MECHANOELECTRIC FEEDBACK IN THE MAMMALIAN HEART

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Bachelor of Science, Physiology (Hons)

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Margaret Helen Kelly

(Sept 6, 1944 – May 30, 2001)

This thesis is dedicated in loving memory of my mother who without I would not have been here

General Table of Contents

Abbreviations:

APD	Action Potential Duration
CICR	Calcium-induced Calcium Release
DAD	Delayed After Depolarisation
EAD	Early After Depolarisation
ECG	Electrocardiogram
EDLVP	End Diastolic Left Ventricular Pressure
EDV	End Diastolic Volume
ESLVP	End Systolic Left Ventricular Pressure
HR	Heart Rate
KSAC	Potassium-selective SAC
LV	Left Ventricle
LVP	Left Ventricular Pressure
MAP	Monophasic Action Potential
MEF	Mechano-electric feedback
NSAC	Non-selective SAC
RMP	Resting Membrane Potential
SAC	Stretch Activated ion Channel
SR	Sarcoplasmic Reticulum
VF	Ventricular Fibrillation
Vm	Membrane Potential

ACKNOWLEDGEMENTS

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I would like to express my sincere thanks to Mr Craig Maier and Mr Rick Carlson of Diamond Cut productions for free DC Audio Restoration Tools (DC-Art) software and participation in beta testing of new programs and restoration algorithms. Throughout my PhD, DC-Art software has provided me with many hours of enjoyable music and the great personal satisfaction that accompanies restoring recordings of long forgotten artists. As such, these artists form the theme to the present thesis. Examples of restored tracks for each artist can be found at http://www.kellyaustralia.com

Lastly, I would like to thank my family and friends for putting up with my behavioural fluctuations over the past years, not to mention the divergent music tastes.

ABSTRACT

Stretch of cardiac muscle is known to activate various physiological processes that result in changes to cardiac function, contractility and electrophysiology. To date, however, the precise relationship between mechanical stretch and changes in the electrophysiology of the heart remain unclear. This relationship, termed mechano-electric feedback (MEF), is thought to underlie many cardiac arrhythmias associated with pathological conditions. These electrophysiological changes are observed not only in the whole heart, but also at the single cardiomyocyte level, and can be explained by the presence of stretch-activated ion channels (SACs). Most investigations of the actions of stretch have concentrated on these sacrolemmal ionic currents thought responsible for the proposed MEF-induced changes in contractility. While these studies have provided some useful insight into possible mechanisms, the inappropriate use of solutions and non-physiological degrees of stretch, may have caused somewhat misleading results. Currently, little is known about the involvement or contribution of non-selective or K^+ selective SACs to the normal cardiac cycle. Here, I investigate the concept that stretch-induced changes in cardiac electrophysiology (MEF) are important in normal cardiac cycle and demonstrate the effects of stretch on the Frank-Starling mechanism (stretch induced increases in cardiac contractility) while pharmacologically manipulating stretch-activated ion currents. Experiments were conducted using a number of agents known to influence stretch-activated channels either in a positive or antagonistic manner. Results proved somewhat negative toward MEF theory with only substantial or pathological levels of stretch being able to elicit any electrophysiological change in the heart. Furthermore, where electrophysiological changes were associated with pathological stretch they were not consistently modulated by stretch-activated ion channel activators or blockers. Of equal importance was the observation that smaller levels of myocardial stretch associated with positive changes in contractility via the Frank-Starling mechanism were not associated with any electrophysiological changes in the Langendorff perfused heart (as observed by monophasic action potentials) nor in isolated muscle preparations (as observed through transcellular membrane potential recordings). As such, the present research undertaken in this thesis confirms an absence of electrophysiological changes with stretch except under extreme conditions suggesting that MEF is not a robust and necessarily repeatable phenomenon in the mammalian heart.

Declaration

I declare that this thesis does not incorporate, without acknowledgment, any material previously submitted for a degree or diploma in any university. I also declare that to the best of my knowledge it does not contain any materials previously published unless noted below, or written by another person except where due reference is made in the text.

Signed:

Declaration

Some of the material in this thesis has been published in the following papers and presentations:

An introduction of the ideas covered in this thesis appeared in

Kelly, DR (2003). Investigation of mechano-electric feedback and the Frank-Starling relationship in the heart: The function of stretch-activated ion channels in the heart, University of Adelaide Report & Presentation.

Parts of the isolated atrial and papillary muscle chapter have been presented;

Kelly, DR and Saint DA (2004). Absence of Mechano-Electric Feedback in isolated rat atrial tissue. Findings presented at the International Society for Heart Research (ISHR) Conference, Brisbane

Some of the Endocardial-Epicardial chapter appeared in;

Kelly, DR, Mackenzie L and Saint DA (2005). Effect of temperature on stretch-induced cardiac action potential shortening in the rat heart: involvement of TREK-1. Findings presented at the Australian Physiological Society, Canberra.

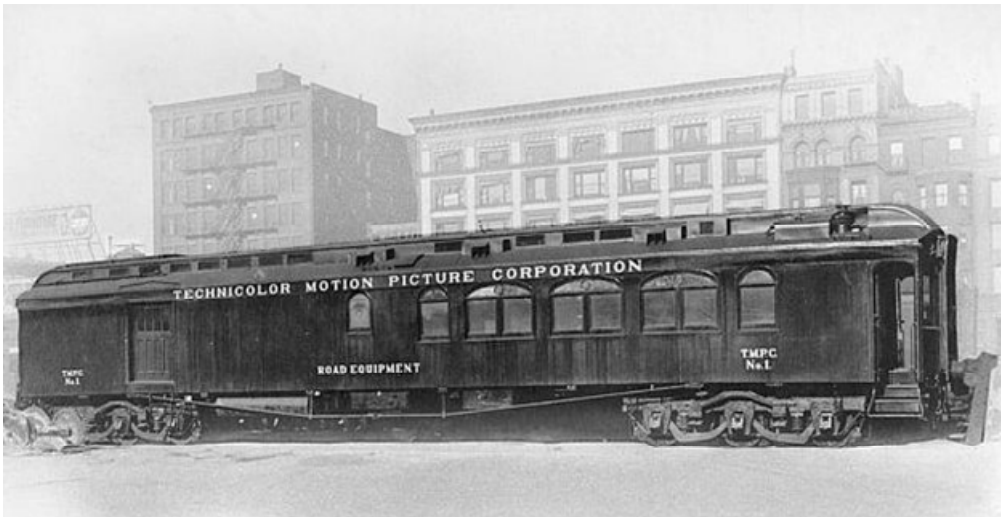
Mackenzie L, Kelly, DR and Saint DA (2005). More than one type of stretch activated channel contributes to the action potential duration in guinea pig. Findings presented at the Australian Physiological Society, Canberra.

Some of the Endocardial-Epicardial chapter appeared in;

Kelly D, Mackenzie L, Hunter P, Smaill B, Saint DA (2006). Gene expression of stretch-activated channels and Mechano-electric feedback in the heart. Clin Exp Pharmacol Physiol. 2006 Jul;33(7):642-8.

CHAPTER 1:

INTRODUCTION



We all start somewhere
Technicolor Motion Picture Corporation, founded in 1915, produced some of the 1st full length colour films in the Technicolor rail cart (above).

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INTRODUCTION

This chapter introduces the complex topic of mechano-electric feedback (MEF) in the heart as well as the content of the research reported in this thesis. As such, the chapter is divided into three main sections. The first of the sections gives a brief outline regarding the interpretation of MEF and the context in which the term is used throughout the thesis as well as the motivation for the work undertaken. This section will give a general overview of current knowledge and indicate potential deficiencies in current MEF theory. The second section of this chapter outlines the structure of the thesis. Finally, the last section describes the research contributions of the present thesis.

MOTIVATION

Cardiovascular diseases are a major health concern across the western world. Moreover, left ventricular function abnormalities resulting from cardiovascular diseases are a strong predictor of sudden death in patients and often result in ventricular tachyarrhythmia and secondary ventricular fibrillation (VF). Whilst VF is associated with ischemic conditions and the development of infarcts, the underlying electrophysiological changes and role of mechano-electric feedback (MEF) in contributing to the generation of these arrhythmogenic states remains largely unknown.

Mechano-electrical feedback (MEF) is used to describe the phenomenon whereby changes in myocardial loading or stretch of the ventricles and atria modulate cardiac electrophysiological properties (Lab, 1999). This can be observed as changes in heart rate (as demonstrated by Bainbridge in 1915) or alterations in the cardiac action potential and resultant electrocardiogram (ECG). Thus, MEF is thought to contribute to arrhythmogenesis in patients with impaired heart function and abnormal filling pressures as a result of various cardiovascular pathologies including hypertension.

Given the surge of interest in MEF in the heart during the past two decades, one would expect that all the anomalies regarding stretch-induced changes in the cardiac action potential would have been thoroughly investigated. Investigations have ranged from *in vivo* animal models (Link *et al.*, 1998; Link *et al.*, 1999; Garan *et al.*, 2005) through to stretch of individual isolated cardiomyocytes using modern patch clamping techniques (Gannier *et al.*, 1994; White *et al.*, 1995; Gannier *et al.*, 1996; Hongo *et al.*,

1996; Cooper *et al.*, 2000; Kamkin *et al.*, 2000a; Belus & White, 2003; Isenberg *et al.*, 2003; Kamkin *et al.*, 2003b; Riemer & Tung, 2003). Thus, MEF has been investigated at all levels within the mammalian system and at different levels of complexity. However, conclusive evidence as to the function of MEF and its integration into normal cardiac function remains quite elusive despite its predicted applications (Lab, 1999; Vemuri *et al.*, 1999). Whilst recent research has developed theoretical mathematical models regarding the physiological function of MEF within the heart (Kohl *et al.*, 1998; Kohl *et al.*, 1999; Garny & Kohl, 2004; Kohl *et al.*, 2006), physiological studies of MEF in the whole isolated heart have provided diverging results between studies. At the cellular level, MEF theory presents certain problems and can be considered discontinuous with other experimental methods. For example, while stretch-activated ion channels thought to be responsible for MEF can be demonstrated in isolated cells (Le Guennec *et al.*, 1991; Sasaki *et al.*, 1992; Gannier *et al.*, 1994; White *et al.*, 1995; Gannier *et al.*, 1996; Hongo *et al.*, 1996; Cazorla *et al.*, 1997; Kamkin *et al.*, 2000a; Riemer & Tung, 2000; Belus & White, 2003; Isenberg *et al.*, 2003; Kamkin *et al.*, 2003b; Riemer & Tung, 2003), calculating or comparing the level of cellular stretch required to activate these currents to those experienced by individual cells in the whole heart throughout the cardiac cycle is made difficult due to unfolding of "slack" membrane on cardiomyocytes in the whole heart model (Khol *et al*, 2003). In addition, many of these experiments were performed at non-physiological temperatures which may bias stretch-induced channel activity and hence the final result regarding the role of MEF in the functional mammalian heart (Maingret *et al.*, 2000a; Patel & Honore, 2001). Moreover, the number of cells that fail to respond to changes in tension across the cellular membrane via various methods can be quite high (50 % or more) (White *et al.*, 1995; Riemer & Tung, 2000, 2003) and many can also respond to the same stretch with differing results (White *et al.*, 1995). This observation, however, is somewhat consistent with similar effects seen *in vitro* (Babuty & Lab, 2001).

