger\) sham versus post-MI; \(\dagger\) sham versus 10 mg Kg\(^{-1}\) DITPA post-MI; \(\ddagger\) post-MI versus 10 mg Kg\(^{-1}\) DITPA post-MI; \(P<0.05\).
FIGURE 5.3. Effect of DITPA on Kv channel subunit immunoreactive proteins encoding the transient outward current in the right ventricle following myocardial infarction.

Panel A shows levels of Kv4.2 proteins from brain (Br), sham (S1, S2, S3), post-MI (V1, V2, V3) and 10 mg Kg\(^{-1}\) DITPA post-MI (D1, D2, D3) hearts. Panel C shows the levels of Kv1.4 proteins from brain (Br), sham (S1-S3), post-MI (V1-V3) and 10 mg Kg\(^{-1}\) DITPA post-MI (D1, D2, D3) hearts. The bar graphs shows mean changes in Kv4.2 (panel B) and Kv1.4 (panel D) in sham (open bars, n=3), post-MI (broken bars n=3) and 10 mg Kg\(^{-1}\) DITPA post-MI (solid bars, n=3) hearts. § sham versus post-MI; ‡ post-MI versus 10 mg Kg\(^{-1}\) DITPA post-MI; P< 0.05.
FIGURE 5.4. Effect of DITPA on the inward rectifier ($I_{K1}$) current in right ventricular myocytes following myocardial infarction. Panel A shows normalized $I_{K1}$ traces derived from sham (panel A), post-MI (panel B), 3.75 mg Kg$^{-1}$ DITPA post-MI (panel C), and 10 mg Kg$^{-1}$ DITPA post-MI (panel D) myocytes. The current traces show $I_{K1}$ traces which were elicited by 500 ms voltage steps from -130 mV to -60 mV from a holding potential of -80 mV. The $I_{K1}$ was normalized to membrane capacitance and plotted against voltage for sham (■), post-MI (●) 3.75 mg Kg$^{-1}$ DITPA post-MI (▲) and 10 mg Kg$^{-1}$ DITPA post-MI (▼) myocytes (Panel E). The steady state current measured at the end of the test pulse in the presence of barium (0.3 mM) was subtracted from the current evoked at the same voltage step in the absence of barium. Myocytes were depolarized every 10 s. — indicates 0 pA/pF. ¶ sham versus post-MI; $P < 0.05$. 
FIGURE 5.5. Effect of DITPA on the L-type calcium current ($I_{Ca,L}$) in right ventricular myocytes following myocardial infarction. Representative normalized $I_{Ca,L}$ are derived from sham (panel A), post-MI (panel B), and 10 mg Kg$^{-1}$ DITPA post-MI (panel C) myocytes. The current traces show cadmium-sensitive difference currents (0.3 mM CdCl$_2$) elicited by 500 ms voltage steps to -40, 0, +10 and +30 mV from a holding potential of -80 mV. The $I_{Ca,L}$ was normalized to membrane capacitance and plotted against voltage for sham (■), vehicle-treated post-MI (●) and 10 mg Kg$^{-1}$ DITPA-treated post-MI (▼) myocytes (Panel D). Myocytes were depolarized every 5 s. — indicates 0 pA pF$^{-1}$. No significant difference was observed in myocytes from sham, post-MI and 10 mg Kg$^{-1}$ DITPA post-MI groups.
FIGURE 5.6 Effect of DITPA on action potentials in right ventricular myocytes following myocardial infarction. Representative action potential traces are derived from sham (panel A), post-MI (panel B), 3.75 mg Kg⁻¹ DITPA post-MI (panel C) and 10 mg Kg⁻¹ DITPA post-MI (panel D) myocytes. The bar graph in panel E shows mean changes in action potential duration at 50% and 90% repolarization in sham (white bars, n = 21), post-MI (gray bars n = 15), 3.75 mg Kg⁻¹ DITPA post-MI (broken bars, n = 16), and 10 mg Kg⁻¹ DITPA post-MI (black bars, n = 18) myocytes. Action potentials were elicited by a brief (5 ms) supra-threshold injection of depolarizing current (2 x threshold) applied at 0.1 Hz. --- indicates 0 mV. § sham versus post-MI; † sham versus 10 mg Kg⁻¹ DITPA post-MI; ‡ post-MI versus 10 mg Kg⁻¹ DITPA post-MI; * sham versus 3.75 mg Kg⁻¹ DITPA post-MI; $P < 0.05.$
FIGURE 5.7. Effect of DITPA on monophasic action potentials in the right ventricular free wall following myocardial infarction. Recordings are obtained from hearts derived from sham (panel A), vehicle-treated post-MI (panel B), and 10 mg Kg⁻¹ DITPA-treated post-MI (panel C) animals. The bar graph in panel D shows mean changes in action potential duration at 50% and 90% repolarization in sham (white bars, n=21), post-MI (gray bars n=15), and 10 mg Kg⁻¹ DITPA post-MI (dark gray bars, n=18). § sham versus post-MI; † sham versus 10 mg Kg⁻¹ DITPA post-MI; ‡ post-MI versus 10 mg Kg⁻¹ DITPA post-MI; P<0.05.
CHAPTER 7. DISCUSSION

The discussion of the results is provided at the end of each manuscript. In this section I will provide an overview of the findings and propose future studies in the area. I will discuss certain aspects of ventricular remodeling in cardiac hypertrophy and failure, discuss the regional variations of $I_{1o}$ down-regulation, discuss the contribution of specific $K^+$ channel genes on $I_{1o}$, discuss the consequence of $I_{1o}$ down-regulation on calcium handling, discuss the possibility that $I_{1o}$ downregulation may contribute to disease progression, and discuss the role of thyroid hormone on restoring $I_{1o}$ expression.

Neurohormonal activation and ventricular remodeling and disease progression

It is widely believed that myocyte stress in the form of stretch and ischemia triggers a neurohormonal activation involving the adrenergic and renin-angiotensin systems. These neurohormones increase the expression of “early” genes such as c-fos, c-myc and egr-1 (Yue et al., 1998; Gidh-Jain et al., 1998; Larsen et al., 1998; Izumo et al., 1988) which are associated with cellular hypertrophy. Tonic stimulation of neurohormones increases the expression of a number of other “late” genes including $\beta$-myosin heavy chain and skeletal actin leading to further remodeling of the surviving myocytes (Swynghedaw, 1999; Dunnmon et al., 1990). The difference in the expression characteristic between the activation of early and late genes is simply due to their intrinsic rate of turnover. For example, a gene with a high rate of turnover would be expected to show significant changes in its expression in the early phases of the disease. On the other hand a gene with a slow turnover would be expected to show little changes in its expression pattern in the early phases of the disease.
Compensated (adaptive) cardiac hypertrophy versus cardiac failure

Myocardial hypertrophy is an early milestone during the course of heart failure and is an important risk factor for subsequent cardiac morbidity and mortality. Since cardiac hypertrophy and failure can be interrelated, it is often difficult to separate these disease states. There is a broad gap between identifying the regulation of genes associated with cardiac hypertrophy and understand how these genes can lead to cardiac abnormalities and failure. Several differences in molecular and biochemical markers may fill in this gap, but the time course of the disease seems to be important since some molecular markers can change with disease progression. For my studies, the eighth week time point is representative of compensated hypertrophy in the rat model of coronary artery ligation (Hasenhuss 1998). The rats appear to be in a state of compensated hypertrophy since there were no physical signs of heart failure, mortality and ascites. At the mechanical level (i.e. excitation-contraction coupling), several studies have demonstrated a reduction of sarcoplasmic reticulum expression and function over prolonged disease states causing a progressive deterioration of contractile function (Yue et al., 1998; Sethi et al., 1997; Yoshiyama et al., 1997; Arai et al., 1996; Afzal and Dhall, 1992). For example, SERCA2a expression was found to be unchanged during compensated hypertrophy at 3 weeks (Huang et al., 1999) and 6 weeks (Yoshiyama et al., 1997) but was significantly decreased during heart failure at 3 months following myocardial infarction (Yoshiyama et al., 1997). The SR Ca²⁺ content was reported to be increased in the right ventricle in rats and rabbits at eight weeks, but was either unchanged or significantly decreased at 16 weeks following myocardial infarction.
Furthermore, mitochondrial oxygen metabolism and tissue ATP levels which regulate SR Ca\(^{2+}\) function begin to decrease after the 8 week time point and worsen until the 12\(^{th}\) week (Sanbe and Tandonaka 1993). Aside from reduction in SR function, excitation-contraction coupling mechanisms is depressed in animal models of heart failure (O'Rourke et al., 1999; Gomez et al., 1997). Gomez and colleagues reasoned that the reduction in [Ca\(^{2+}\)]\(_i\) amplitude may be related to a defect in E-C coupling which may be attributed of a geometric distortion of the space between L-type calcium channels and ryanodine receptors (Gomez et al., 1997). In compensated cardiac hypertrophy, [Ca\(^{2+}\)]\(_i\) amplitude is increased as demonstrated in spontaneous hypertensive rats, a model of cardiac hypertrophy without the presence of cardiac failure (Shorofsky et al., 1999; Brooksby et al., 1993).

At the molecular level, differential activation of mitogen activated protein kinase (MAPkinase) pathways and growth factors can possibly explain whether the cardiac myocyte will undergo adaptive cardiac hypertrophy or fail (Hunter and Chien 1999 with references therein). For example, the two isoforms of p38, namely α and β, can provide important switches in the pathways between apoptosis and adaptive hypertrophy. Furthermore, calcineurin activity is increased in adaptive cardiac hypertrophy, but attenuated in cardiac hypertrophy whereas SAPkinase activation is unchanged in hypertrophy and is markedly elevated in advanced heart failure (unpublished observations from the laboratories of Drs. Hajjar, Force and Molkentin).
Action potential prolongation phenotype

Action potential duration prolongation is a prominent feature observed in diseased and hypertrophied myocardium. During the process of remodeling, a reduction in I\textsubscript{to} expression and action potential prolongation occur in the MI model (Kaprielian et al., 1999; Rozanski et al., 1998; Qin et al., 1996) and a number of other models of cardiac hypertrophy (Bryant et al., 1999; Gomez et al., 1997; Bryant et al., 1997; Kaab et al., 1996). Accumulating evidence from my thesis indicate that I\textsubscript{to} downregulation occurs as early as 48 hours after the induction of myocardial infarction which is consistent with data obtained by others (Yao et al., 1999). The rapid onset of the I\textsubscript{to} downregulation may be due to the high turnover rate of Kv\textsubscript{x} genes in the myocyte. A discussion on the regulation of I\textsubscript{to}-based Kv\textsubscript{x} genes is provided below. Irrespective of the mechanism, downregulation of I\textsubscript{to} causes a prolongation of action potential duration. APD prolongation can elevate [Ca\textsuperscript{2+}]\textsubscript{i} and increase cardiac contractility in the surviving hypertrophied myocytes as a compensatory mechanism for the loss of myocytes. APD prolongation can elevate [Ca\textsuperscript{2+}]\textsubscript{i} and together with neurohormones can activate MAP kinase pathways and induce cellular hypertrophy. APD prolongation can also provide a substrate for the development of cardiac arrhythmias.