Regardless of the theoretical complexities and assortment of effects associated with stretch, some form of MEF is known to exist. In recent years, it has been shown in numerous animal experiments that stretch of the myocardium or direct distension of cardiac muscle strips can lead to significant electrophysiological changes (Franz, 1996). Present literature suggests that the major electrophysiological changes involve:

- A shortening of the action potential duration (APD)
- Depolarisation in the diastolic resting membrane potential (RMP),
- A reduction in action potential amplitude,
- Ectopic beats that originate from depolarisations in RMP during prolonged or extreme stretch.

It should be noted, however, that many of these observations are made utilising different techniques, each specifically unique with its own advantages and disadvantages. For example, APDs can be measured in single isolated cardiomyocytes with reasonable ease, although these experiments were usually at room temperature and at a pacing frequency far below that normally experienced. Similarly, durations can be measured using intracellular impalements (transcellular recordings) of isolated cardiac tissue strips, blind intracellular impalement of the whole heart (although extremely difficult) or indirectly through the use of monophasic action potentials (MAPs). However, depolarisations in resting potential and reductions in action potential amplitude are only observable through direct access to a cell's interior which enables the exact recording of the potential difference developed across the cell membrane. By contrast, the development of after depolarisations and ectopic activity is easier to monitor with MAPs and ECGs because they are less likely to suffer a loss of recording quality upon sudden movements of an additional contraction. Thus, no one technique it best suited for any of the observations and each has its advantages and disadvantages, which will be discussed later, dictating our ability to accurately demonstrate MEF induced changes in the electrophysiology of the heart and its cells.

In order to overcome some of the limitations of each of the methodologies used to evaluate MEF, in this thesis a variety of techniques have been utilised in an attempt to obtain a more comprehensive evaluation of MEF in the whole heart during the normal cardiac cycle. Each method chosen will present an aspect, slightly different from the

next, contributing to the developed theory of MEF in the whole heart during the normal cardiac cycle. The impetus behind implementing these various methods was to develop a reasonably solid understanding and foundation for the function of MEF in the mammalian heart.

STRUCTURE

In chapter 2, past and current literature is reviewed in detail. It should be noted however, that due to the complexity of the topic each of the experimental chapters will provide an individual introduction and literature review of its own to aid the reader in the reasoning behind and justification of the experiments performed. Chapter 3 presents the basic methodology used in the treatment and preparation of animals used throughout all experiments undertaken in this thesis. It is specifically focused on Langendorff experiments but provides detail on the treatment of animals and preparation of physiological preparations. Chapter 4 is composed of preliminary experiments that helped to define the ideal conditions to facilitate MEF observations. Chapters 5-8 deal with the effects of physiological levels of stretch throughout the cardiac cycle in the Langendorff heart using MAP electrode methods and various conditions. These chapters place great focus on the possible involvement of MEF in the Frank-Starling length-tension relationship of the heart. Chapter 7 introduces the technique of using extracellular electrodes to record simultaneously both endocardial and epicardial changes in heart electrophysiology. In addition, these chapters describe the use of various drugs used to modulate MEF-induced changes in cardiac electrophysiology. In chapters 9 & 10, intracellular recording techniques are used to further investigate the responses to stretch observed in the previous chapters using isolated atrial and papillary muscle strip preparations. Chapters 11 & 12 examine another possible involvement of MEF-induced changes in the electrophysiology of the heart, namely, The Gregg effect. Chapter 13 details the phenomenon of rapid stretch-induced ectopic beats in both the Langendorff heart and *in vivo* as positive verification for the presence of MEF. Chapter 14 provides a discussion and vision regarding the results obtained in earlier chapters and a comparison with current and past literature. This chapter also presents a comprehensive conclusion based on the outcomes of all experiments and suggestions for further research.

RESEARCH CONTRIBUTION & AIMS

This thesis makes several main contributions to the current knowledge of MEF involvement in the heart, namely:

- Demonstration of MEF using different types of electrodes (suction and contact) to detect changes in cardiac electrophysiology under various conditions and with physiological and pathological levels of stretch
- The demonstration and comparison of MEF across epicardial and endocardial layers of the ventricular free wall.
- The role of various stretch-activated ion channels, both K^+ selective and non-selective in stretch-dependent changes in electrophysiology of the left ventricle, (making use of various pharmacological modulators: streptomycin, riluzole, chlorpromazine and temperature).
- Lastly, the demonstration of MEF by various means.
    - Rapid stretch induced changes (ectopic inducing), slower physiological changes, perfusion related changes.
    - Contact and transmural MAP recordings.
    - Trans-cellular or intracellular recordings

Through the use of various techniques and preparations, this dissertation explores how MEF may be involved in the normal cardiac cycle. This thesis not only contributes to the body of evidence suggesting a lack of MEF in the normal cardiac cycle, but examines the possible physiological mechanisms behind the phenomenon and explores its involvement in pathological conditions.

CHAPTER 2: LITERATURE REVIEW

The Dancing Divinity
British actress, dancer & singer of the 20s and 30s: Jessie Matthews
(March 11, 1907 - August 19, 1981)

Chapter 2

Chapter 2 - Table of Contents

INTRODUCTION

Changes in myocardial load result in rapid changes to contractile activity through various mechanisms. These mechanisms include intrinsic mechanisms (the Frank-Starling relationship) and extrinsic mechanisms (the autonomic nervous system with the former enabling rapid adaptation of cardiac function to changes in load. This fine balancing of the contractile activity of the heart to changes in cardiac function enables the heart to operate in an efficient manner during periods of varying demand. In addition to these mechanical and chemical mediators, however, the precise relationship between mechanical stretch and changes in the electrophysiology of the heart remain unclear. This relationship, termed mechano-electric feedback (MEF), is thought to underlie many cardiac arrhythmia associated with pathological conditions and may even supply an important connection between the mechanical environment and electrophysiological activity of cardiomyocytes (Franz, 1996; Lab, 1996; Kohl *et al.*, 1999; Lab, 1999; Kohl *et al.*, 2001; Franz & Bode, 2003; Janse *et al.*, 2003; Knoll *et al.*, 2003; Kohl & Ravens, 2003; Ravelli, 2003; Ravens, 2003). These electrophysiological changes are observed not only in the whole heart, but also at the single cardiomyocyte level, and can be explained by the presence of stretch-activated ion channels (SACs). Most investigations of the actions of stretch have concentrated on these sarcolemmal ionic currents thought responsible for the proposed MEF-induced changes in contractility. Whilst these studies have provided some useful insight into possible mechanisms, the inappropriate use of solutions and non-physiological degrees of stretch, render the results somewhat difficult to interpret. In addition, due to the technical difficulties associated with mechanically stretching isolated cardiomyocytes during recording of changes in cell membrane properties, it remains unsurprising that there are conflicting results. As such, currently, little is known about the involvement or contribution of non-selective or K^+ selective SACs to the normal cardiac cycle.

Ventricular Loading as a Trigger for Arrhythmia

Left ventricular function abnormalities resulting from cardiovascular diseases are a strong predictor of sudden death in patients and often result in ventricular tachyarrhythmia and secondary ventricular fibrillation (VF). Whilst VF is associated with ischaemic conditions and the development of infarcts, the underlying electrophysiological changes and role of MEF in helping to generate these arrhythmogenic states remains largely unknown. Reduced oxygen supply to myocardial tissues through narrowed or blocked coronary arteries can lead to reduced performance, myocardial infarction and altered distribution of tension or stress across myocardial tissue. This process alone may promote the development of boundaries where MEF may originate in the heart.

The effects of mechanical interventions on the heart have been recognized for some considerable time. This has primarily resulted from reports of sudden cardiac death associated with chest impact not associated with any physical damage to the heart or it surroundings (Kohl & Ravens, 2003). This is thought to have given birth to the theory of MEF in the animal model and is readily tested by stretching or loading the ventricle of an isolated, perfused heart preparation. Stretch of the ventricles during the cardiac cycle is generally tested in the laboratory under one of two different conditions. Of most physiological relevance is preload, the load that cardiomyocytes are subject to at the time of greatest End Diastolic Volume (EDV) and is usually described in terms of End Diastolic Left Ventricular Pressure (EDLVP). Increases in EDLVP or volume bring about an increase in cardiac contractility, a phenomenon known as the "Frank-Starling" mechanism of the heart. Afterload, that is the stress that the myocardium is under while contracting, has slightly different meanings depending on the model used. Under physiological conditions (*in vivo* or in a working heart preparation), after a brief period of isovolumic contraction, the heart muscle shortens against a more or less constant pressure (ie systolic pressure) once the aortic valve opens. In the usual Langendorff preparation, used in the present experiments, the entire contraction phase is isovolumic, with no appreciable shortening of the contracting muscle.

In working heart models which more accurately simulate the *in vivo* function of the heart, MEF theory has been supported by the observation that changes in EDV (and hence EDLVP) abbreviate the cardiac action potential (Hansen, 1993). In addition to this effect, in the Langendorff heart model, transient increases in EDLVP or pre-load

can produce stretch-induced depolarisations of the ventricular muscle in isolated perfused hearts (Hansen, 1993; Zabel *et al.*, 1996). Further to these effects, early investigations using transmembrane (transcellular) recording techniques of stretched papillary muscles describe reductions in action potential amplitude (Penefsky & Hoffman, 1962).

Excitation-Contraction Coupling

Iin order to best understand the mechanics of MEF in the heart, it is first important to understand the basics of the cardiac cycle. Cardiac contractions are brought about by a process referred to as excitation-contraction coupling. This is the process by which a wave of depolarization conducted across the cardiac tissue through specialized conducting cells initiates a series of intracellular events resulting in Ca^{2+} release and activation of the contractile apparatus. Depolarisations that travel along the conducting pathways are spread across the cardiomyocyte membranes to the intracellular compartment by means of transverse tubules (t-tubules). This depolarisation is rapidly conducted by fast acting voltage gated Na^{+} channels which aid in propagating the wave of depolarization. As a result, L-type voltage gated Ca^{2+} channels on the t-tubule membranes also activate upon membrane depolarisation initiating the process of calcium-induced calcium release (CICR). This is the process whereby extracellular Ca^{2+} that passes into the cardiomyocyte through L-type Ca^{2+} channels activate Ca^{2+} release channels (also known as Ryanodine receptors) on the sarcoplasmic reticulum (SR) of the cell. This results in a large Ca^{2+} influx of SR Ca^{2+} into the intracellular space that then activates the regulatory enzymes (Troponin C) involved in the second process, cross-bridge cycling. The final result is movement of thick and thin filaments of the contractile apparatus and the resultant force develops. Contractile activity is terminated when outward voltage activated K^{+} currents increase, repolarising the membrane and when Ca^{2+} is removed from the intracellular space by means of SR Ca^{2+}-ATPase pumps that transport Ca^{2+} back into the SR, or Ca^{2+} removal by the sarcolemmal membrane (primarily involving the Na^{+}-Ca^{2+} exchange mechanism). As such, cardiac contractile activity is always preceded by a wave of depolarisation-induced excitation or electrical activity that can be readily observed by various means. Due to the involvement of the various ion movements, MEF is thought to be able to alter this process at many levels, namely through changes in Na^{+}, Ca^{2+} and K^{+} conductance during the cardiac action potential. The end result would bring

about modulation of the cardiac action potential shape, resulting in changes to CICR and hence, the level of contractile activity (see Figure 1, below).

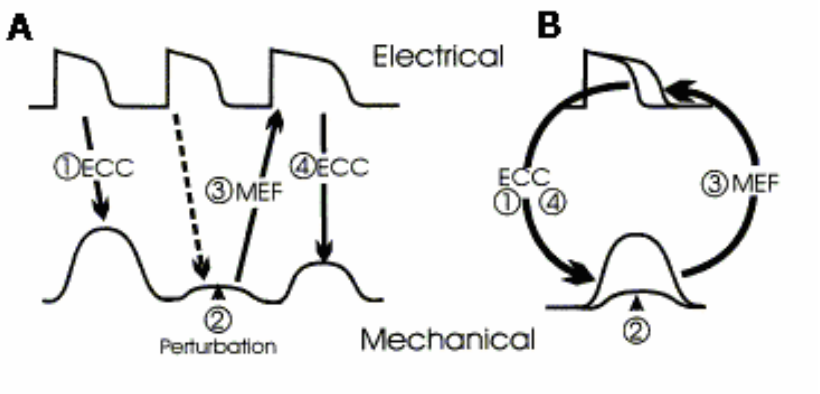

Figure 1: Cellular Regulation Invoking MEF

Diagrammatic interaction of action potentials and contraction. A. Rough time series. B — series depicted as a feedback loop. After the electrical event normal excitation contraction coupling (solid and dashed arrows at (1) ECC) produces a myocardial mechanical change (large mechanical trace with solid arrow). Associated with the next ECC (dashed lines), there is a perturbation at (2). In this illustration it is a load reduction (small mechanical trace), e.g. drop in venous return or blood pressure — decreased stretch. This feeds back (3) MEF) to prolong the action potential. This prolongation tends to increase the force ((4) ECC — repeat left loop in B). (Lab, 1999)

METHODS OF OBSERVING MEF

The various electrode techniques

Monophasic action potentials (MAPs) are one of the methods by which changes in the excitation of cardiac tissue can be observed. MAPs were first defined by what is known today as an injury potential, the changes in electrical excitation observed between an injured region of the heart and a functional region. In theory, injury to the tissue results in the death of cardiomyocytes creating a low resistance access path to the surrounding cells. Thus, when an electrode is placed in such a position and compared to a 2nd electrode placed on a functional area on the surface of the heart, a monophasic action potential (MAP) signal is generated. In the mid 1960s, Korsgren *et al.* (Korsgren *et al.*, 1966) further developed the suction MAP electrode technique which greatly simplified the generation of MAP signals as no myocardial lesion was required to obtain the recording. Instead, suction applied to a small portion of the

myocardial surface produced the cellular inactivation required to obtain the MAP recording. This technique was refined over the years by various labs to the point where MAP recordings became an inherent part of cardiac electrophysiology. In recent years the method of MAP recordings has undergone further development (Franz *et al.*, 1989; Merkely *et al.*, 1998), whereby simple contact between the myocardium and 2 electrodes generate stable high quality MAPs.

Although entirely different from intracellular measurements of changes in membrane potential, the time course of repolarisation of the MAP is near identical to that of action potentials obtained by transmembrane or transcellular techniques (Hoffman *et al.*, 1959; Levine *et al.*, 1986). Despite this fact, passionate debate still exists regarding the theory and electrical function behind the MAP electrode (Franz *et al.*, 1986; Franz, 1999; Knollmann *et al.*, 2002; Kondo *et al.*, 2004; Tranquillo *et al.*, 2004; Franz, 2005; Coronel *et al.*, 2006; Moore & Franz, 2007). It would appear from the most recent literature, however, that the MAP signal is generated from the electrode in contact with non-injured areas of the heart (Kondo *et al.*, 2004; Okamoto *et al.*, 2006), a combination of the two (Franz, 2005) or most recently, the "depolarising" electrode (the electrode that exerts pressure on the myocardium, contains KCL or is associated with suction) (Coronel *et al.*, 2006).

With the debate regarding the bioelectrical theory behind MAP generation, one may wonder why MAP electrodes are used in preference to standard micro-electrode techniques. The simple answer is that MAP electrodes have several distinct advantages over classical ECG and transmembrane recording techniques (Franz, 1994; Coronel *et al.*, 2006). The main advantage of MAPs over ECGs being that the MAP signals recorded are the summation of action potentials from a much smaller population of cells rather than from the whole heart. As such, MAPs give an indication of the rate of cellular repolarisation and unlike ECG recordings; they are not heavily distorted by the dispersion of action potentials around the heart by time, distance and conduction resistances. As a result, MAP recordings exhibit electrical phenomena otherwise hidden in ECG recordings. More importantly, MAPs are reasonably easily established using contact electrode techniques (Franz, 1999).

By contrast, intracellular impalement techniques that produce transcellular or transmembrane potentials (otherwise known as transmembrane action potentials), provide all possible electrophysiological information about the repolarisation state of

individual cardiomyocytes. The biggest disadvantages of this method, however, is the ease with which the recording electrode is broken by vigorous contractile activity and that the cellular membrane must be damaged to gain access to the intracellular space, both problems not encountered with MAP electrodes (Franz, 1994). Another disadvantage is their susceptibility to movement induced artifacts, loss of seal quality and resultant recording. This is more so for patch clamp and whole cell recording techniques of individual isolated cardiomyocytes during stretch. In patch-clamp recordings of stretch activated currents and electrophysiological changes, stretch of the cellular membrane could reversibly increase non-specific leak currents into the cell, that may simulate the presence of SAC ion currents. Due to the variability of this technique, it is impossible to predict the amplitude of such stretch-induced leak currents and account for them in final recordings (Crozatier, 1996). Whilst MAP recordings are also subject to movement artifacts, they are less so than intracellular or trans-membrane techniques.

The main advantage of MAP techniques over similar impalement techniques is the application of the method to the intact beating heart. However, despite the usefulness of the method, there remain several major drawbacks:

(1) MAP recordings have an arbitrary amplitude and resting membrane potential (RMP) – neither which reflect the true membrane voltages obtained with intracellular impalement techniques. The amplitude of MAP recordings is relative to the number of cells inactivated or the amount of damage created/force applied to the myocardium. (2) As mentioned previously, the MAP is a summation of a finite number of cells (although significantly less than an ECG). As such, the MAP signal reflects action potentials from many cells determined by the region of the actual recording electrode (Leirner & Cestari, 1999).

MEF at the Cellular Level

As discussed, it is well known fact that myocardial stretch brings about increases in cardiac contractility, this is readily seen by increasing end diastolic volume (Frank-Starling or length tension relationship) and even increases in coronary flow (Gregg effect). A variety of mechanisms both intrinsic and extrinsic have evolved to control the heart's function in response to changing demands placed on it by the body. However, for the heart to be able to rapidly adapt on a beat-to-beat basis, this must

involve a mechanism inherent to the cardiomyocytes that make up the myocardial tissue and this mechanism must be sensitive to changes in stress occurring in the muscle during both systole and diastole (Hanck & Jewell, 1985). The before mentioned changes in electrophysiology in response to altered myocardial loading, in theory, requires the presence of ion channels sensitive to stretch (Kim, 1993; Kohl & Sachs, 2001) and these are likely to generate electrical changes able to be observed using MAP and transcellar recording techniques. Importantly, authors have observed stretch-related depolarisations (Franz *et al.*, 1989; Zabel *et al.*, 1996) and correspondingly, the opening of poorly selective cation channels in isolated cardiomyocytes (Craelius *et al.*, 1988; Akay & Craelius, 1993; Hu & Sachs, 1996; Kamkin *et al.*, 2000a; Zhang *et al.*, 2000; Kamkin *et al.*, 2001; Belus & White, 2003; Kamkin *et al.*, 2003b; Kamkin *et al.*, 2003c) using both electrode methods. Thus, stretch-activated ion channels (SACs) on the cell membrane and the ion currents they pass serve as the Mechano-sensors for the cardiomyocyte. SACs, through their inherent ability to change open probability in response to mechanical stimuli are able to convert changes in mechanical loading or stretch into alterations in cellular ion movements (Kohl *et al.*, 2006). Of the various SACs known, two forms are at least known to exist on the membrane of cardiomyocytes enabling complex stretch-induced changes in electrophysiology to occur. However, to understand their effects on the cardiomyocyte, it is important to revisit the basic ionic processes that accompany the normal cardiac cycle.

Simple Electrophysiological Theory

All mammalian cells are separated from their surroundings by a phospholipid bilayer (plasma membrane). Due to the presence of proteins that form specific ion channels, the semi-permeable nature of this plasma membrane enables the formation of an electrical potential between the inner and outer compartments. The cytoplasm within the cell contains a host of complex anions (mainly proteins) and simple ions such as K^+ and Cl^-, to which the membrane is selectively permeable based on the presence and functional status of ion channels and carriers. These simple ions are able to move across the cell membrane in a direction determined by their relative concentration gradients and the potential difference across the cell membrane. Under resting conditions, the Na^+/K^+ ATPase exchanger maintains a low $[Na^+]_i$ and high $[K^+]_i$. Due to the imbalanced stochiochemistry of this transporter, ($3Na^+$ removed for each $2K^+$

retained), in conjunction with the presence of impermeable intracellular complex anions a negative membrane potential is maintained. As a result, increases in Na^+ permeability through the opening of Na^+ permeable channels on the plasma membrane enable Na^+ ions to diffuse down their concentration gradient into the cell, carrying positive charges that depolarize the cell interior with respect to the extracellular space. Likewise, opening of K^+ permeant channels enables K^+ ions with a positive charge to leave the cell by moving down their concentration gradient making the cell interior more negative with respect to the extracellular space. Whilst these are the two major depolarising and repolarising currents respectively, other ions such as Ca^{2+} can also play a role in altering membrane potential (Vm) via similar mechanisms and changes in plasma membrane permeability to these ions during CICR and cellular contraction.