1) Elevation in [Ca\textsuperscript{2+}]\textsubscript{i}:

APD prolongation causes elevation in [Ca\textsuperscript{2+}]\textsubscript{i} as a result of increasing calcium current through L-type Ca\textsuperscript{2+} channels as demonstrated in Chapter 3 (Kaprielian et al., 1999) and other studies (Volk et al., 1999; Bouchard et al., 1995; Brooksby et al., 1993). Data in progress in the laboratory by Rajan Sah (personal communication) demonstrate that APD lengthening especially in the early phase of repolarization seem to be the most
important in causing elevations in \([\text{Ca}^{2+}]_i\). This result is not surprising since calcium channels are open during that phase of the action potential corresponding to approximately 25-50% repolarization. Extensive mathematical modeling is required to simulate variations in APD\(_{25-50}\) and predicting \([\text{Ca}^{2+}]_i\). While the elevation in \([\text{Ca}^{2+}]_i\) transient amplitude is due to an increase in \(I_{\text{Ca,L}}\), other cellular mechanisms may also cause a rise in \(\text{Ca}^{2+}\) transient amplitude such as changes in \(\text{Na}^+/\text{Ca}^{2+}\) exchange activity, a decrease in the affinity of contractile proteins to calcium and changes in the expression and function of sarcoplasmic proteins, irrespective of the changes in action potential duration. These parameters were not examined in this study but several groups have demonstrated that the properties of myofilaments (Holt et al., 1998; Anversa 1993) and SR function are not changed in post-MI myocytes. Regardless, elevations in \([\text{Ca}^{2+}]_i\) may provide a compensatory mechanism whereby cardiac contractility is improved. In this regard, experiments performed in the laboratory by Z. Kassiri (MSc Thesis- University of Toronto Press) show that peak twitch force is elevated in isolated trabeculae derived from post-MI animals compared to their sham counterparts. These changes in calcium handling observed in my studies represent the early events occurring during compensated hypertrophy.

2) Arrhythmogenesis

In the normal heart, the heterogeneity in repolarizing currents guards the myocardium against torsades de pointes and re-entry type arrhythmias (Tomaselli et al., 1994). Following myocardial infarction, prolongation of APD and a loss of the APD heterogeneity occurs as a result of the regional modulation of \(I_{\text{to}}\) as reported in Chapter 4 which is consistent with other animal models of cardiac disease (Bryant et al., 1999;
Gomez et al., 1997. The mechanism of generating arrhythmias is complex and probably involves the loss of K⁺ channel heterogeneity as well as action potential prolongation. Nuss and colleagues (1999) recently suggested that electrical changes at the single cell level provide a basis for generating arrhythmias. In this regard, the attenuation of Kᵥ1.1- (London et al., 1998) and Kᵥ2.1 (Xu et al, 1999)-based K⁺ currents in transgenic mice result in action potential prolongation and triggered activity in single cell recordings to premature ventricular beats in electrocardiographic recordings. These results are consistent with an increased propensity to develop and to sustain cardiac arrhythmias. While we did not study arrhythmias in this study, they can be detected as early as 4 weeks following myocardial infarction (Qin et al., 1996).

3) Role of APD prolongation in cardiac hypertrophy and failure: a unified hypothesis

We hypothesize that reduction in Iₒ prolongs action potential duration resulting in important elevations in [Ca²⁺]ᵢ which may activate calcium-dependent pathways such as MAPKinase and calcineurin pathways. The activation of these stress pathways can contribute to the disease progression. Since Iₒ downregulation and APD prolongation is a consistent finding in various animal models of cardiac hypertrophy and failure, we developed a strategy to create transgenic mice overexpressing the N-terminal fragment of Kᵥ4.2 and inhibit Iₒ by a dominant-negative mechanism (Wickenden et al., 1999). Electrophysiological analysis revealed that Iₒ was reduced and action potential duration was prolonged at 2-4 weeks of age. Interestingly, these electrical changes were associated with the development of cardiac hypertrophy. These mice developed cardiac failure by 10-12 weeks of age as evidenced by marked changes in molecular and cellular
remodeling and poor hemodynamic performance. Furthermore, I found that \( I_{n0} \) downregulation occurs as early as 48 hours after the induction of myocardial infarction (unpublished observations figure IX.) which is consistent with data obtained by others (Yao et al., 1999). These results support the notion that APD prolongation secondary to reduction in \( I_{n0} \) may be important in the progression of cardiac disease.

It must be remembered that the mere presence of action potential prolongation alone does not cause heart failure, since a variety of animal models and humans with long Q-T syndrome exhibit prolonged action potentials without the presence of heart failure (O’Rourke et al., 1999; Shorofsky et al., 1999; Keating and Sanguineti 1996). Therefore, the progression of cardiac hypertrophy to failure may not be explained by action potential prolongation alone, but probably involves a complex interaction of calcium with signaling pathways and continual alterations in gene expression. Furthermore, prolongation of action potential does not necessarily result in elevations in \( \text{Ca}^{2+} \) transient amplitude. In this regard, it was previously shown that \( \text{Ca}^{2+} \) transients amplitude is decreased in the pacing dog model of heart failure despite the presence of prolonged action potentials (O’Rourke et al., 1999, Maltsev et al., 1998). These findings are not unexpected since SERCA2a expression is decreased in that particular model. Considering the complex cascade of physiological, neurohormonal, and biochemical abnormalities associated with heart failure, it is difficult to assess the time period when the heart will no longer be able to compensate for the initial cardiac insult and instead plunge into cardiac failure.
Role of hormones on signal transduction: The important role of intracellular calcium.

Hormones such as angiotensin II (Ang II), endothelin-1 (ET-1), and norepinephrine (NE) known to be elevated in diseased hearts can increase the expression of a number of embryonic genes such as c-fos, c-myc and egr-1 but can also activate a variety of intracellular kinases including protein kinase A, protein kinase C, tyrosine kinase mitogen-activated protein kinases (Force et al., 1999; Hunter and Chien 1999; Bogoyevitch et al., 1993; 1994; 1996). Briefly, these hormones can bind to their respective receptors and activate second messenger systems to elevate intracellular calcium through a G-protein transduction mechanism. These hormones are collectively referred to as G protein coupled receptor agonists (GPCR). These agonists bind to their respective receptors which are coupled through Gq family. The family of G proteins activate phospholipase C-β which catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). DAG activates PKC activity while IP3 increases [Ca2+]i by promoting the release of Ca2+ from the endoplasmic reticulum (for review Dorn et al., 1999). As mentioned above, another possibility by which [Ca2+]i is increased is via downregulation of K+ channel and action potential prolongation.

Aside from its primary role in excitation-contraction coupling, Ca2+ is an attractive candidate for the induction of embryonic genes and activation of signal transduction pathways including a variety of intracellular kinases such as calmodulin kinase, protein kinase C, mitogen-activated protein kinases and phosphatases such as calcineurin (Force et al., 1999; Hunter and Chien 1999; Sugden 1999; Molkentin et al.,
In support of this hypothesis, GPCR activation has been shown to induce the expression of the early genes (Larsen et al., 1998; Izumo et al., 1988) which may be attenuated by the addition of calcium channel blockers (Grohe et al., 1994). Furthermore, direct elevations of extracellular calcium ([Perreault et al., 1994], treatment with calcium ionophores (Ito et al., 1991) can elevate [Ca^{2+}]_i and induce cardiomyocyte hypertrophy. Membrane depolarization can also increase Ca^{2+} influx into cells resulting in activation of Ca^{2+}-calmodulin dependent protein kinases and MAP kinase pathways (Enslen et al., 1996). Interestingly, chelation of Ca^{2+} can completely abolish agonist-evoked MAP kinase activation (Yamazaki et al., 1997; Sadoshima et al., 1995). While chelation of Ca^{2+} has been shown to abolish agonist-induced MAP kinase activation and abrogate the hypertrophic response, other studies have shown that targeted inhibition of SAPKInase (Choukroun et al., 1998; 1999) and calcineurin (Taigen et al., 2000) pathways can also attenuate agonist-induced cardiomyocyte hypertrophy.

The progression of cardiac hypertrophy to cardiac failure is possibly linked to these elevations in intracellular calcium, but a trigger of other events such as activation of apoptotic pathways may be important. Furthermore, elevations in [Ca^{2+}]_i can activate specific signaling pathways resulting in alteration in gene expression in a maladaptive way. For example, reduction of the gene regulating SR function (i.e. SERCA2a) can alter the contractile cellular function. Also excessive elevations in [Ca^{2+}]_i can lead to abnormal contractions and increase the propensity for the development of arrhythmias.

At the present time, it is unclear whether the cardiac myocyte can discriminate between the calcium involved in the normal processes of excitation-contraction coupling and the calcium involved in the induction of hypertrophic/apoptotic pathways. As such
several questions remain unanswered. Are there two distinct pools for intracellular calcium for the diverse cellular processes? Which calcium is important, namely systolic or diastolic levels or oscillations? Are elevations in \([\text{Ca}^{2+}]_i\) as a result of action potential prolongation sufficient to activate \(\text{Ca}^{2+}\)-sensitive signaling pathways and induce cardiac hypertrophy? Data from confocal microscopy shows that calcium entry into myocardial cells occurs in microdomains (Cheng et al., 1993; Gomez et al., 1997). In this regard, it was recently proposed that a \(\text{Ca}^{2+}\)-sensitive signaling system may exist in cardiac myocytes and may be important in the induction of hypertrophy (Olson and Molkentin, 1999). Furthermore, intracellular calcium oscillations have been shown to modulate gene transcription (Dolmetsch et al., 1997). Based on the data from this thesis and other work, I have drawn a schematic representation of the events leading up to cardiac hypertrophy and failure (Figure VI). It demonstrates the possible link between neurohormonal factors, \(I_{\text{to}}\) reduction, action potential prolongation, elevations in intracellular calcium, alterations in gene expression and activation of stress pathways involved in hypertrophy and apoptosis.
Molecular basis of $I_{to}$ and its relationship to electrical recordings

Several $K^+$ channel $\alpha$ subunits such as $Kv1.4$, $Kv4.2$ and $Kv4.3$ generate transient outward currents similar to $I_{to}$ and have been detected in rat cardiac myocytes at the mRNA and protein level (Dixon and McKinnon, 1994; Barry et al., 1995). Using RT-PCR, other $K^+$ channel $\alpha$ subunits such as $Kv3.3$ and $Kv3.4$ encoding for transient outward-like currents have been identified (Ohya et al., 1997). However, these subunits are not expected to contribute to rat cardiac $I_{to}$, since the expression of these genes at the mRNA and protein level is very low in the rat heart (Dixon and McKinnon, 1994). For normal biogenesis and function of $I_{to}$ channels, four compatible $\alpha$ subunits from a given subfamily must co-assemble together (Li et al., 1992). For example, $Kv4.2$ $\alpha$-subunits can either form homotetramers or can co-assemble with $Kv4.3$ $\alpha$-subunits to form heterotetramers. Similarly, $Kv1.4$ $\alpha$-subunits can either form homotetramers or can co-assemble with delayed rectifier encoding genes such as $Kv1.2$, $Kv1.5$ to form heterotetramers (Po et al., 1993). $Kv4$ homo or heterotetramers channels display fast recovery from inactivation kinetics similar to native $I_{to}$ expressed in rat ventricular myocytes. On the other hand, $Kv1$ homo or heterotetramers exhibit slowed recovery kinetics (Po et al., 1993; Tseng-Crank et al., 1990) that can reach 15-20 seconds. RNAse protection and western blot assays suggests that $Kv4.x$ and not $Kv1.x$ are major contributors to cardiac $I_{to}$ in rat ventricular myocytes (Barry et al., 1995; Dixon and McKinnon, 1994). Furthermore, the use of dominant-negative and anti-sense strategies against $Kv4.x$ genes (Johns et al., 1997; Fiset et al., 1997) have revealed that $I_{to}$ is predominately encoded by $Kv4.2/4.3$ gene products. Therefore, the importance of the
Kv1.4 channel and its contribution to cardiac $I_{\text{to}}$ in the adult rat appears to be minimal in comparison to Kv4.2/4.3.

**Regional modulation of Kv4.x and Kv1.x-based $I_{\text{to}}$ in normal and post-MI myocytes**

The duration of the cardiac action potential is not uniform throughout the mammalian heart where the action potential duration (APD) is of longer duration in endocardial myocytes compared to epicardial myocytes (Litovsky et al., 1988; Furukawa et al., 1990; Clark et al., 1993; Fedida and Giles 1991, Li et al., 1998; Bailly et al., 1997). These differences in action potential duration explain the electrical heterogeneity that exists in the normal myocardium and is attributed to a higher density of the calcium-independent transient outward current ($I_{\text{to}}$) in epicardial myocytes compared to endocardial myocytes (Litovsky et al., 1988; Fedida & Giles 1991; Antzelevitch et al., 1991; Benitah et al., 1993; Clark et al., 1993; Li et al., 1998). Specifically, it seems that the heterogeneity of Kv1.x and Kv4.x expression explains the differences in $I_{\text{to}}$ density and APD across the ventricular wall and between the different myocardial regions. The available evidence suggests that Kv4.x is predominately expressed in epicardial myocytes, while Kv1.x is predominately expressed in endocardial myocytes. In this regard, a large gradient of Kv4.2 expression exists across the ventricular myocardium with the highest expression observed in the epicardium and the lowest expression observed in the endocardium (Brahmajothi et al., 1999; Dixon and McKinnon, 1994). The reason for the regional difference in $I_{\text{to}}$ density and action potential duration is not well understood but may be due to the role of the specific myocyte in cardiac excitation-contraction. It is possible that endocardial cells are primarily responsible for cardiac contraction whereas epicardial cells are primarily responsible for cardiac conduction.
Therefore, it is conceivable that the prolonged action potentials in the endocardium as compared to the epicardium may serve a role to regulate cardiac contractility. In my studies, I did not specifically record from epicardial and endocardial myocytes but electrophysiologic and molecular evidence indicate that right ventricular myocytes are mostly of epicardial origin while septal myocytes are mostly of endocardial origin (Wickenden et al., 1999; Brahmajothi et al., 1999; Dixon and McKinnon, 1994; Clark et al., 1993). We reported that Kv4.2 expression (detected at the mRNA and protein levels) and I_o density is higher in the right ventricular wall compared to the septum (Wickenden et al., 1999). Kv1.4 expression is also regionally regulated with a large predominance in the atria (Wang et al., 1999; Dixon and McKinnon 1994) and in the ventricular endocardium (Brahmajothi et al., 1999; Dixon and McKinnon, 1994) compared to other regions of the heart.

Following myocardial infarction, Kv4.2 expression is drastically reduced and Kv1.4 is increased in right ventricular myocytes. This change in the molecular profile of K^+ channel genes is expected to reduce I_o density and slow the recovery kinetics which was readily observed in right ventricular myocytes. In contrast to right ventricular myocytes, Kv4.2 is not significantly reduced which was corroborated with our observation that I_o density was not significantly reduced in the septum. The reason for the regional modulation I_o may be related to the fact that Kv4.2 expression is relatively low in the septum compared to the right ventricle of the normal heart. Another possibility may be due to differences in pressure prior to and after myocardial infarction which can effectively change loading conditions on the myocytes or to inherent differences in the expression of G protein coupled receptors between the two regions. Following
myocardial infarction, the loss of left ventricular myocytes leads to a drop in left ventricular cardiac output and a reduction in left ventricular pressure. Over a prolonged period of time, the drop of left ventricular pressure can increase the loading condition on the right ventricle (i.e. by way of damming of blood from the left atrium to the pulmonary circulation). Therefore, the right ventricle is faced with a higher pressure following infarction of the left ventricle and the right ventricular cells respond to the insult by prolonging action potential duration and in essence convert their action potential phenotype to that observed in left ventricular myocytes prior to infarction. The left ventricular pressure does not increase but decrease which may explain why \( I_{\alpha} \) expression and prolongation of action potential duration are less severe in the septum compared to those observed in right ventricular.

Our results also indicate that Kv4.2/4.3 expression are reduced while Kv1.4 expression is increased following myocardial infarction. While the reduction of Kv4.2/4.3 explains the observed reduction in \( I_{\alpha} \) density, the increase in Kv1.4 expression is expected to somewhat offset the changes leading to insignificant changes in \( I_{\alpha} \) density.

Again, the role of Kv1.4 α-subunits in encoding adult \( I_{\alpha} \) is known to be less prominent compared to Kv4.2/4.3 α-subunits and its increased expression may not compensate for the large reduction in Kv4.2/4.3 expression.

**Kvx expression in cardiac development and disease**

In the mammalian heart, \( I_{\alpha} \) density is low during embryonic life and is increased during the course of development. Furthermore, the expression profile of specific Kvx genes is quite different. In neonatal rat ventricular myocytes, Kv1.4 expression is high and decrease during development (Guo et al., 1998; Wickenden et al., 1997). On the
other hand, Kv4.2 expression is low and is increased during development (Guo et al., 1998; Wickenden et al., 1997). Following myocardial infarction, Kv4.2/4.3 expression is markedly reduced while Kv1.4 expression is increased which closely resemble the expression pattern observed in neonatal rat ventricular myocytes. Therefore, a recapitulation of the fetal gene program is not unique to structural and contractile proteins but is also extended to K⁺ channels (Wickenden et al., 1997; Xu et al., 1996). In this study, although Kv1.4 mRNA and protein were detected, the total Iₒ density is probably comprised almost entirely of Kv4.x gene products. The increase of Kv1.4 expression may explain the emergence of slowed recovery kinetics for Iₒ in post-MI myocytes which is also evident in neonatal myocytes (Wickenden et al., 1997). Iₒ channels open following a step depolarization and quickly enter into an inactivated (non-conducting) state and require channel repolarization to recover from the inactivated state for subsequent openings. Iₒ measured under my recording conditions (where step depolarizations are provided every 5 seconds) may not include any contribution from Kv1.x channels to the overall current density since the recovery from inactivation lasts more than 20 seconds. In other words, Kv1.x-based Iₒ may not be accounted in our recordings since data acquisition was faster than the time required for the channels to open and recover from inactivation.

**Mechanism of Iₒ downregulation**

The mechanisms involved by which K⁺ channel gene expression occurs was not examined in this Thesis. In this section, I will present some arguments for the mechanisms by which Iₒ–based gene regulation occurs and propose experiments that may help address these issues. Despite the fact that reduction in Iₒ density is a hallmark
finding in a variety of animal models of heart disease and human heart disease. Reduction in the expression of $I_{to}$-based $K^+$ channel genes is believed to involve extrinsic and intrinsic factors. Extrinsic stimuli include neurohormonal factors which are known to be elevated in heart disease. Several studies have shown $I_{to}$ down-regulation may be induced by a number of agonists including $\alpha$-adrenergic (Parker et al., 1999; Guo et al., 1998; Gaughan et al., 1998; Fedida and Bouchard, 1992; Braun et al., 1990; Apkon and Nerbonne, 1988), angiotensin II (Nagatomo et al., 1995), endothelin-1 (Guo et al., 1998; Shimoda et al., 1998; Damron et al., 1993), growth factors (Guo et al., 1998; Heath et al., 1998; Liu et al., 1998) and other paracrine factors released from cardiac non-myocytes (Guo et al., 1998). As mentioned above, these agonists through GPCR activation can increase PKC activity. Interestingly, downregulation of Kv1.4-based $I_{to}$ was originally proposed to occur as a result of activation of protein kinase C which can phosphorylate specific amino acid residues and inactivate the channels, thereby decreasing the availability of channel opening (Murray et al., 1994). More recently, PKC was reported to decrease Kv4.2 and Kv4.3 based $I_{to}$ which was not related to channel inactivation (Nakamura et al., 1997). At the present time the mechanism by which PKC can inhibit $I_{to}$ is unclear. One interesting possibility may be related to the fact that GPCR activation can elevate intracellular calcium which can in turn activate signal transduction pathways and reduce $K^+$ channel gene expression.

Aside from GPCR activation, cardiomyocyte stretch can also reduce $K^+$ channel gene expression involving the paracrine release of angiotensin II by non myocytes such as fibroblasts (Guo et al., 1998). It was recently reported that unidentified paracrine factors released from cardiac nonmyocytes can downregulate Kv4.2-based $I_{to}$ (Guo et al.,
An increase in cardiac afterload can also reduce $I_{to}$ by causing specific reductions in Kv4.x expression (Takimoto et al., 1997). The mechanism by which stretch can reduce $I_{to}$ is unclear but one study showed that stretch can also increase $[Ca^{2+}]$. (Gannier F and Le Guennec JY, 1996). Caution must be taken when extrapolating results from myocyte stretch studies to the *in vivo* hypertrophic and failing heart since the loading conditions may be quite different. In summary, down-regulation of $I_{to}$ in the setting of heart disease may be complex since intrinsic (i.e. stretch) and extrinsic (i.e.neurohormones) factors are involved both of which can increase intracellular calcium and activate various signaling pathways.