As such, in cardiomyocytes the action potential is shaped by a combination of ion channels on the plasma membrane that alter ionic permeability in a specific and regulated fashion. The upstroke phase is generated by a large and rapid increase in Na^+ permeability that enables Na^+ ions to diffuse into the cell by means of voltage gated Na^+ channels. This results in a rapid change in membrane potential to near 20 mV. This depolarization also triggers the opening of voltage gated L-type Ca^{2+} channels and a range of voltage gated K^+ channels. The former enable Ca^{2+} to diffuse into the cell, increasing $[Ca^{2+}]_i$ and initiating calcium-induced calcium release (CICR) that ultimately activates the contractile apparatus. Opening of K^+ channels is generally much slower, and contributes primarily to the repolarisation phase (termination of contraction) but also to action potential morphology. All these ion currents are regulated by their respective concentration gradients as well as the membrane potential. As such, the driving forces for each of the currents alters over time as the membrane potential swings from negative resting membrane potentials (RMP) of ~ -80mV to positive potentials of 0-20 mV. In addition to the presence of voltage gated Na^+, K^+ and Ca^{2+} channels are Na^+-Ca^{2+} exchangers in the plasma membrane. The main function of these exchangers is in Ca^{2+} maintenance. Like the Na^+-K^+ exchanger, the Na^+-Ca^{2+} exchanger is electrogenic in that 3 Na^+ ions are moved for every Ca^{2+} ion. In addition, the direction of this exchanger is reversible enabling an inward or outward current, the polarity of which depends on the electrochemical gradients and Vm. In general, during the initial action potential upstroke, the Na^+-Ca^{2+} exchanger enables Ca^{2+} movement into the cell, whilst increasing $[Ca^{2+}]_i$ brought about by CICR and inward L-type Ca^{2+} currents force the exchanger into reverse mode, removing

Ca^{2+} from the cell and producing a depolarising inward Na^{+} current that tends to lengthen the action potential in later stages. The action potential itself is terminated ready for the next event once the cell is repolarised by outward K^{+} currents and removal of intracellular Ca^{2+} via the Na^{+}-Ca^{2+} exchanger and sarcoplasmic Ca^{2+} pumps (refer to Figure 2 below).

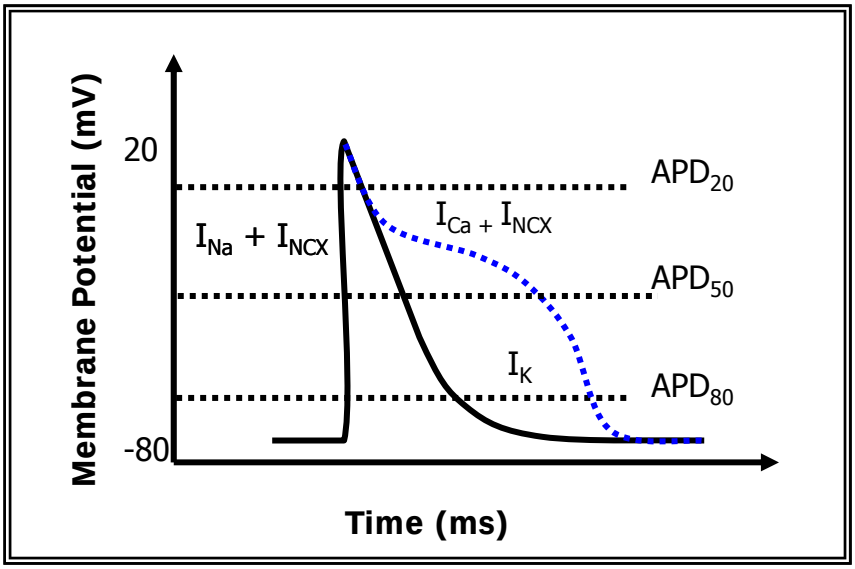

Figure 2: Cardiac Action Potential

Diagram showing ionic fluxes involved in (1) depolarisation, I_{Na}, I_{NCX} (2) initiation of Calcium-induced Calcium release, I_{Ca}, I_{NCX}, and (3) repolarisation, I_K. As would be expected, additional ion currents allowing movement of Ca^{2+}, Na^{+} or K^{+} across the membrane via stretch-activated ion channels would alter the action potential morphology. Action potential duration (APD) is generally measured at 20, 50 and 80 % of repolarisation as denoted by $APD_{20,\ 50}$ and APD_{80}. Black trace shows typical rat action potential, whilst the blue trace denotes a guinea-pig action potential.

Non-Selective Stretch-Activated Cation Channels (NSACs)

Because a range of stretch-activated ion channels exist that are able to respond to an array of conditions including osmotic cell swelling, the remainder of this review focuses on channels activated by uniaxial stretch and its cellular equivalents since this model most accurately reflects stretch of cardiac tissue (Brady, 1991).

Based on the simple electrophysiological theory presented above and a proposed reversal potential of between 0 and -36 mV, non-selective stretch-stretch activated cation channels (NSACs) generally depolarize the cell when activated (Sasaki *et al.*,

1992; Hu & Sachs, 1996; Kamkin *et al.*, 2000a; Zeng *et al.*, 2000; Zhang *et al.*, 2000; Isenberg *et al.*, 2003). If myocardial stretch is applied during diastole when the transmembrane potential is negative (-80 mV) relative to the equilibrium potential of the NSAC (-36 mV), then an inward current (termed I_{NSAC}) will flow through the channels and produce a depolarisation of the cardiomyocytes' membrane. This depolarisation has been described previously (Craelius *et al.*, 1988; Bustamante *et al.*, 1991; Kamkin *et al.*, 2000a; Isenberg *et al.*, 2003) and has been shown by some authors to rapidly adapt and by others to show no adaptation properties at all (Sasaki *et al.*, 1992; Hu & Sachs, 1996; Bett & Sachs, 2000b, a; Zeng *et al.*, 2000). Activation of I_{NSAC} which ranges in size from 21-25 pS (and even as large as 120 pS have been quoted) leads to an influx of Na^+ and Ca^{2+} and an observable change in their intracellular concentrations with increasing $[Na^+]_i$ favouring further Ca^{2+} accumulation via the Na^+-Ca^{2+} exchange mechanism (Sigurdson *et al.*, 1992; Kondratev & Gallitelli, 2003). Furthermore, the stretch-induced depolarisation in Vm can either prolong the action potential, or if large enough, it may cause the cardiac myocyte membrane potential to depolarise to threshold activating the normal chain of events that accompany excitation-contraction coupling. With this in mind, the major current carried by NSACs is Na^+ (Youm *et al.*, 2006) and full activation is observed at 20-30 mmHg pipette suction (Ruknudin *et al.*, 1993). Despite this observation, whilst stretch has been shown to increase diastolic $[Ca^{2+}]_i$ measured by indo-1 and fura-2 in guinea pigs (Le Guennec *et al.*, 1991; White *et al.*, 1995), the same cannot be said for adult rat myocytes (Hongo *et al.*, 1996).

Others have found the reversal potential of I_{NSAC} to be nearer 0 mV (Kamkin *et al.*, 2000a; Isenberg *et al.*, 2003) which has obvious considerations regarding ion movements during the cardiac cycle. Consequently, it can be argued that a reduction in the action potential duration at 20% repolarisation (APD_{20}) when the membrane is near 0 mV and the Ca^{2+} plateau potential. This will inherently inhibit Ca^{2+} entry via both voltage activated L-type Ca^{2+} channels and reverse mode of the Na^+-Ca^{2+} exchange mechanisms. As such, one would expect an abbreviation of APD to accompany a reduction in action potential amplitude. The main concern for the present research is that many of the studies on isolated cells have been performed on immature cells lines. Of the articles published utilizing adult ventricular cardiomyocytes, it was strongly emphasized that every cell needed to undergo continuous stretch for at least five minutes before any mechanosensitive response was

observed (Bett & Sachs, 2000b). This is a direct contradiction to the rapid response of NSACs in other isolated cellular preparations mentioned above and would tend to suggest processes other than direct ion fluxes across the cellular membrane through NSACs are occurring in response to stretch. As such, it remains possible that the effects are a result of membrane degradation at either the mechanical probe or patch pipette sites used to stretch single cardiac myocytes, an observation previously reported to accompany changes in resting membrane potential in isolated myocyte preparations (White *et al.*, 1993; Tavi *et al.*, 1998). This is a possibility strongly supported by the observations that mechanosensitive currents were easily elicited when cells were stretched under manual control, but not when under computer control, even if large excursions/tension are applied (Bett & Sachs, 2000b). Others have also reported a loss of membrane stability upon mechanical stimulation which could induce SAC-like leak currents (Hu & Sachs, 1996). In addition to these effects, single channel recordings of I_{NSAC} have not successfully been demonstrated in the adult rat cardiac myocyte despite repeated attempts (Zeng *et al.*, 2000). The reasons for this discrepancy in the ability to demonstrate whole cell I_{NSAC} in adult cardiac myocytes and the current at the single channel level remain unclear but have been proposed to relate to the location of NSACs in the T-tubules or changes in channel property upon patch formation (Zeng *et al.*, 2000). The latter could be considered unlikely since other stretch channels are readily demonstrated at the single channel level in the adult rat cardiac myocyte (Duprat *et al.*, 2000; Terrenoire *et al.*, 2001; Niu & Sachs, 2003; Tan *et al.*, 2004).

Research from other investigators suggests that the electrophysiological effects produced when the loading of cardiomyocytes is altered may be mediated by length-dependent modulation of intracellular Ca^{2+} concentration (Allen & Kurihara, 1982). Rises in intracellular Ca^{2+} either through NSACs (Sigurdson *et al.*, 1992; Kondratev & Gallitelli, 2003) or from the contractile apparatus in response to rapid release and changes in sarcomere Ca^{2+} affinity (Allen & Kurihara, 1982) may invoke myocardial depolarization through the Na^{+}-Ca^{2+} exchange current (Spencer & Sham, 2003). This leads to another MEF theory altogether.

Part of the discrepancy in results may relate to the methods used to demonstrate the presence of MEF and NSACs which have ranged from isolated cell preparations (Nakagawa *et al.*, 1988; Gannier *et al.*, 1994; Wang *et al.*, 1996; Isenberg *et al.*, 2003; Kondratev & Gallitelli, 2003) to whole heart preparations both *in vitro* (Hansen *et al.*,

1991; Stacy *et al.*, 1992; Hansen, 1993; Wang *et al.*, 1994; Zabel *et al.*, 1996; Todaka *et al.*, 1998; Eckardt *et al.*, 2000) and *in vivo* (Franz *et al.*, 1989; Horner *et al.*, 1994; Wang *et al.*, 1994; Satoh & Zipes, 1996; Karunanithi *et al.*, 1999; Takagi *et al.*, 1999).

Of importance in the stretch-induced effects on isolated cardiomyocytes are observable membrane depolarisations (sometimes leading to spontaneous electrical activity) as well as $[Na^+]_i$ accumulation (Franz *et al.*, 1992; Isenberg *et al.*, 2003; Kondratev & Gallitelli, 2003). Stretch in general reduces the plateau of guinea pig action potentials (Figure 2: Cardiac Action Potential), and results in longer APDs at 80-90% repolarisation (Isenberg *et al.*, 2003). However confusion still exists regarding the effect of stretch on $[Ca^{2+}]_i$. In adult rat ventricular myocytes and ventricular trabeculae, stretch failed to induce increases in diastolic $[Ca^{2+}]_i$ (Hongo *et al.*, 1996; Kentish & Wrzosek, 1998) whist others have observed a decrease in diastolic $[Ca^{2+}]_i$ during stretch (Alvarez *et al.*, 1999) accompanied by an increase in the Ca^{2+} transient. Further complicating the involvement of NSACs on $[Ca^{2+}]_i$ is the observation that stretch doubled diastolic $[Ca^{2+}]_i$ concentrations in isolated mouse ventricular myocytes (Kondratev & Gallitelli, 2003). Likewise, whilst Na^+ is the major carrier current of NSACs (Youm *et al.*, 2006), little or no change in $[Na^+]_i$ is observed in rat ventricular myocytes (Hongo *et al.*, 1996). In theory, however, if NSACs operate within the physiological range of stretch, then cell membrane stretch through the function of NSACs should provide a depolarising current at most membrane potentials except during the initial rapid peak depolarization cause by I_{Na}.