The regulation of Kv4 gene expression

While some hormones/growth factors modulating $I_{to}$ expression are beginning to emerge, the mechanism regulating the expression of these $K^+$ channel genes is/are not known. Clearly, ribonuclease protection assays and western blotting reveal that Kv4.2/4.3 are reduced. These techniques reveal information on the end-product but provides little evidence on the mechanism by which the specific gene is down-regulated. Gene expression is accomplished by the transfer of genetic information from DNA to RNA molecules and then from RNA to protein molecules. Clearly, the reduction in gene expression can be due to several mechanisms such as a decrease in the rate of transcription, a reduction in mRNA stability and an increase in mRNA degradation. The first step in transcription is the binding of RNA polymerase to a DNA molecule in the promoter region. If a hormone or growth factor regulates transcription, it must somehow signal the DNA. This can be achieved with transcriptional factors (i.e DNA, RNA or proteins) which can bind to specific sequences in the promoter region known as repressor
or enhancer elements thereby altering the synthesis of RNA. Hormones can also increase the expression of these transcriptional factors to amplify the signal. Suffice it to say, that the sequence of the entire promoter region of Kv channels is not completely deciphered. However, a preliminary report reveals that the promoter of Kv4.2 contains a repressor element (TGGGGTAAAC) and a CArG-Box which may influence transcription (Kong et al., 1999 AHA meeting abstract Atlanta, Georgia, 1999). Gene transcription is a continual process and probing the rate of transcription is difficult unless the internal controls of gene transcription are inhibited. The rate of transcription of a particular Kvx gene may be tested by extracting cell nuclei and quickly arresting the internal transcriptional machinery. $^{32}$P-labeled nucleotides and several reaction buffers are added to the extracted nuclei. Newly synthesized $^{32}$P-labelled mRNA is extracted and cross-hybridized with denatured (i.e. single-stranded) cDNA under identical loading condition. Any difference in the intensity of cDNA-mRNA hybrid end-products between sham and post-MI samples will reveal information on the rate of transcription. Another mechanism whereby K$^+$ channel gene expression may be reduced is by reducing mRNA stability and/or increasing mRNA degradation. Since mRNA is continuously produced, it is difficult to distinguish a reduction in mRNA synthesis from an increase in mRNA degradation by simply examining the mRNA levels by Northern blots or ribonuclease protection assay as used in these studies. One way of probing this possibility is by disturbing the system and examining the outcome. For example, one can examine the stability of mRNA by arresting transcription (i.e. actinomycin D or RNA polymerase inhibitor) in sham and post-MI tissues and extracting mRNA at different time points. The extracted mRNA can then be quantified with Northern blotting under equal loading conditions using a gene
that is very stable and is not itself inhibited by treatment with actinomycin D such as 18S rRNA (Liu et al., 1997). If the stability of mRNA of a particular KvX gene is decreased following myocardial infarction, serial analysis should reveal a reduction in mRNA levels in the post-MI samples compared to the sham samples. With respect to this study, since the changes in mRNA level are correlated with changes at the protein level, it is likely that the regulation of these K+ channel genes is not due to post-transcriptional and post-translational processes.

**Role of thyroid hormone on I\textsubscript{to} expression**

We reported that thyroid hormone treatment increases I\textsubscript{to} density by increasing the expression of the Kv4.2 K+ channel gene. Thyroid hormone analog used in the present study was found to increase Kv4.2 expression and decrease Kv1.4 expression in infarcted hearts, essentially reversing the expression profile of the K+ channel genes to those observed in non-infarcted hearts. Thyroid hormone can re-induce the expression of genes in normal adulthood which can be beneficial in the treatment of heart disease where a variety of embryonic genes are known to be expressed. Aside from its role in mediating a switch from the neonatal β-MHC isoform to the adult α-MHC isoform, thyroid hormone treatment to cultured neonatal rat ventricular myocytes can decrease the expression of Kv1.4 neonatal isoform and increase the expression of Kv4.2 adult isoform (Guo et al., 1998; Wickenden et al., 1997). This finding may be important to prevent life-threatening arrhythmias and to prevent disease progression resulting from action potential prolongation. If action potential prolongation alone causes the progression and propagation of the disease, then restoration of a normal electrical phenotype should reverse these changes. Studies may be conducted on the prolonged use of thyroid
hormone in different stages of cardiac hypertrophy and failure. These studies may be complicated since thyroid hormones are non-specific agents and can alter several metabolic and biochemical parameters at the cellular level. In this regard, caution must be taken to target and amplify thyroid hormone action to the heart and prevent its action on other peripheral tissues. Recently, several enzyme families known as monodeiodinase isoenzymes have been identified and can modulate transcription by regulating the availability of the active hormone T3 to the peripheral tissues (Kohrle et al., 1999). Three monodeiodinase isoenzymes can convert the prohormone L-thyroxine (T4) to a thyromimetically active tri-iodo-thyronine (T3) by simply removing an iodine atom. An interesting possibility is the creation of transgenic mice that express the deiodinase enzyme under the control of the mouse α-MHC promoter and examine whether the induction of heart disease in these mice can prevent the down-regulation of Ito. The creation of these mice will allow us to learn the role of Ito in disease progression without the peripheral effects. These mice are being generated in the laboratory (personal communication Dr. Backx).

**Mechanism of action of DITPA**

The actions of the L-thyroxine (T4) analog, DITPA remain unknown, but a speculation of the possible mechanism of action is presented. The differential effect of DITPA on these K+ channel genes indicates the complexity of the action of this agent and may involve the presence of several elements. However, similar changes at the mRNA and protein level suggest that the regulation of KvX genes must be regulated at the transcriptional level. Similar to T4, DITPA, is probably inactive and is converted to the active hormone, T3 by the deiodinase enzyme. The product of this enzyme reaction
probably interacts with nuclear receptors for T3 known as thyroid receptor (TR). These receptors can bind to regulatory regions of genes known as thyroid hormone response elements (TRE) and modify their expression. One possible example may be related to the activation of thyroid receptor (TR) by the thyroid hormone which can act as a transcriptional activator in the case of Kv4.2 or silencer in the case of Kv1.4. The mechanism of action is presented based on a previously published model for another gene (Kim et al., 1999). Optimal activation by TR requires synergism with activators bound to a specific region in the promoter known as thyroid response elements (TRE) receptor element (Kim et al., 1999). TR may also associate with a corepressor complex, resulting in silencing transcription (Kim et al., 1999). This process requires protein-protein and protein-DNA interactions and DNA looping (Kim et al., 1999). Molecular studies are warranted to examine the mechanism of thyroid hormone on the regulation of Kv channel gene expression. To accomplish this task, several steps must be undertaken. First the entire promoter region of the specific Kv channel gene must be sequenced. Next, using site directed mutagenesis and deletion analyses, I would examine whether TRE sequences exist in the promoter. Several types of TRE have already been identified which consist of short sequences (less than 20 bp) that bind to the thyroid receptor. Using mutational and deletional analyses, one can create a plasmid containing the Kv gene and the modified promoter. The plasmid may then be transfected to cell lines that lack the Kv gene to quantify transcription using Northern blotting and ribonuclease protection assays in the presence and absence of thyroid hormone. Another gene known to be transcriptionally regulated by thyroid hormone is the α myosin heavy chain (α MHC). In our studies, we find that thyroid hormone analog increases the expression of α MHC in post-MI rats.
consistent with previous results (Izumo et al., 1987). In this regard, two thyroid response elements (TRE) have been identified in the proximal promoter region of the murine α-MHC (Rindt et al., 1995). It is possible that thyroid response elements also exist in the promoter region of specific Kvx genes.
LIMITATIONS OF THE STUDY

The advantages and disadvantages of using the rat animal model

The progress made in our understanding of the mechanisms governing cardiac hypertrophy would not have been possible without the creation of a number of animal models of cardiac hypertrophy. Rat models of cardiac hypertrophy have been used extensively to study the mechanisms of cardiovascular disease and pharmacological interventions such as the present study. Studying rat models provides an advantage over human hearts mainly due to their availability. Studies with diseased human hearts (i.e. end-stage) often represent an end-point of the disease process and non-homogeneous group with different etiologies such as ischemic cardiomyopathy and dilated cardiomyopathy. However, the effects of $I_o$ reduction in these rats might not be applicable to humans and extrapolation of these data to patients with heart disease must be made with caution. Compared to the rat, $I_o$ density is lower and action potential duration at 50% repolarization is prolonged by 10 fold (Li et al., 1998) in human cardiac myocytes. Second, calcium removal from the cytosol is predominated by the activity of the sarcoplasmic reticulum calcium pump and $Na^+/Ca^{2+}$ exchange activity is less important in the rat compared to man (Bers, 1991). These differences in basic electrical and calcium handling properties may result in substantial differences in the mechanisms of disease.
Experimental conditions

As a general rule, there are limitations to a variety of techniques in scientific investigation. Without discussing the strengths and weakness of the techniques used in my studies, I will review some of the issues on the experimental conditions that were raised by the reviewers during the submission of the manuscripts. First, I must mention that a majority of electrophysiologic studies were conducted at room temperature (19-21 °C) which was not a limitation since the main focus of my studies were merely comparative in nature. While warmer temperatures may provide direct physiological relevance to the beating in vivo myocardium, it may also result in a poor voltage-control of the different ion channel measured in the course of these studies. Ion channel kinetics during warmer conditions (at 37 °C) are so fast that the peak of the current (I\text{peak}) may be masked by the capacity transient overshoot thereby making it difficult to resolve for differences in current density. Monophasic action potential recordings were recorded for the thyroid hormone study to simply approach physiological conditions where the temperature was set at 37 °C. The reviewers also raised the issue of rate of stimulation, since the rat heart is between 300 to 400 beats per minute. Many of the parameters in these studies cannot be performed at high frequencies of stimulation especially for the Ca\textsuperscript{2+} transient recording where spontaneous calcium release and overload may occur. Furthermore, there are other issues revolving the gating of some ion channels which are slowed and fast rates of stimulation would change the size of the current. The studies conducted during the course of my training cannot answer all the questions on calcium handling mechanisms. For instance, the activity or expression of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger was not studied and may be studied by another candidate in the laboratory. This is
important since changes in the Na+/Ca2+ exchanger alone may affect action potential duration, calcium handling and contraction in these myocytes. Previous studies have examined Na+/Ca2+ exchange activity in the presence or absence of prolonged action potentials (Bouchard et al., 1995). Our experiments were carried out in conditions to minimize, albeit, eliminate the contribution of the exchanger.