As such, it is thought these channels are responsible for the development of after depolarisations and trigger responses or arrhythmia (Craelius, 1993). Early after depolarisations (EADs) are those that appear before termination of the previous action potential and generally only occur when resting membrane potential is more negative than -10 mV. By contrast, delayed or late after depolarisations (DADs) occur after resting membrane potential is reached. Likewise, Ca^{2+} overloading of cells either through SACs, changes in Troponin C affinity for Ca^{2+} upon quick release, or other mechanisms can all induce after depolarisations through reverse mode Na^+-Ca^{2+} exchange activity. Transport of Ca^{2+} out of the cells via the Na^+-Ca^{2+} exchanger generates a depolarising inward Na^+ current (due to the 3:1 stochiometry) that can also elicit these ectopic responses.

Potassium-Selective Stretch-Activated Cation Channels (KSACs)

In addition to NSACs, other channels have also been suggested, and even demonstrated, to be stretch activated. These include the inward rectifier K^+ channels (Isenberg *et al.*, 2003) and outwardly rectifying K^+ currents (Maingret *et al.*, 1999b), with the latter suggested to represent TREK-1 current also found in cardiomyocytes (Maingret *et al.*, 1999b). Others have found stretch sensitive Ca^{2+} channels in the endocardial endothelium of rat papillary muscles (Kohler *et al.*, 1998) or have reported that stretch reduces other standard currents such as I_{CaL} (Matsuda *et al.*, 1996; Isenberg *et al.*, 2003). More debatable are the observations that stretch may activate K_{ATP} channels (Van Wagoner, 1993) and components of the delayed rectifier current (Wang *et al.*, 1996).

Formerly reported in adult rat atrial cells (Kim, 1993) and embryonic chick cells (Ruknudin *et al.*, 1993; Hu & Sachs, 1996; Kawakubo *et al.*, 1999) potassium-selective SACs (KSACs) exist in two forms having either a small conductance of around 25 pS or a larger conductance of near 100 pS, have a reversal potential close to the RMP and have been reasonably well studied (Kim, 1993; Maingret *et al.*, 1999b; Terrenoire *et al.*, 2001; Tan *et al.*, 2002; Kim, 2003; Niu & Sachs, 2003; Tan *et al.*, 2004). Unlike NSACs, these KSACs are outwardly rectifying and their reported sensitivity to negative pipette pressures varies considerably between studies with half maximal activation having been quoted as low as 1.5 mmHg suction, making them more sensitive to mechanical loading than NSACs (Kim, 1993; Terrenoire *et al.*, 2001; Niu & Sachs, 2003). In addition, some have reported that these KSACs (likely TREK-1 channels) display adaptation within a short period, whilst others have found this not to be the case (Tan *et al.*, 2002; Niu & Sachs, 2003; Tan *et al.*, 2004). Unlike NSACs, there are no specific inhibitors of KSACs or TREK-1 channels. Instead, K^+-channel blockers, or amphiphile-induced membrane curvature must be used to alter channel properties. The cationic amphiphile Chlorpromazine is used to inhibit TREK-1 channels. Chlorpromazine consists of a charged hydrophilic and uncharged hydrophobic domain, and therefore interacts with the inner and outer leaflets of the membrane bilayer. As such, chlorpromazine mimics cell shrinkage and inactivates TREK-1 channels. The obvious problem with this method is that it may stimulate or inhibit other ion channels involved in cell volume regulation (Baumgarten & Clemo, 2003). Moreover, one must

wonder if TREK-1 channels are stimulated by changes in cell volume rather than axial stretch *per se*.

The other major defining difference between NSACs and KSACs is that these channels also respond to a variety of chemical stimuli including inhalational anaesthetic agents, poly-unsaturated fatty acids, phospholipids and even modulation through phosphorylation (Patel *et al.*, 1998; Maingret *et al.*, 1999b; Patel *et al.*, 1999; Maingret *et al.*, 2000b; Maylie & Adelman, 2001; Patel & Honore, 2001; Patel *et al.*, 2001; Terrenoire *et al.*, 2001; Patel & Honore, 2002). For a review of TREK-1 function and properties refer to (Lesage & Lazdunski, 2000; Lesage, 2003).

METHODS USED TO VISUALISE SACS

It should be noted as with all cellular experiments that the pressures applied to the patch pipette or stretching stylus for mechanical stimulation of isolated cardiomyocytes are not comparable to those pressures found in the ventricular wall of the heart. As such, it remains uncertain if the properties of SACs in isolated membrane patch clamp experiments accurately reflect their contribution to MEF in intact cardiac tissues. Complementary to this, treatment of cells with pharmacological agents known to disrupt the cytoskeleton system such as cytochalasin D or colchicin reduce the sensitivity of NSACs suggesting the cellular matrix/cytoskeleton are important in transferring deformation to changes in ion channel function, a process disrupted by the very process of patch clamping (Isenberg *et al.*, 2003; Kamkin *et al.*, 2003b). In addition, TREK-1 has been demonstrated to have profound effects on the actin network architecture in transfected cells. Conversely, the actin cytoskeleton represses TREK-1 mechano-gating suggesting that cross-talk between TREK-1 and the cytoskeleton may have an important role in the control of cellular excitability (Lauritzen *et al.*, 2005). As such, the function of SACs in isolated cardiomyocytes using patch clamp experiments where the cytoskeleton is knowingly disrupted in an unpredictable manner may not necessarily reflect their global function in the whole cell let alone the whole heart.

NSAC Modulators

Among the problems of characterising SACs at the single cell level are problems associated with modulating their function pharmacologically. Gadolinium (Gd^{3+}), a trivalent lanthanide was first established as a blocker of NSACs in *Xenopus Oocytes*

(Yang & Sachs, 1989) and has since been a popular choice in the past for "selective" inhibition of NSACs and suppression of stretch-induced ectopic activity in the concentration range of 10-40 μM (Bustamante *et al.*, 1991; Stacy *et al.*, 1992; Ruknudin *et al.*, 1993; Tavi *et al.*, 1996; Hu & Sachs, 1997; Takagi *et al.*, 1999; Bode *et al.*, 2000; Cooper *et al.*, 2000; Zeng *et al.*, 2000; Zhang *et al.*, 2000; Nicolosi *et al.*, 2001; Kamkin *et al.*, 2003b; Nicolosi *et al.*, 2004). However, these results should be considered with great care especially when used in the presence of bicarbonate and phosphate buffers (Boland *et al.*, 1991) since the free concentration of Gd^{3+} in these solutions is "vanishingly low" and thus failure to detect or observe effects of Gd^{3+} on SAC-related mechanisms does not itself provide credible evidence regarding the role of SACs (Caldwell *et al.*, 1998). This is of some concern since there have been a number of publications involving Gd^{3+} in the presence of bicarbonate and phosphate buffers not to mention the use of Gd^{3+} *in vivo* (Ovize *et al.*, 1994; Takano & Glantz, 1995; Takagi *et al.*, 1999) where the concentrations quoted are thus unlikely to be reliable and reflective of the free Gd^{3+} concentration (the form proposed to block SACs). Others have also noted differences in the function and actions of Gd^{3+} depending on the physiological solution used (Lacampagne *et al.*, 1994; Ward & White, 1994). These facts are of considerable importance when interpreting results such as those obtained by the administration of Gd^{3+} (10 μM) in the whole animal model (Penefsky & Hoffman, 1962; Ovize *et al.*, 1994; Takano & Glantz, 1995; Takagi *et al.*, 1999) given the actual free concentration of Gd^{3+} proposed to interact with NSACs is much lower.

In addition to the effects of unwanted Gd^{3+} precipitation in physiological buffers through binding of anions (Caldwell *et al.*, 1998), Gd^{3+} has also been shown to have an array of effects at low concentrations on other ion channels and transport mechanisms found on cardiomyocytes and is described as highly non-specific (Pascarel *et al.*, 1998). Of interest was the observation that Gd^{3+} is a potent L-type Ca^{2+} channel blocker in isolated guinea pig cardiomyocytes with an EC_{50} of 1.4 uM and complete block at 10 μM (Lacampagne *et al.*, 1994), a concentration well within the concentration range used by many studies to inhibit NSACs (Ruknudin *et al.*, 1993; Hu & Sachs, 1997). Other studies have also identified I_{Ca} and I_{Na} inhibitory effects of Gd^{3+} (Boland *et al.*, 1991; Li & Baumgarten, 2001). Further to these observations, others have shown Gd^{3+} to block the K2P channels such as TRAAK and TREK-1 (Maingret *et al.*, 1999a; Maingret *et al.*, 2000b), delayed rectifier potassium current with an EC_{50} of 24 μM whilst not inhibiting stretch-induced increases in $[Ca^{2+}]$ (Hongo *et al.*, 1997).

More recently, Gd^{3+} has also been shown to inhibit the Na^{+}-Ca^{2+} exchange current with an IC_{50} concentration as low as 20 μM (Zhang & Hancox, 2000). Of particular interest is the considerable variation in the concentration of Gd^{3+} required to inhibit NSACs, with concentrations of up to 100 μM having been shown to inhibit only 90% of I_{NSAC} and to be only partially reversible upon washout (Zeng *et al.*, 2000) As such, results obtained regarding the function of NSACs in the presence of Gd^{3+} should be considered carefully and these previous findings taken into consideration when interpreting results. These findings are likely to explain the differences in the effects of Gd^{3+} on action potentials and stretch responses by several authors (Hansen *et al.*, 1991; Tavi *et al.*, 1996; Hongo *et al.*, 1997; Takagi *et al.*, 1999; Bode *et al.*, 2000; Nicolosi *et al.*, 2001). As such, the non-specific actions of Gd^{3+} mean that the Gd^{3+}-sensitive currents observed in patch clamp experiments are not necessarily due to NSACs alone, likewise, one can not be assured that Gd^{3+} is only blocking NSACs in isolated tissue and whole heart preparations even at low concentrations.

With this in mind, streptomycin was considered a better and more selective blocker of NSACs as others have shown complete block at concentrations ranging from 30 to 200 μM (Gannier *et al.*, 1994; Slinker & Tobias, 1996; Kamkin *et al.*, 2000a; Belus & White, 2003; Isenberg *et al.*, 2003; Kondratev & Gallitelli, 2003; Nicolosi *et al.*, 2004). Although streptomycin is considered by some to be somewhat non-specific and can block other ion channels including I_{Ca}, I_{Kr}, and I_{Ks}, the concentrations at which the IC_{50} of these effects occur is well beyond that used to effectively block NSACs (Belus & White, 2002). Only one known drug derived from the venom of a Chilian tarantula (grammastola toxin) is known to selectively block NSACs in chick ventricular myocytes (Suchyna *et al.*, 2000). However, the active peptide, GsMTx4, is expensive and difficult to obtain in quantities suitable for a series of whole heart experiments.