RECENT PROGRESS, PROSPECTS AND CHALLENGES

Down-regulation of I_{io} and the consequent prolongation of action potential duration is a hallmark condition in cardiac hypertrophy as observed in numerous studies. In this thesis, I was able to demonstrate a pathway by which a downregulation of K+ channels can increase intracellular calcium. As mentioned above, this particular mechanism may not necessarily be applicable to human heart failure since other calcium handling proteins such as SERCA 2a, phospholamban and sodium-calcium exchange are all affected. Nevertheless, calcium channels and the coupling to ryanodine receptors may a mechanism by which calcium is elevated in cardiac hypertrophy and during the transition to cardiac failure. Figure VII shows a plot of systolic [Ca^{2+}] with respect to voltage-clamp depolarizations of varying duration. The mean data demonstrates a steep portion of the plot at depolarizations with pulses of 400 msec or less in duration which is relevant to the rat with respect to action potential waveform and a lesser steep portion for voltage clamp depolarizations greater than 400 msec in duration which is more consistent with human action potential waveforms. Therefore, the mechanism by which action potential prolongation causes a rise in Ca^{2+} transient amplitude on rat ventricular myocytes must be tested on human ventricular myocytes. While the experiments performed in figure VII may be indicative of an increase in the loading of the
Figure VII. The effect of varying the duration of cellular depolarization on calcium handling.

$[Ca^{2+}]_i$ were recorded on sham myocytes stimulated with square wave pulse of varying durations from a holding potential of -80 mV to +10 mV. A shows $[Ca^{2+}]_i$ in response to voltage-clamp pulses ranging from 25 ms to 1000 ms. B shows a plot of mean systolic $[Ca^{2+}]_i$ plotted against the duration of voltage clamp pulses.
Figure VIII. The role of trigger action potential under controlled loading conditions on calcium handling. [Ca$^{2+}$]$_i$ were recorded in sham myocytes stimulated with 9 square wave pulse (100 ms in duration from a holding potential of -80 mV to +10 mV) followed by a sham (solid line) and post-MI (broken line) action potential.
sarcoplasmic reticulum during steady state stimulation, it appears that that one pulse is sufficient to observe a change in Ca\(^{2+}\) transient amplitude. Figure VIII shows an experiment where myocytes were delivered with short and long action potential under equal loading conditions. These results suggest that the trigger pulse is deterministic of systolic [Ca\(^{2+}\)]\(_i\) in the rat. These experiments are presently being performed by Rajan Sah in the laboratory.

Models to study the role of I\(_{\alpha}\) and associated K\(^+\) channel genes in cardiac hypertrophy and disease progression

In the past few years, hypertrophy and failure models have been studied in murine models where specific genes were knocked out or over-expressed to induce or rescue a heart failure phenotype. It is possible to disrupt, or “target” a gene of interest in a mouse by replacing it with a mutated sequence early in embryogenesis (conventional transgenesis), at a specific time point during development (inducible or conditional transgenesis) and adenoviral gene transfer. In this way, introduction of new genes or knockdown endogenous genes may be directly targeted to the heart by generating constructs containing the gene of interest under the control of MLC-2v promoter. Transgenic mice have been generated by expressing a dominant-negative Kv4 \(\alpha\) subunit with a point mutation in the pore region of the channel (Barry et al., 1998) or truncation of the N-terminal fragment (Wickenden et al., 1999) leading to a functional knockdown of I\(_{\alpha}\). I\(_{\alpha}\) downregulation and APD prolongation was observed in either transgenic mice. However, the transgenic mice expressing the point mutation showed no loss of function phenotype while the transgenic mice expressing the truncated Kv4.2 displayed a disease phenotype progressing to failure. Unfortunately, transgenic approaches are not applicable
to mammalian systems in vivo without manipulating the germline. For example, the differences in the phenotype may be due to differences in the level of transgene expression or developmental issues such as differences in the timing and genetic backgrounds of the mice.

Recently, recombinant adenoviral vectors have been used to introduce exogenous genes and manipulate expression of K\(^+\) channel genes. Adenoviruses have been shown to be highly efficient vehicles for transferring one or two genes simultaneously into myocardium in vivo and in vitro (Hajjar et al., 1997; Hajjar et al., 1998). Data in progress shows that suppression of I\(_{o}\) in cultured neonatal cardiac myocytes by adenoviral gene transfer of dominant-negative Kv4.2 constructs can downregulate I\(_{o}\), prolong action potential duration and promote cellular growth (data not shown, collected by Zameh Kassiri -PhD candidate in the laboratory). Interestingly, treatment with cyclosporin and calcium channel blockers can attenuate the cellular growth indicating that action potential prolongation may activate calcium dependent signaling pathways such as MAPKinases and the calcineurin pathways. Taken together, these data support out current hypothesis of the role of I\(_{o}\) in the development of hypertrophy and disease progression.

The role of I\(_{o}\) downregulation on signaling pathways in cardiac hypertrophy and failure?

While I\(_{o}\) downregulation can induce cellular growth, the transduction signals that are involved in this process is unclear. In this regard, several questions remain unanswered. Does reduction of Kv4.2 expression cause activation of stress pathways (i.e MAP kinase and calcineurin) and contribute to the onset of hypertrophy? Is the reduction
of Kv4.x-based $I_{to}$ an epiphenomenon due to the altered neurohormonal environment and does it contribute to the process of heart failure? Can we prevent the activation of MAPKinase pathways and cardiac hypertrophy by introducing Kv4.x-based $I_{to}$ in post-MI animals? What is the role of intracellular calcium as a result of action potential prolongation on these pathways. Certainly, it is conceivable that elevations in intracellular calcium may contribute to the observed cellular hypertrophy in this model by stimulating growth through the activation of several stress pathways (Hunter and Chien, 1999). Future studies are required to demonstrate the role of elevated intracellular calcium in myocytes with prolonged action potentials on these pathways. To do so, we first need to understand the relationship between basal fluctuations in intracellular calcium in the normal heart and the changes in calcium cycling observed in disease models and its role on the activation of second messenger systems such as PKA, PKC, calmodulin kinase, tyrosine kinase and calcium-dependent stress pathways such as calcineurin and MAP kinase pathways. For example, calcineurin may be sensitive to local changes in calcium rather than global calcium within the cell. An interesting series of experiments would involve an intricate manipulation of the expression of K$^+$ channel genes and activation of these calcium dependent signaling pathways in cultured myocytes. Recent developments in cardiac adenoviral gene transfer will enable us to perform manipulations of K$^+$ channel gene expression and test hypotheses of disease mechanisms \textit{in vitro} and \textit{in vivo}. Gene transfer techniques have already been used to rescue the SERCA2a gene which are known to be downregulated in heart failure (Del Monte et al., 1999; Hajjar et al., 1997a, 1997b, 1998, He et al., 1999). Furthermore, specific MAP kinases have been shown to be implicated in endothelin-induced cardiac
hypertrophy (Choukroun et al., 1998; Choukroun et al., 1999; Force et al., 1999). During my post-doctoral studies, I intend to pursue my working hypothesis and test the role of K+ channel gene encoding the Ito channel on the activation of signaling pathways and disease progression. In this regard, ion channels are already known to play a critical role in mechanical stress-induced cardiomyocyte hypertrophy by activation of Raf-1 and MAPKinases (Yamazaki et al., 1998). Identifying molecular mechanisms by which K+ channels contribute to cardiac hypertrophy and propagate disease may be a daunting task since there are more than 2000 protein kinase genes in the vertebrate genome. The investigation of calcineurin, MAPkinases, protein kinase C, protein kinase B (or Akt), and transcription factors such as NFAT3 (Force et al., 1999) may be a good start since they are already known to be upregulated in hypertrophied hearts. Our ability to engineer dominant-negative viruses to inhibit selected pathways will be important to carefully dissect the mechanisms of hypertrophy and will hopefully lead to identification of novel pathways by which ion channels can mediate cardiac hypertrophy and even failure.
Figure IX. Time course of downregulation of the transient outward current following myocardial infarction. Serial current-voltage plots for the transient outward current at 24-36 hours (panel A), 3-5 day (panel B), 8 weeks (panel C) and 24 weeks post-MI (panel D).
REFERENCES

Textbooks:


Original articles:


44. Bogoyevitch MA, Glennon PE, Andersson MB, Clerk A, Lazou A, Marshall CJ, Parker PJ, Sugden PH. Endothelin-1 and fibroblast growth factors stimulate the mitogen-


95. Fabiato A. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *Am J Physiol.* 1983;245:C1-14.

96. Fedida D, Braun AP, Giles WR. Alpha 1-adrenoceptors reduce background K+ current in rabbit ventricular myocytes. *J Physiol (Lond).* 1991;441:673-84.


104. Fiset C, Clark RB, Shimoni Y, Giles WR. Shal-type channels contribute to the Ca2+-independent transient outward K+ current in rat ventricle. *J Physiol (Lond).* 1997;500:51-64.


218. Milano CA, Dolber PC, Rockman HA, Bond RA, Venable ME, Allen LF, Lefkowitz RJ. Myocardial expression of a constitutively active alpha 1B-adrenergic


343. Woo SH, Lee CO. Role of PKC in the effects of alpha1-adrenergic stimulation on Ca2+ transients, contraction and Ca2+ current in guinea-pig ventricular myocytes. Pflugers Arch. 1999;437:335-44.


PUBLICATIONS ARISING FROM THIS THESIS

Published Papers: 6  Papers Submitted/Under Revision: 1  Abstracts: 7

Manuscripts in peer-reviewed journals


Manuscripts submitted/under revision

1. **R. Kaprielian**, R. Sah, A.D. Wickenden & Peter Backx. Regional changes in K+ currents and [Ca2+]i in control and hypertrophied rat ventricular myocytes.
Abstracts


REGIONAL CONTRIBUTIONS OF Kv1.4, Kv4.2 AND Kv4.3 TO THE TRANSIENT OUTWARD K⁺ CURRENT IN RAT VENTRICLE

A.D. WICKENDEN, T.J. JEGLA, R. KAPRIELIAN AND P.H. BACKX

Regional contributions of \(Kv1.4\), \(Kv4.2\), and \(Kv4.3\) to transient outward \(K^+\) current in rat ventricle

A. D. Wickenden,1 T. J. Jegla,2 R. Kaprielian,1 and P. H. Backx1

1Department of Medicine, Centre for Cardiovascular Research, and the Toronto Hospital, University of Toronto, Toronto, Canada M5G 2C4; and 2ICagen Incorporated, Durham, North Carolina 27703

Wickenden, A. D., T. J. Jegla, R. Kaprielian, and P. H. Backx. Regional contributions of \(Kv1.4\), \(Kv4.2\), and \(Kv4.3\) to transient outward \(K^+\) current in rat ventricle. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1599-H1607, 1999.—The aim of the present study was to assess differences in transient outward potassium current \(I_o\) between the right ventricular free wall and the interventricular septum of the adult rat ventricle and to evaluate the relative contributions of \(Kv4.2\), \(Kv4.3\), and \(Kv1.4\) to \(I_o\) in these regions. The results show that \(I_o\) is composed of both rapidly and slowly recovering components in the right wall and septum. The fast component had a significantly higher density in the right free wall than in the septum, whereas the slow component did not differ between the two sites. \(Kv4.2\) mRNA and protein levels were also highest in the right wall and correlated with \(I_o\) density, whereas \(Kv4.3\) was expressed uniformly in these regions. The kinetics of the rapidly recovering component of \(I_o\) in myocytes was similar to that recorded in tsa-201 cells expressing \(Kv4.2\) and \(Kv4.3\) channels. \(Kv1.4\) mRNA and protein expression correlated well with the density of the slowly recovering \(I_o\), whereas the recovery kinetics of the slow component were identical to \(Kv1.4\) expressed in tsa-201 cells. In conclusion, \(Kv1.4\), \(Kv4.2\), and \(Kv4.3\) differ between regions in rat hearts. Regionally specific differences in the genetic composition of \(I_o\) can account for the region-specific properties of this current.

**METHODS**

Isolation of adult rat ventricular myocytes. Adult rat ventricular myocytes were isolated as previously described (27) with minor modifications. Briefly, rats (\(\approx\) 250 g, Sprague-Dawley, Charles River) were heparinized and killed (under 75 mg/kg pentobarbital sodium) by cervical dislocation. The heart was removed and retrogradely perfused for 3 min with Tyrode solution of the following composition (mM): 132 NaCl, 5.4 KCl, 1 CaCl\(_2\), 1.2 MgSO\(_4\), 10 HEPES, and 10 d-glucose, pH 7.4. The hearts were then perfused with nominally calcium-free Tyrode for a further 5 min, followed by perfusion with Tyrode solution containing collagenase (type II, 0.6 mg/ml, Boehringer-Mannheim), protease (type XIV, 0.05 mg/ml, Sigma Chemical, St. Louis, MO), and CaCl\(_2\) (25 \(\mu\)M). Once digestion was complete (typically 7-9 min), hearts were perfused for an additional 5 min with enzyme-free high-K\(^+\) solution of the following composition (mM): 120 potassium glutamate, 20 KCl, 20 HEPES, 1 MgCl\(_2\), 10 d-glucose, and 0.5 K-EGTA. The entire right ventricular free wall (from 5 hearts) and interventricular septum (from 4 hearts) were removed under a dissecting microscope and minced in high-K\(^+\) solution. Cells were liberated with gentle mechanical agitation, filtered through nylon mesh, and stored in high-K\(^+\) solution until required. Only calcium-tolerant, quiescent, rod-shaped myocytes with clear cross striations were selected for electrophysiological recordings. All experimental protocols conformed with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.
(NIH) [DHHS Pub. no. (NIH) 85–23, revised 1996]. The protocols were approved by the Animal Care Committee of the Research Institute at the Toronto Hospital.

tsa-201 cell culture and transfection. tsa-201 cells were maintained in MEM supplemented with 10% fetal bovine serum and gentamicin (50 μg/ml) in an incubator at 37°C with a humidified atmosphere of 5% CO₂. Medium was replaced every 48–72 h. Twenty-four hours before transfection, tsa-201 cells were harvested by brief trypsinization (0.5 mg/ml in phosphate-buffered saline) and replated at a density of 3 × 10⁶ cells per 35-mm culture dish. Cells were transfected using Lipofectamine reagent (GIBCO BRL) according to the manufacturer’s instructions. Cells were incubated for a period of 5 h with a mixture of 10 μl Lipofectamine, 1 μg green fluorescent protein (GFP), and 0.5–1 μg pRCCMVKv4.2, 1 μg pcDNA3Kv4.3, 0.03–1 μg pGW1HKv1.4, or a similar amount of vector alone in OPTI-MEM serum-free media. Twenty-four to forty-eight hours after transfection, cells were prepared for electrophysiological evaluation. Cells were removed from the culture dish by brief trypsinization (as described in Isolation of adult rat ventricular myocytes), collected by centrifugation (1,000 rpm, 5 min), and replated in growth medium at low density. All reagents for cell culture were purchased from GIBCO BRL.

Electrophysiological recording. Adult right ventricular myocytes were placed in a bath and perfused (~1 ml/min) with extracellular Tyrode solution of the following composition (mM): 140 NaCl, 2 CaCl₂, 4 KCl, 1 MgCl₂, 6 H₂SO₄, 10 glucose, 10 HEPES, and 0.5 CaCl₂ (for myocyte recordings only). pH 7.4 with NaOH. Electrophysiological recordings from tsa-201 cells were made with the cells adhered to the 35-mm culture dishes in which they were plated. Culture medium was replaced with extracellular solution immediately before recordings. Pipette tips were heat polised to a resistance of 1–3 MΩ when filled with an intracellular solution of the following composition (mM): 140 KCl, 1 MgCl₂, 6 H₂SO₄, 10 EGTA, 10 HEPES, and 5 MgATP, pH 7.2–7.3 with KOH. In some experiments with cultured cells a KF-based pipette solution was used to improve seal stability. The composition (mM) of the modified Tyrode (KF) solution was 100 KF, 40 KCl, 5 NaCl, 2 MgCl₂, 10 HEPES, 5 EGTA, and 5 glucose, pH 7.2–7.3 with KOH. Recovery kinetics were similar with KCl- and KF-based intracellular solutions. All recordings were made at room temperature (22–24°C) within 12 h of cell isolation (myocytes) or replating (tsa-201 cells). Successfully transfected tsa-201 cells were identified by their green fluorescence under appropriate conditions.

After membrane rupture, the capacitance transient was integrated on-line to estimate cell capacitance as a measure of cell size. Uncompensated series resistance was 4.0 ± 0.3 MΩ (n = 20) for recordings from right ventricular myocytes and 3.8 ± 0.4 MΩ (n = 19) for recordings from septal myocytes. Series resistance compensation was 81.0 ± 2.4% (n = 20) for recordings from right ventricular myocytes and 73.0 ± 2.6% (n = 19) for recordings from septal myocytes. Outward currents were induced with 500-ms depolarizing pulses to 60 mV from a holding potential of −80 (myocytes) or −100 (tsa-201 cells) mV. In myocyte recordings, a brief prepulse (−40 mV for 30 ms) was used to inactivate Na⁺ current. In all studies, Iᵣ was defined as peak current elicited by the depolarizing voltage step minus the steady-state current remaining at the end of a 500-ms voltage step. Current-voltage relationships were constructed by eliciting a series of depolarizing steps (−60 to +60 mV) in 20-mV increments from the holding potential. Recovery from inactivation was measured using a double-pulse protocol. From the holding potential, cells were depolarized by 500 ms. Cells were returned to the holding potential for 10 ms to 20 s, and then a second 500-ms depolarizing pulse was applied. The magnitude of Iᵣ, induced by the second pulse is expressed as a percentage of Iᵣ, induced by the first pulse. Monoeponential or biexponential functions were used to fit recovery from inactivation data. For biexponential fits

\[ Iᵣ(t) = A_{fast}(1 - \exp(-\tau_{fast}t)) + A_{slow}(1 - \exp(-\tau_{slow}t)) \]

where \( A_{fast} \) and \( A_{slow} \) are the amplitudes of the fast and slow components for recovery, \( t \) is the time spent at the recovery potential, and \( \tau_{fast} \) and \( \tau_{slow} \) are the time constants for recovery of the fast and slow components, respectively. When the recovery data was fit to a monoexponential, \( A_{fast} = A_{slow} = A \) and \( \tau_{fast} = \tau \).

For myocyte studies, the repetition interval was >20 s to allow complete repriming of slowly recovering currents. In tsa-201 studies, the repetition interval was set at 15 s for Kv4.2 and Kv4.3 and >20 s for Kv1.4.

Preparation of total RNA. Hearts were removed rapidly, and the right ventricle and septum were isolated, rinsed briefly in 0.9% NaCl (wt/vol), and snap frozen in liquid nitrogen. Ventricular tissue was homogenized in Trizol reagent (GIBCO) and RNA precipitated with isopropyl alcohol. The integrity of RNA samples was confirmed by the presence of sharp bands after brief electrophoresis through a 1% agarose gel. The concentration of RNA was measured spectrophotometrically and confirmed by agarose gel electrophoresis. RNA was isolated independently from four adult rat hearts.

RNA protection assays. RNA protection assays were performed using an RPAII Ribonuclease Protection Assay Kit (Ambion, Austin, TX) as previously described (27). The Kv4.2 and Kv4.3 probes were kindly provided by Dr. David McKinnon (State University of New York at Stony Brook) and have been described previously (5). A 429-bp fragment of rat Kv1.4 (kindly provided by Dr. David McKinnon) was subcloned into pGEM11 (Hind III–Nsi I) to make a Kv1.4 probe capable of protecting a 331-bp fragment of Kv1.4 mRNA. The cyclinphil probe was purchased from Ambion. Abundance of mRNA transcripts was quantified by densitometry (Bio-Rad GS670 Imaging densitometer). Signals were normalized to a cyclinphil internal standard to ensure that findings were not influenced by minor variations in loading. Absolute cyclinphil levels (densitometric units) were not significantly different between right ventricle and septum in the present study (right ventricular cyclinphil levels were 129 ± 20% of septal levels; n = 24; P > 0.05, two-tailed, paired t-test), indicating that this gene was expressed uniformly between these regions. Right ventricular wall mRNA levels for each rat were normalized to mRNA levels in the septum of the same animal.

Isolation of protein from right ventricular wall and septum. Hearts were removed rapidly, and the right ventricle and septum were isolated as described in Isolation of adult rat ventricular myocytes, rinsed briefly, and homogenized in buffer A [0.32 M sucrose, 5 mM Tris (pH 7.4)] containing protease inhibitors phenylmethylsulfonyl fluoride (100 μM), o-phenanthroline (1 mM), iodoacetamide (1 mM), and benzamidine (1 mM). Homogenate was centrifuged to remove particulate matter. Membranes were collected from the supernatant by centrifugation at 27,000 g for 45 min and resuspended in buffer C (0.32 M sucrose, 5 mM HEPES solution containing protease inhibitors as described above). Membrane protein was isolated independently from three to five adult rat hearts. Protein concentration was determined by the Lowry method with minor modifications (11), and the
membranes were aliquoted and stored at -70°C until further use. Rat brain membranes were prepared as described previously (9). After protein determination, rat brain membranes were aliquoted and frozen in liquid nitrogen.