Methods of Demonstrating SACs

The presence of SACs in cardiomyocytes has been demonstrated using various methods ranging from isolated ventricular myocytes through to changes in end diastolic pressure or volume of the left ventricle (EDLVP) in the whole heart. The main issues with working at the cellular level are in applying the mechanical forces to the myocyte required to activate the channels. Whilst various methods have been applied from carbon/glass styli and measuring changes in sarcomere length (Belus & White, 2003; Isenberg *et al.*, 2003), ferric oxide beads (Glogauer *et al.*, 1995), and membrane

patch suction/stretch (Craelius, 1993), the physiological relevance of these methods cannot be determined. Moreover, the great technical difficulty associated with these methods (Nishimura *et al.*, 2004) and the susceptibility to leak currents developing (White *et al.*, 1993; Hu & Sachs, 1996; Tavi *et al.*, 1998) makes these experiments somewhat difficult to conduct and maintain a stable recording during stretch (Brady, 1991). These factors confounded with the observations that stability of stretched isolated cardiomyocyte preparations last 10 minutes at best (Nishimura *et al.*, 2004) and reported NSAC currents require at least 5 minutes to develop (Bett & Sachs, 2000b) suggest there is a very fine window for observing a true NSAC current. With this in mind, initial experiments demonstrated variable effects of stretch on both action potential configuration and resting membrane potential (White *et al.*, 1993). Despite the efforts of others, no single channel recordings of NSACs exist in the isolated adult cardiac myocyte, and as such, it is impossible to accurately deduce the effectiveness of modulating agents such as Gd^{3+} and streptomycin on these channels.

Relevance to Pathological Conditions

As discussed, SACs are thought to form the transduction mechanism between mechanical and electrophysiological processes in the heart. In addition, SACs have been proposed to play a role in mechanical and electrophysiological remodelling in both cardiac hypertrophy (Bustamante *et al.*, 1991; Nicolosi *et al.*, 2001; Nicolosi *et al.*, 2004) and arrhythmic states (Franz *et al.*, 1992; Stacy *et al.*, 1992; Bode *et al.*, 2000; Kamkin *et al.*, 2000b; Kiseleva *et al.*, 2000). In theory cardiac hypertrophy and ischemic zones place increased mechanical stress on individual cardiomyocytes leading to the activation of SACs in the sacrolemmal membrane (Franz *et al.*, 1992; Kamkin *et al.*, 2000b). As described and demonstrated by others, the opening of NSACs in practice leads to an influx of Na^+ and Ca^{2+} ions which then have the potential to trigger other intracellular processes including electrophysiological changes (MEF) and altered protein synthesis that may lead to altered cell activity and growth. In support of these theories, recent research has demonstrated that inhibition of stretch-activated currents with Gd^{3+} (10 μM) and streptomycin (40 μM) can restore contractile function associated with dilated cardiomyopathy (Nicolosi *et al.*, 2004)

Commotio cordis is another stretch-related phenomena whereby blunt impacts to the chest can result in sudden death without obvious reason at autopsy (Futterman & Lemberg, 1999; Link, 2003; Link *et al.*, 2003). Since the presence of SACs was first

identified in the mammalian heart, a number of studies have been conducted *in vivo* to demonstrate their involvement in *commotio cordis* (Kohl *et al.*, 2001; Li *et al.*, 2004; Garan *et al.*, 2005). The concept of SACs was applied to the situation where by blunt impacts to the chest wall could initiate ectopic beats by the processes described above and develop into ventricular arrhythmias. However, whilst modelling of the process suggests a role for NSACs in the phenomenon (Li *et al.*, 2004), *in vivo* experiments demonstrate a lack of NSAC modulation (Garan *et al.*, 2005) or a role for K_{ATP} channels (Link *et al.*, 1999) instead.

Whilst the existence of MEF has been well established as far back as the 1930s, the mechanisms of arrhythmia induction through SACs and MEF by stretch remain poorly defined. Mechanisms involving the function of MEF have been readily proposed, but studies fail to find adequate functional relevance, even in pathological models such as hypertrophy (Jauch *et al.*, 1994). All of these observations make isolated interpretations of MEF difficult to extrapolate back to functional relevance.

CONCLUSION

The proposed effects of stretch on the heart are many and may not necessarily involve SACs (Sung *et al.*, 2003). Changes include reductions in action potential conduction velocity and special dispersion (Ravelli, 2003) , the development of additional depolarisations (Eckardt *et al.*, 2001; Ravelli, 2003), and reductions in refractoriness (Eckardt *et al.*, 2001). These can all contribute to action potential conduction abnormalities, whereby ectopic activity induced by additional, stretch-induced depolarisations may initiate arrhythmic activity. Although multicellular preparations have been used in the past to provide insight into the importance of MEF in the whole heart or cardiac tissue, the present series of experiments aims to use pharmacological modulators of SACs with the hope of providing more specific information on the contribution of mechanosensitive channels in normal heart function/physiology. Whilst the results obtained will be unable to provide information about the specific behaviour of the channels, it will provide results more readily extrapolated back to the whole animal than isolated myocyte preparations.

Nonetheless, the contribution of SACs to electrophysiological changes that are associated with the induction and incidence of cardiac arrhythmias would make them an appealing pharmacological target for the prevention of developing life threatening arrhythmias in patients with left ventricular function abnormalities resulting from

cardiovascular diseases. This, however, only holds true if the responses can be adequately modulated and selectively targeted using pharmacological agents and forms the main framework for the reseach presented in this thesis.

Thus, study of the effect of stretch on cardiac electrophysiology has produced quite variable results. Whilst the present experiments appear superficially quite similar, there are a number of significant differences. These differences can be categorized into

- Animal model of choice, the rat and guinea pig.
- Tissue of choice, – only the left ventricle and left ventricular papillary muscles.
- Methods of stretch application, left ventricular balloon or axial stretch of papillary muscles.
- Methods of recording electrophysiological or changes in action potential morphology – namely MAP (various forms) and transmembrane/intracellular impalements.

Lastly, the research directions are different in that various methods and conditions are imposed on preparations in an attempt to modulate MEF responses and elucidate the role of SACs rather than merely recording the presence of SACs or stretch-induced changes in action potential morphology. Stretch was applied via various means and using a common model. The main approach was to implement various methods to demonstrate MEF and the functional contribution of SACs and MEF throughout the cardiac cycle. In addition, part of the present study aimed at investigating heterogeneous contribution of SACs to MEF across the heart wall. Consequently through the present design undertaken a number of issues are addressed because conditions (namely solutions, drug concentrations, source of animals) are maintained constant and thus not likely to differentially affect outcomes.

CHAPTER 3: GENERAL METHODS

Cabaret dancer & singer of the 20s and 30s: Joésphine Baker
(June 3, 1906 – April 12, 1975)

Chapter 3 - Table of contents

INTRODUCTION

The aims of the majority of experiments undertaken in this thesis were to demonstrate the presence and role of MEF in the normal cardiac cycle of the mammalian heart using both rat and guinea-pig models. Pharmacological agents known to affect the function of TREK-1 channels (KSACs) and SACs were added to the perfusate of Langendorff heart preparations to modulate stretch induced changes in various cardiac parameters. In Chapters four and five, changes in cardiac electrophysiology (as recorded by monophasic action potentials, MAPs) upon stretch and during the Frank-Starling (length/tension-force) relationship were investigated using both surface and transmural MAP electrode techniques.

In this chapter, the method of setting up Langendorff perfused rat and guinea pig hearts are described. The chapter consists of a brief history of the Langendorff model, followed by the apparatus and procedures required to setup and perform a functional Langendorff experiment. The chapter uses preliminary experiments to explore intrinsic properties of the Langendorff-perfused rat heart so that the conditions for best observing MEF could be used. The chapter then describes the method with which MEF-induced changes in the cardiac action potential is examined. Here, three types of electrodes and their construction are detailed as well as a brief indication of how they will be used. Finally, the chapter gives a description of the general findings and conditions used for Langendorff experiments performed in later chapters.

BACKGROUND

Langendorff Perfused Heart

The Langendorff perfused heart was first pioneered by Oscar Langendorff more than a century ago. With this method, he was able to maintain the health of an isolated heart through the use of a physiological saline solution that was pumped into the heart via the aorta (Zimmer, 1998). As would occur in the normal heart, this results in a build up of back pressure in the aorta. This causes the aortic valve to close and forces the perfusate through the coronary vessels. This would then supply the cardiomyocytes with the nutritional environment suitable for survival for several hours. This *in vitro* method of maintaining heart activity is termed "retrograde" perfusion because the perfusate flows backward through the aorta and into the coronaries (but not into the ventricles) rather than in the normal situation where blood enters the aorta and coronary vessels from the left ventricle. Without question, the main importance of Oscar Langendorff's technique is that it enables the examination of various cardiac parameters; both electrophysiological and functional, that are often otherwise impractical *in vivo*. This is because the heart is able to be maintained in an artificial environment, and the ventricles remain empty throughout the cardiac cycle. The major limitation of this method is that the saline perfusate carries only a fraction of the oxygen compared to that of blood. As such, regardless of perfusion flow rate and tubing lengths, the saline perfused Langendorff heart is always somewhat hypoxic and prone to developing oedema to some extent compared to the more costly and complex blood perfused equivalent.

Since in a typical Langendorff perfusion the heart is not perfused via the normal pathway but retrogradely, the intra-ventricular pressure (left ventricular pressure [LVP]), must be measured using a fluid-filled balloon attached to a catheter inserted into the left ventricle. In this setup, the balloon forms part of a closed system from which pressure measurements can be made, allowing LVP to be determined. The balloon is attached to a fluid-filled catheter and connected to a pressure transducer and bridge amplifier. The balloons required for these experiments need to be ultra thin, highly flexible and made to a size such that the balloon is sufficiently large to allow it to be fully inflated to greater than the size of the stretched ventricular lumen without itself exerting any pressure. In the case of pathological stretch experiments a diastolic pressure of 50 mmHg or greater was required without being altered by the

elastic nature of the rubber balloon. Commercial latex balloons are limited in their ability to meet these criteria and the requirement of high diastolic pressures used in the majority of these experiments, so purpose made balloons were made from ultra thin condoms (Ansell, Japan). To aid in the selection of balloon size (or volume), the thickness of the LV free wall and volume of the LV cavity of both rats and guinea-pigs was determined by injecting two-pack fast set dental grade hydrophilic vinyl (TAKE-1, Kerr USA) into the left ventricle via the atrio-ventricular valve to obtain a pliable vinyl mould of the entire left ventricular cavity (including finer details of vessels, papillary muscles and trabeculae). This mould was used to gauge the approximate size of the LV in the rat and guinea pig for subsequent experiments. Thus, the saline perfused Langendorff heart forms an excellent compromise in the whole heart model of MEF especially in relation to electrophysiological recordings and ability to apply stretch. The major advantage of the isolated perfused Langendorff heart in respect to MEF is the absence of complications imposed on responses by circulating neurohormones, systemic or peripheral circulation changes and influence by the autonomic systems through sympathetic and parasympathetic (vagal) stimulation. Another major advantage is that experiments such as those relating to stretch-induced electrophysiological changes that would normally jeopardize the condition and survival of an *in vivo* model become less of a concern as they have no effect on animal welfare.