**Western blot analysis.** For Western blot analysis, 10- to 50-µg (heart) or 3.5- to 10-µg (brain) aliquots of freshly thawed membrane protein were tritratated in 2X SDS sample buffer containing β-mercaptoethanol, heated for 5 min at 95°C, and centrifuged to pellet any insoluble debris. Supernatant proteins were then resolved by electrophoresis on 7.5% SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose (0.45 µm hole, 3-7.5). The blots were then blocked in Tris-buffered saline (TBS) containing 5% nonfat dried milk, washed with TBS, and probed with anti-Kv antibody raised in rabbit (Dr. O. T. Jones, Department of Pharmacology, University of Toronto) at a 1:10,000 dilution in blocking solution. After consecutive washes with TBS containing 0.05% Tween-20 and TBS alone, the blots were incubated for 2 h with secondary antibody [horse-derished peroxidase-conjugated donkey anti-rabbit (Amersham), 1:4,000 in blocking solution], rewashed, and developed by enhanced chemiluminescence (ECL, Amersham). Gel loading was checked by staining total proteins with Ponceau S, and molecular masses were determined using prestained markers (Kaleidoscope, Bio-Rad). Densitometric analysis of bands was done with a Bio-Rad model GS-670 imaging densitometer.

The procedure by which the anti-Kv.4.1 antibody was produced has been described in detail previously (27). Peptide Kv.4.2N, corresponding to a region of Kv.4.2 that shares little identity with Kv.4.3 (amino acid residues 23-42 of Kv.4.2, 14-54 of Kv.4.3), was synthesized by solid-phase fluorenylmethoxycarbonyl chemistry (Vetrogen, London, ON, Canada) and coupled to keyhole limpet hemocyanin (KLH) using the heterobifunctional crosslinker N-(maleimidobutyryloxy)sulfosuccinimide ester (Pierce). After being dialyzed against PBS, the KLH conjugate was injected into New Zealand White rabbits at multiple subcutaneous sites by Berkeley Antibody (Richmond, CA). Antisera were collected and tested by ELISA using microtiter plates coated with Kv.4.2N peptide, and the IgG was enriched by affinity chromatography on protein A agarose (MAPS kits, Bio-Rad). The specificity of the serum was determined by immunoblots with rat brain membranes exactly as previously described (21).

**Plasmid constructs.** The long splice variant of human Kv.4.3 (10) was amplified from a hippocampus cDNA library (Clontech) using overlap extension. First, three overlapping pieces covering the entire Kv.4.3 coding sequence were amplified using three primer pairs: 5'-TTCTACGATCTTCACCATG-GGCCGCGGAGTTGCCGCTGCT-3' and 5'-GCAGGGC-ATCGAATCTCTTGTGCTTCTGCCGTGTC-3'; 5'-CACCA-AGAATCTGACCTCGTCTCGCAGTAATCTCG-3'; 5'-GCAGAATCTGACCTCGTCTCGCAGTAATCTCG-3'; and 5'-GCAGAATCTGACCTCGTCTCGCAGTAATCTCG-3'. Overlap extension was performed using 20 ng of each amplified fragment, using the first and last primers. The overlap product was subcloned into pC5DNA (Invitrogen), and the insert was confirmed by sequencing.

**Plasmid pRC/CMV/Kv.4.2.** containing the entire coding region of rat Kv.4.2 was obtained from Dr. J. Nerbonne (Washington University, St. Louis) with kind permission from Dr. L. Jan (University of California, San Francisco). Full-length rat Kv.4.2 was kindly provided by Dr. M. M. Tamkun (Vanderbilt Medical Center, Nashville, TN). A BamHI/SalI fragment containing the entire coding region of Kv.4.1 was subcloned into BglII/SalI pGWI1 (British Biolabs). A plasmid encoding jellyfish GFP was kindly provided by Dr. Jeremy Nathan (Johns Hopkins University).

**Statistics.** All data are expressed as means ± SE. Statistical significance was determined using a suitable (hetero- or homoscedastic) unpaired two-tailed t-test. For RNA protection assays and Western blot analysis, the null hypothesis (i.e., that mRNA/protein levels were not different in septum and right wall) was tested using paired two-tailed t-tests. A P < 0.05 was considered significant.

**RESULTS**

I\(a\) in right ventricular free wall and septum. I\(a\) density (at 60 mV) was 29.5 ± 3.0 (n = 20 cells from 5 hearts) and 15.9 ± 3.0 (n = 19 cells from 4 hearts) pA/pF in myocytes isolated from the right ventricular free wall (cell capacitance 139.3 ± 8.0 pF) and the interventricular septum (cell capacitance 167.0 ± 7.2 pF), respectively. When comparing the biophysical properties of these currents, we focused exclusively on the kinetics of recovery from inactivation because the rate of recovery from inactivation has previously been shown to vary between different regions of the left ventricle and at different developmental stages (22, 27). Furthermore, dramatically different rates of recovery from inactivation have been observed between Kv1.4 and the Shal-related K\(^+\) channels Kv4.2 and Kv4.3 in Xenopus oocytes (6, 14, 19, 20, 25), thereby potentially providing an electrophysiological fingerprint for the different channel types. Recovery from inactivation was measured using the double-pulse protocol shown in the inset to Fig. 1C and described in METHODS. In these studies we used a very low stimulation frequency (0.05 Hz) to ensure sufficient time for all currents to completely recover (see below). In both the right ventricular free wall and interventricular septum the recovery from inactivation of I\(a\) was dominated by a rapid component. Complete recovery from inactivation, however, frequently required very long interpulse intervals, resulting in a biphasic time course of recovery. The slowly recovering component of I\(a\) was observed in less than one-half of the cells isolated from the right ventricular free wall (7/20) and in virtually all cells isolated from the septum (17/19). The biphasic nature of recovery from inactivation for I\(a\) is clearly visible from the voltage-clamp recordings displayed in Fig. 1 for myocytes from the right wall (Fig. 1A) and septum (Fig. 1B). For both cells, I\(a\) recovered rapidly to ~80% of the control level, but complete recovery required prolonged interpulse intervals. The plots of normalized current against interpulse interval for the same cells are shown in Fig. 1, A (right wall) and D (septum). These figures show that the rapid phase of recovery was complete within 200 ms but that full recovery required an interpulse interval of 20 s. Consistent with the presence of a slowly recovering component of I\(a\), the amplitude of I\(a\) was decreased in some cells during repetitive, high-frequency stimulation. This is shown in Fig. 2, C and D, in which voltage-clamp recordings from a septal cell show that the amplitude of I\(a\) was reduced by 20% during repetitive stimulation with 500-ms depolarizing pulses at a rate of 1 Hz.
Fig. 1. Biphasic recovery from inactivation of transient outward potassium current ($I_o$) in myocytes isolated from adult rat heart. Recovery from inactivation was measured using double-pulse protocol shown in inset in C. Typical voltage-clamp recordings showing recovery from inactivation of $I_o$ in a right ventricular free cell (A) and a septal cell (B), with increasing interpulse intervals (10, 20, 40, 100, 200, and 600 ms, 1, 2, and 20 s) are shown. Note that although recovery is dominated by a rapid component, complete recovery required prolonged interpulse intervals in both cells. $I_o$ (peak current — current remaining at end of 500-ms pulse) elicited by second pulse was normalized to that elicited by first pulse and plotted against interpulse interval in C (right wall) and D (septum). Recoveries were best fit with a biexponential function, with fast components accounting for 80.2% of the recovery in right wall and 85.4% in septum [time constant for recovery of fast component ($\tau_{fast}$) = 26.7 and 49 ms for right wall and septal cells, respectively], with a slow component [time constant for recovery of slow component ($\tau_{slow}$) = 3.478 and 12.772 ms for right wall and septal cells, respectively] accounting for the remainder.

On average, the rapidly recovering component of $I_o$ accounted for 92.9 ± 2.5% ($n = 15$) of $I_o$ in the right ventricular cells and 81.6 ± 3.1% ($n = 19$) of $I_o$ in the septal cells. The density of the fast component was significantly larger in the right ventricular cells (27.6 ± 3.2 pA/pF, $n = 15$) compared with the septum (13.6 ± 2.9 pA/pF, $n = 19$; $P = 0.003$). The $\tau_{fast}$ were also significantly different between the right ventricular free wall (24.2 ± 1.4 ms, $n = 15$) and the septum (33.3 ± 3.1 ms, $n = 19$; $P = 0.012$). The density of the slow component, on the other hand, was not different between regions (2.4 ± 0.98 pA/pF in right wall compared with 2.2 ± 0.6 pA/pF in septum; $P = 0.85$). The $\tau_{slow}$ were 3.527 ± 983 ms ($n = 7$) in right ventricular free wall cells and 4,561 ± 1,090 ms (n = 17) in cells from the septum. These values were not significantly different ($P = 0.58$).

Recovery from inactivation of $K_{v4.2}$-, $K_{v4.3}$-, and $K_{v1.4}$-based currents in a mammalian cell line. To understand the molecular basis of the rapidly and slowly recovering components of $I_o$ in the right wall and septum, we measured the recovery kinetics of candidate $K^+$-channel gene products ($K_{v4.2}$, $K_{v4.3}$, and $K_{v1.4}$) after transient transfection of mammalian tsA-201 cells. Cells transfected with $K_{v4.2}$ (Fig. 3B), $K_{v4.3}$ (Fig. 3C), or $K_{v1.4}$ (Fig. 3D) expressed robust, transient outward-like currents. Similar currents were never recorded from nontransfected cells or cells transfected with vector DNA only (although these cells do express a small endogenous delayed rectifier-like current; Fig. 3A). Figure 3, E and G, shows typical voltage-clamp recordings of recovery from inactivation of $K_{v4.3}$- and $K_{v4.2}$-based currents, respectively. As shown, recovery from inactivation for both these gene products was
relatively rapid, with the normalized current versus interpulse interval being fit with monoexponential functions with time constants of 84 (Kv4.3, Fig. 3F) and 51.4 (Kv4.2, Fig. 3H) ms. Similar observations were made in a total of six cells per group. On average, recovery from inactivation was slower for Kv4.3 (mean ± SE \( \tau \) value was 141 ± 23 ms) compared with Kv4.2 (\( \tau \) value was 73.1 ± 9.3 ms; \( P = 0.02 \)). The recovery kinetics of Kv4.3 and Kv4.2 based currents expressed in tsa-201 cells resembled the rapid component of \( I_{to} \) measured in myocytes. These findings suggest that Kv4.3 and/or Kv4.2 may underlie the rapidly recovering component of \( I_{to} \) in these regions.

Recovery from inactivation of Kv1.4-based currents was relatively slow in tsa-201 cells, as illustrated in Fig. 3, I and J. Kv1.4-based currents typically required interpulse intervals of \( \geq 20 \) s to fully recover from inactivation. The plot of normalized Kv1.4 current against interpulse interval shown in Fig. 3J was well fit with a monoexponential function with a \( \tau \) equal to 1,784 ms. Similar observations were made in a total of six cells (\( \tau = 3,307 \pm 902 \) ms). Clearly, the recovery kinetics of Kv1.4 expressed in tsa-201 cells was similar to the recovery kinetics of the slow component of \( I_{to} \), supporting the notion that Kv1.4 contributes to \( I_{to} \) in right ventricular and septal cells.