In the present thesis, two animal models were chosen. Of the two models, the majority of experiments were performed on the rat heart since it is the best characterized and most readily suited to the Langendorff perfusion system (AD Instruments, Australia). The major limitation of the rat heart is its extremely short action potential duration and absence of a plateau phase. This limitation was overcome by the use of Guinea-pigs as the 2nd model. Guinea-pigs were chosen for their significantly longer action potential duration and morphology more similar to the human action potential.

LANGENDORFF SETUP & CALIBRATION

Intra-ventricular Balloon Construction

The common technique used to measure ventricular contractile force is a fluid-filled balloon inserted into the left ventricle, which measures isovolumic contractions (contractions against the set volume of the intra-ventricular balloon). The balloon was made slightly larger than the maximum expanded volume of the ventricle to avoid the effects of measuring resistance, and hence, developed pressure of the balloon to stretch. Balloons of slightly varying sizes were made, and the best chosen to suit each individual heart. This was done by cutting the last 3-4 cm off the tip of an non-lubricated condom (or one that had been cleaned free of lubricant) and pulling it over a piece of rigid (thick walled) polyethylene tubing of similar size used to cannulate the aorta. Sliding the condom down to the tip of the catheter a suitable distance altered the size of the "inflating" portion of the balloon. Care was taken to ensure folds did not cross and block the catheter opening. Condoms were then fixed in place on the plastic catheter by a series of knots and windings with either fine silk suture of enameled wire before being over filled (inflated) with de-ionised water and tested for air and water tightness. Air bubbles were removed by pinching the top of the balloon (pushing any air bubbles below the cannulation point) and then pulling back on the syringe. Because the presence of air in any part of the LVP recording system causes damping of the pressure signal, extreme care was taken to remove any air bubbles from the balloon, calibrated syringe and transducer setup. The process of over-inflation and pinching was continued until all bubbles were visibly removed from the balloon and cannula.

Functional balloons were left over-inflated and kept in dark, dry airtight containers lined with tissue and silica gel beads (sigma) when not in use. Between experiments, balloons were rinsed with milli-Q water and dried. On average, balloons treated in the above manner would last approximately 12-15 experiments or more unless punctured. Other useful functions derived from contractile measurements include the first derivative, dP/dt, a determinant of the rate of change of developed pressure and the integral of pressure as an index of work. Heart rate was monitored from the force measurements or monitored independently through the MAP recordings.

Calibration of Pressure and Flow Recordings

Two pressure recordings were made for the majority of experiments, the Left ventricular pressure as recorded from the intra-ventricular balloon, and the coronary perfusion pressure (the resistance or back pressure developed to the flow of perfusate through the coronary vessels of the heart). In both cases, physiological pressure transducers (MLT844, AD Instruments Australia) were placed level with the heart in respect to the ground. The zero pressure point for the LV balloon was taken by using a "U-tube" configuration. A short length of flexible tubing was filled with air-free water, one end attached to the balloon-pressure transducer setup and the other, open end, placed at the approximate height of the heart on the Langendorff setup. This was used to zero the DC offset in Chart V (AD Instruments, Australia) and to set the 0 mmHg pressure. Once zeroed, pressure calibrations were then performed by comparing pre-set points to a digital pressure gauge (World Precision Instruments, USA). For perfusion pressure, 0 mmHg was taken as the pressure in the absence of the heart and with a flow rate of 12-14 ml/min through the cannula of the Langendorff system. Both left ventricular and coronary perfusion pressure recordings were calibrated for pressure readings between 0 and 150 mmHg.

Flow rate was calibrated for coronary flow rates between 10 and 15 ml/min as outlined in the AD Instrument Langendorff setup guide.

Ethics and Animal Care

Animals used throughout the studies undertaken in this thesis were cared for according to the National Health and Medical Research Council (NHMRC) *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*. All experimental procedures were subject to prior approval by The University of Adelaide Animal Ethics Committee. Male Sprague Dawley rats (350-500 g) and male Guinea Pigs (400-800 g) were maintained in the Adelaide Medical School Animal house until use. Room temperature was maintained at 24 ^{o}C, constant humidity (55 %) and animals were subject to a standardised 12 hr light/dark cycle. Animals had free access to food and water at all times and limited to a maximum of 4 rats per cage.

Anaesthetisation of Animals

Rats were anaesthetised by intraperitoneal (i.p.) injection of 75 mg/kg Ketamine and 0.5 mg/kg Domitor (medetomidine) in 2000 units of heparin as outlined by the NHMRC guidelines. Guinea-pigs (450-600 g) were anaesthetised with 80 mg/kg Ketamine and 0.5 mg/kg Domitor in 3000 units of heparin delivered subcutaneously just bellow the base of the head. Where required, additional doses of Ketamine were given where animals showed signs of light anaesthesia. Depth of anaesthesia was determined prior to surgery in the following order:

- Absence of righting reflex (the ability of the animal to re-orientate using coordinated muscle activity when placed on its back),
- palpebral reflex (the eyelid reflex as initiated by gentle touch of the cornea) and finally,
- pain withdrawal reflex (initiated by squeezing between the toes with a pair of forceps).

With this method, full anaesthesia was generally achieved within 5 minutes for rats, 10-15 minutes for guinea-pigs. Animals were not allowed to regain consciousness following anaesthesia as hearts were rapidly excised and placed in ice cold (4°C) HEPES buffer (see pg 39). As such, all animals died under anaesthesia by this method.

Surgical Preparation Of The Heart

Rats (350-500g) were anaethetised by i.p. injection of Ketamine (75 mg/kg), Domitor (0.5 mg/Kg) mixed with 2000 UI of the anticoagulant heparin (*Pfizer*, Australia). Once deeply anaesthetized, a sternotomy was performed by cutting and retracting the ribcage, and the cavity was flooded with cold perfusate. Firstly, a transabdominal incision was made to expose the thoracic cavity and to reveal the diaphragm of the rat. The thorax was opened by either a single incision along one side of the sternum or a bilateral incision along the lower sides of the last to 1st ribs of the thoracic cage before being forced open or reflected over the animal's head respectively, clearly exposing the heart and thymus. At this point, the thoracic cavity was flooded with ice-cold HEPES buffer (pH 7.35) to reduce intrinsic heart rate and possible ischemic damage to the heart. The thymus was then pulled upward with blunt forceps before being carefully cut away from the aortic junction. Following removal of the thymus, fine suture silk was threaded under the descending branch of the aorta (at the junction with the ascending aorta) by passing a pair of sharp curved forceps under the descending portion of the aorta. With the suture silk passed under the aorta, the descending aorta was tied off to later aid in manipulation of the ascending aorta during cannulation. The heart was then gently cupped between the fingers (in order to avoid injury) and lifted prior to cutting the vena cava, pulmonary vessels and finally, the aorta above the branch and knot. Once removed, the heart was immediately immersed in ice-cold Ca^{2+} free HEPES buffer and taken for immediate cannulation. The process of rapidly cooling the heart is known to improve cardiac parameters and lessen ischemic damage during heart removal (Gauthier *et al.*, 1998; Sutherland & Hearse, 2000). Throughout the procedure, heart activity was monitored, and if the intrinsic heart rate was considered too fast, the cavity was again flooded with ice-cold solution to arrest heart activity. As the heart is a highly metabolically demanding organ, it requires a constant supply of oxygen and nutrients. Therefore, the time taken to remove and mount the heart is very important. At physiological temperatures, an extended period (> 30 seconds) of reduced oxygen and/or nutrients will significantly affect cardiac tissue, in particular its survival and experimental responses, hence ice cold HEPES buffer (for stable pH) was used throughout the excision procedure.

Following removal, a small incision was made on the side of the ascending aorta at the branch where it was tied. Blunt forceps were then used to hold one side of the

incision, whilst the hole was opened for cannulation by pulling gently on the suture silk. During cannulation, a slow stream of perfusate was permitted to flow through the aortic cannula, removing any air bubbles from the aorta prior to cannulation of the heart. The aorta was cannulated with a blunt-ended 21 Gauge needle and perfused at constant flow in the Langendorff mode. During cannulation, it was ensured that the tip of the aortic cannula did not reach the base of the aorta to avoid restriction of coronary perfusion or damage to the aortic valve. Once satisfactorily in place, the aorta was tied onto the cannula, taking care not to block the vena cava, following which the perfusate flow rate through the heart was increased to obtain a perfusion pressure of at least 50-60 mmHg. In addition to the standard perfusion apparatus a cellulose acetate filter (pore size 4.5 μm, Sartorius, Germany) was placed in the perfusion line to remove any particulate impurities before reaching the heart. Following successful cannulation, any remaining fatty and lung tissue on the heart surface were removed.

Measurement of Contractile Force

Once properly perfused (as observed by maintenance of perfusion pressure) the left atrium was cut away from the base of the heart forming a clear entrance to the left ventricle. The balloon in its deflated state (under ~-60-100 mmHg negative pressure) was inserted into the left ventricle through the atrio-ventricular valve with the aid of a blunt probe. Once in the ventricle, the balloon was partially inflated (by injecting fluid through the catheter with a calibrated syringe) to achieve a resting EDLVP of approximately 20-25 mmHg (to ensure the balloon had completely unfolded) before returning back to 0-5 mmHg. At this point perfusion was either left in the constant flow mode or switched over to constant perfusion pressure and the contractility of the heart was monitored and allowed to stabilise. By convention, hearts were considered acceptable if systolic pressure reached ~60 mmHg or greater for a left ventricular diastolic pressure of ~ 0-5 mmHg at a flow rate of 10 ml/min or better and constant perfusion pressure of 60-70 mmHg. Perfusion pressures were generally kept within this range since changes in the perfusion pressure or flow rate are known to also affect contractility (see Chapter regarding the Gregg effect).

Pacing

Hearts were artificially paced using two purpose made silver-chlorided electrodes pressed against the outer wall of the right atrium. Pacing was used to maintain a standard contractile response, stable action potential duration and metabolic demand. Hearts were paced with stimuli rates of 4 or 5 Hz (as noted) that exceeded the natural cardiac pacemaker rate (< 4 Hz). Where necessary, the sinoatrial node was crushed or the right atrium excised to eliminate contribution of the primary intrinsic pacemaker. Pacing voltage was set at 2-3 times the voltage required to capture (pace) the heart with duration of 10 milliseconds using bi-polar pulses to inhibit ionisation at the pacing electrodes. A second electrode, the MAP electrode (described later) was set up in a similar manner to that of the stimulus electrode. A metal clip placed around the aorta of the heart formed the ground electrode for MAP recordings where required, but in the majority of experiments, a balanced electrode configuration was used.

Using this setup, EDLVP, ESLVP were easily monitored. Other useful functions derived from these contractile measurements were the first derivative, dP/dt, a determinant of the rate of change of developed pressure and the integral of pressure as an index of work. Heart rate was monitored from the force measurements or monitored through the MAP recordings (discussed elsewhere). It should be noted however, using the Langendorff technique, perfusate solution does not flow via the normal ventricular circulatory pathway. Therefore, this system does not permit the left ventricle to generate pressure-volume work that is representative of typical cardiac function.