Kv4.2, Kv4.3, and Kv1.4 mRNA levels in right ventricular free wall and interventricular septum. To gain further insight into the molecular basis of \( I_{to} \) in the right ventricular free wall and septum, we measured mRNA levels of Kv4.2, Kv4.3, and Kv1.4 in these regions. Figure 4, A, C, and E, shows representative gels from RNase protection assays showing Kv4.2, Kv4.3, and Kv1.4 mRNA levels in the right ventricular free wall, the interventricular septum, and brain. Robust transcriptional expression of Kv4.2, Kv4.3, and
$Kv1.4$ was observed in both the right ventricular free wall and septum. Transcript levels (normalized to the levels of cyclophilin mRNA and to respective mRNA levels in the septum) are plotted in Fig. 4, B, D, and F. The mRNA levels of $Kv4.2$, $Kv4.3$, and $Kv1.4$ tended to be higher in the right ventricular free wall compared with the septum (right ventricular mRNA levels were $248 \pm 46$, $113 \pm 9$, and $133 \pm 26\%$ of those in the septum for $Kv4.2$, $Kv4.3$, and $Kv1.4$, respectively; $n = 4/group$). However, only the $Kv4.2$ levels were significantly different between right ventricle and septum ($P = 0.049$). The higher level of expression of $Kv4.2$ mRNA in the right wall compared with the septum correlated well with the twofold higher density of the rapidly recovering component of $I_o$ in the right wall compared with the septum. Furthermore, the observation that $Kv1.4$ mRNA was readily detected supports the suggestion that the product of this gene may underlie the slowly recovering component of $I_o$.

$Kv1.4$ and $Kv4.2$ protein levels in right ventricular free wall and interventricular septum. Because mRNA levels may not always be predictive of expression at the protein level (28), we also conducted Western blot analyses using specific anti-$Kv4.2$ and anti-$Kv1.4$ antibodies. Figure 5A shows that $Kv1.4$ was detectable in samples isolated from the right ventricular free wall and septum, albeit at considerably lower levels than in brain. In the present study, the anti-$Kv1.4$ antibody labeled two distinct bands, in both brain (seen with reduced exposures; Fig. 5A, inset) and myocyte protein. The molecular masses of these two bands were $\sim 91.5$ and $100.5$ kDa, which are very similar to those previously identified in protein extracted from cos-1 cells transfected with $Kv1.4$ (18) and cultured neonatal rat ventricular myocytes (27). The basis for the presence of these bands is unclear, but studies have established that $Shaker$, $Kv1.3$, and $Kv1.1$ channels have glycosylated and nonglycosylated fractions leading to two distinct bands (4, 18, 23). Both bands could be eliminated by preincubation of the antibody with the peptide against which the antibody was raised ($Kv1.4\text{N} 12 \mu g/\mu l$; Fig. 5B). Taken together, these results suggest that both bands likely represent $Kv1.4$-related proteins. Total $Kv1.4$ immunoreactivity in the right ventricular free wall, although somewhat greater, was not statistically ($P = 0.06$) different from the septum in five hearts studied.

$Kv4.2$ immunoreactivity was also readily detectable in protein samples from the right wall and septum. The anti-$Kv4.2$ antibody labeled a single band with an estimated molecular mass of $72$ kDa (Fig. 5D), similar to that reported previously (1, 28). This labeling appeared to be specific because the band disappeared when the blot was probed with antibody that had been preincubated with the $Kv4.2\text{N}$ peptide (20 $\mu g/\mu l$, Fig. 5E). It is unlikely that the detected band contained contributions from $Kv4.3$ channels because the NH$_2$-terminal $Kv4.2$ peptide sequence used to generate the antibodies had only roughly $50\%$ sequence identity with $Kv4.3$. Furthermore, the predominant (long) isoform of $Kv4.3$, expressed in the heart, has a predicted
molecular mass that is 20% larger than Kv4.2, yet only a single band was detected (13, 24). Nevertheless, we found that Kv4.2-like immunoreactivity in the right ventricular free wall was significantly higher (361 ± 54%, n = 3 hearts; \( P = 0.008 \)) than in the septum, which coincided closely with the mRNA expression of Kv4.2 and correlated with the density of the rapidly recovering component of \( I_o \) in both the septum and right free wall.

**DISCUSSION**

Differences in the density and regulatory properties of \( I_o \) exist between anatomically distinct regions of the rat heart (3, 8, 22). The purpose of the present study was to characterize and compare \( I_o \) in the right ventricular free wall and the interventricular septum of the adult rat ventricle and to evaluate the possible relative contributions of Kv4.2, Kv4.3, and Kv1.4 to \( I_o \) in these regions. In the present study, we found that the density of \( I_o \) was significantly greater in the right ventricular free wall compared with the septum. Recovery from inactivation (using slow stimulation frequencies) revealed two kinetically distinct components to \( I_o \) in both regions. Recovery from inactivation of \( I_o \) was dominated by a rapidly recovering component in both the right ventricular free wall and the interventricular septum. The kinetics of this rapid component were similar to the kinetics of recovery of Kv4.2 expressed in mammalian cells (Refs. 7 and 29 and present study) and to Kv4.3 expressed in mammalian cells (present study) and in Xenopus oocytes (6, 20), suggesting that these Shai-related K⁺-channel genes, either as homomers or heterotetramers, contribute importantly to the rapidly recovering component of \( I_o \) in the right wall and septum. Consistent with this, we found that Kv4.2 and Kv4.3 mRNAs and Kv4.2 protein were robustly expressed in both the right ventricle and the septum. The finding that Kv4.2 mRNA and protein levels were significantly higher in the right wall compared with the septum suggests that differences in Kv4.2 protein expression account for the density of the rapidly recovering component of \( I_o \) between these regions. The observation that Kv4.3 mRNA levels were similar in these regions suggests that Kv4.3 may be proportionately more important in the septum compared with the right wall. Because we found that recovery from inactivation for Kv4.3 is marginally slower than that for Kv4.2, it is possible that differences in the quantities of Kv4.2 and Kv4.3 in different regions of the adult rat heart could explain the finding that the recovery kinetics of the fast component differed between the right wall and the septum, as suggested previously for differences observed between the endo- and epicardium (6).

In addition to the dominant, rapidly recovering component of \( I_o \), a smaller, slowly recovering component of \( I_o \) was also evident in a portion of right wall cells and the septum in the present study. This current appeared...
component was recorded in the H1606 line. Consistent with previous findings, Kv1.4-4 expression was shown predominantly in cells from the endocardial layer of the ferret left ventricle (2). In addition, the slow component of Kv1.4, Kv4.2 expression predominates in the right wall and septum of the adult rat heart, as has been previously suggested for the slow recovering I_{Na} recorded in rabbit ventricle (26). These findings are also consistent with previous studies in human and rat myocardium showing that a slowly recovering I_{Na} is expressed predominantly in cells from the endocardial layer of the left ventricular free wall (12, 22) and that Kv1.4 protein is preferentially expressed in the endocardial layer of the ferret left ventricle (2). In addition, the slowly recovering component of I_{Na} was only detected in a portion of right wall cells. It is possible that the right wall cells displaying the slow component originate from the endocardium portion of the right wall. Further studies are required to address this possibility. Our findings differed from previous studies that failed to detect Kv1.4 protein in adult rat heart (1, 28). It is possible that this discrepancy may be explained by the use of different strains of rats, differences in the methods of protein isolation, and/or by the use of different anti-Kv1.4 antibodies. Although Kv1.4-based currents seem to contribute to I_{Na} under the conditions of the present study (i.e., long interpulse intervals), the functional contribution of such slowly recovering channels under more physiological conditions is unclear. Indeed, it has been suggested that at normal heart rates these currents would be permanently inactivated (15). In fact, however, the behavior of these currents under physiological conditions is very difficult to predict. Temperature, action potential amplitude and/or duration, extracellular K^+, redox, and posttranslational modification could all impact on the degree of inactivation and/or recovery from inactivation and potentially render these currents available at physiological heart rates. Interestingly in this regard, one recent report has shown that Ca/calmodulin-dependent protein kinase II-mediated phosphorylation of the NH2-terminus of Kv1.4 slows the rate of inactivation and accelerates the recovery kinetics of Kv1.4-based currents (17).

In the present study we have sought to correlate gene expression (mRNA and protein) with function (electrophysiology) to identify the molecular correlates of I_{Na} in the right wall and the septum of the rat heart. The RNase protection assay is a powerful technique for the identification of rare messages such as ion channels. However, this technique can only provide information on the relative expression of a given transcript between regions and does not allow for the measurement and comparison of absolute copy number of multiple molecular species within a region. Further studies using alternative molecular techniques (such as quantitative PCR) would be helpful in this regard. Correlations between gene expression and function, although persuasive, also require support using alternative techniques. We are currently examining the possibility of using recombinant adenoviruses to deliver dominant negative constructs into cardiac muscle before cell isolation as an alternative strategy for determining the molecular nature of native currents in the rat heart. In summary, the results of the present study show that I_{Na} is composed of both rapidly and slowly recovering components in the right wall and septum of the rat ventricle. Both Kv4.2 and Kv4.3 probably contribute to the rapid component, and Kv1.4 appears to underlie the slow component of I_{Na}. Kv4.2 expression predominates in the right wall, whereas Kv4.3 and Kv1.4 may be proportionately more important in the septum. Such regional differences in the contribution of Kv4.2, Kv4.3, and Kv1.4 may account for regional differences in the properties and regulation of I_{Na}.

The authors gratefully acknowledge T. Nguyen for help with the RNase protection assays and Western blotting. This work was supported by a grant from the Heart and Stroke Foundation of Ontario (to H. Backx) and a University of Toronto Department of Medicine Post-Doctoral Fellowship (to A. D. Wicken- dan). Funding from the Alan Tiffen Trust and the Centre for Cardiovascular Research for equipment is also gratefully acknowledged. The anti-Kv1.4 and anti-Kv4.2 antibodies and rat brain protein were kindly provided by Dr. O. T. Jones, Department of Pharmacology, University of Toronto and the Playfair Neuroscience Unit, the Toronto Hospital.

Present address of A. D. Wicken-dan: ICAgent Inc., 4222 Emperor Blvd., Suite 460, Durham, NC 27703. Address for reprint requests and other correspondence: P. H. Backx, CCRW 3–802, the Toronto Hospital (General Division), 101 College St., Toronto, Ontario, Canada M5G 2C4 (E-mail: p.backx@utoronto.ca).

Received 27 January 1998; accepted in final form 3 February 1999.

REFERENCES


7. Fiset, C., R. B. Clark, Y. Shimoni, and W. R. Giles. Shal-type channels contribute to the Ca\textsuperscript{2+}-independent transient outward K\textsuperscript{+} current in rat ventricle. J. Physiol. (Lond.) 500: 51–64, 1997.