Perfusate Solutions

BICARBONATE PERFUSATE was equilibrated with carbogen (95 % O_2 and 5 % CO_2, Lynde Gasses, Australia) and consisted of (in mM): $CaCl_2$ 1.5, NaCl 111.0, KCl 4.0, $MgCl_2$ 0.6, $NaHCO_3$ 23.9, NaH_2PO_4 1.2, D-glucose 12.0 at 37 ^oC. Once bubbled with carbogen (or carbanox), pH was stable at 7.35

Calcium Chloride (sigma) was made up as a 1 M stock solution in milli-Q H_2O. A final concentration of 1.5 mM $CaCl_2$ was achieved for perfusate buffers by adding 1.5 ml of the stock solution to every liter of perfusate.

HEPES PERFUSATE was equilibrated with 100 % oxygen where required and contained (in mM): NaCl 134.0, KCl 4.0, $MgCl_2$ 1.2, $CaCl_2$ 1.5, NaH_2PO_4 1.2, Glucose 11.0 and N-

2-hydroxyethylpiperazine-N-2-ethane sulphonic acid (HEPES) 10.0. Solutions were made up fresh, 1x concentration at the volume required, and adjusted to pH 7.35 with 1.0 M NaOH.

Previously it has been observed that the choice of perfusate solution is important as it can affect myocardial performance as well as action potential morphology. (Fulop *et al.*, 2003) showed that HEPES buffered perfusates reduced systolic pressure and the amplitude of the [Ca^{2+}] transients in the Langendorff perfused guinea pig heart. In addition, to the reduction in contractility, action potential morphology of canine and rabbit right ventricular preparations was lengthened in the presence of HEPES buffered solutions. In addition to these observations, it was discovered that the Na^{+}-HCO_3^{-} co-transport mechanism found in rat ventricular cardiomyocytes is electrogenic (Aiello *et al.*, 1998), and thus the absence of HCO_3^{-} from perfusate solutions is likely to affect the cardiac action potential. From preliminary experiments it was found that, in general, Langendorff heart preparations stabilized more quickly when perfused with bicarbonate buffered perfusate (stabilization generally within 10minutes, see Figure 3, pg 42) compared to HEPES buffered perfusate (generally within 20-30 minutes). This is consistent with the observation by (Fulop *et al.*, 2003) that mild intracellular acidification took place over a 20 minute period in the guinea pig heart perfused with HEPES buffered perfusate. As such, bicarbonate buffered perfusate was used for the majority of Langendorff experiments unless stated otherwise. HEPES buffered solution was predominantly used for its stable pH in preparation of hearts for cannulation. Stabilisation of each Langendorff heart following cannulation was defined as the point at which systolic and diastolic pressures reached a plateau (measured in mmHg).

Perfusion of the Heart

The perfusion apparatus was supplied by AD Instruments, Australia and consisted of a perfusate reservoir (500 ml in volume) connected to a perfusate pump (Miniplus 3, Gilson, Anachem, UK). The delivery tube passed through a heated coil and through a heated jacket connected directly to the aortic cannula. Immediately above the aortic cannula and heated water jacket was a junction box consisting of the delivery tube, a line leading to the perfusion pressure transducer as well as an air filled syringe (5-15 ml). The syringe supplied damping to the coronary perfusion pressure line to minimize the pressure oscillations induced in the delivery line by the peristaltic pump action.

Once mounted on the apparatus, the heart would begin to beat strongly within seconds of reperfusion. The pressure of the perfusate was initially between 60-80 mmHg for a flow rate of ~12 ml/min at a cardiac temperature of 36 ^{o}C. Perfusion pressure was chosen to be constant for the majority of experiments. Healthy hearts generally stabilized rapidly and most experiments began within 15 minutes for bicarbonate buffered perfusates, and 20-30 min for HEPES buffered perfusates. With all conditions optimized, the heart was experimentally viable for several hours, although experimental time was reduced as much as possible and usually completed in under an hour. Previous research by Fulop et al (2003) also demonstrated that the ability of drugs to block or induce ectopic beats in the Langendorff heart can and could differ between the two differently buffered perfusates. As such, the majority of Langendorff experiments were limited to the use of Bicarbonate buffered solutions. In addition to these considerations, since the isolated Langendorff perfused heart is an excised, saline perfused preparation, it is also a continuously deteriorating preparation both physiologically and electrophysiologically. As such, experimental durations were kept to a minimum (generally much less than 1 hour).

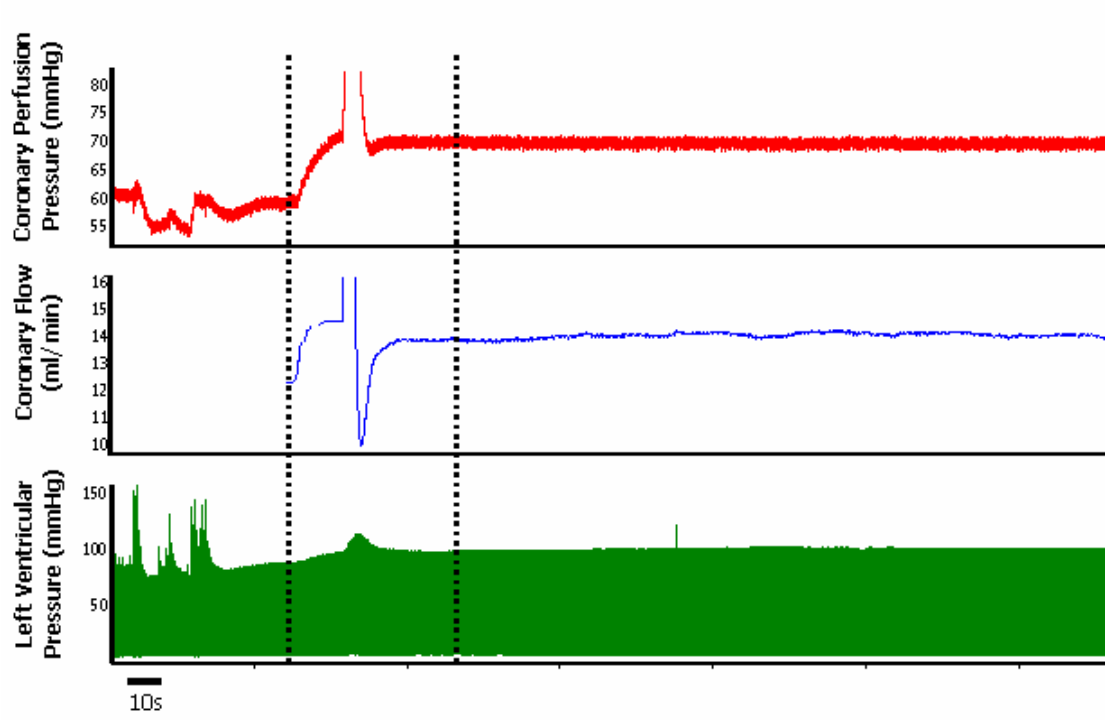

Figure 3: Raw Chart Recording

Figure showing stabilisation and stabilised period for rat heart perfused with Bicarbonate buffer containing 1.5 mM $CaCl_2$. Top Chart (red) denotes Coronary Perfusion Pressure (mmHg) which can bee seen to stabilise at 70 mmHg. Middle (blue) chart denotes Coronary Perfusion Rate (ml/min) seen to stabilise at ~ 13-14 ml/min. Bottom Chart (green) shows left ventricular pressure as recorded by intra-ventricular balloon. End diastolic pressure is ~0 mmHg and systolic pressure has stabilised at > 100 mmHg. 1^{st} Dashed line denotes changeover to constant perfusion pressure from constant flow rate. 2^{nd} Dashed line denotes the start of the stabilised period.

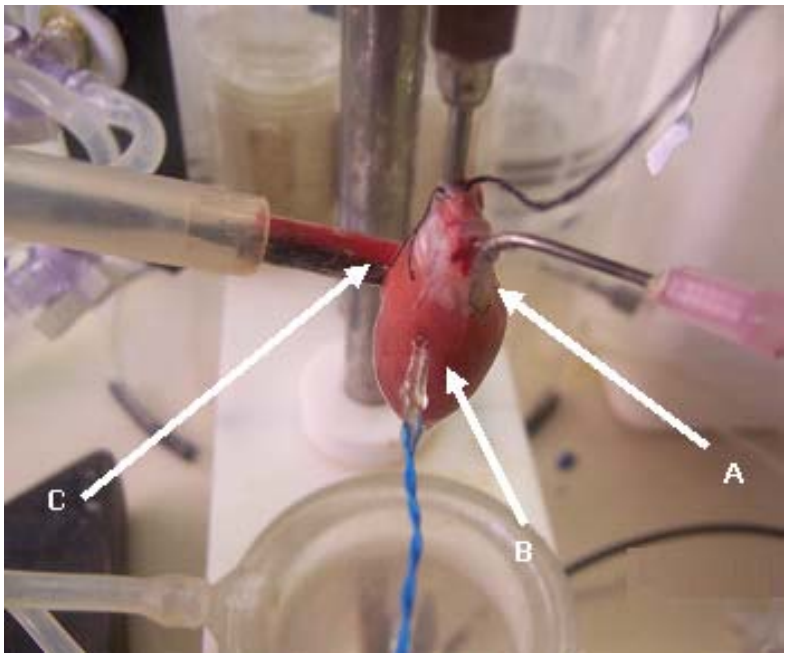

Figure 4: Balloon Placement in Langendorff Perfused Heart

Figure showing placement of balloon through left atrial incision, down through the left atrio-ventricular valve into the lumen of the left ventricle. (A) denotes the point where the balloon and cannula enter the left atrium, (B) a surface recording electrode (explained in detail later) and (C) bipolar, insulated pacing electrodes placed against the right atrium.

Statistical Analysis

Data presented in this thesis are Mean ± SEM unless otherwise stated. In all cases presented, data for a minimum of 10 action potentials was averaged for each experimental condition. Data analysis of these averaged values was most commonly carried out using general linear model (GLM) ANOVAs algorithm unless stated otherwise. Significance was accepted at $p < 0.05$. Statistically significant data was then compared using pair-wise analysis – a Tukey's Post Hoc test was used to determine direction/placement of significance. Statistical Analysis was performed using Minitab 14, academic release (2005).

CHAPTER 4: PRELIMINARY EXPERIMENTS

French singer & songwriter of the 30s - 50s : Charles Trenet
(May 18, 1913 – February 19, 2001)

Chapter 4 - Table of contents

Preliminary Experiment 1: Intrinsic Heart Rate and Basic Conditions

INTRODUCTION

Prior to establishing the model of stretch in the isolated Langendorff heart, the intrinsic rate at which cardiac preparations contracted spontaneously was important to know when determining the desired pacing frequency. The intrinsic heart rate (HR), defined as the spontaneous beating rate of the heart observed in the absence of any sympathetic or parasympathetic nervous input, is observed as a direct consequence of removal of the heart from the rat torso. Such a procedure (outlined in Surgical Preparation Of The Heart) severs both the vagus (parasympathetic) and sympathetic neural input to the heart. As a result, the Langendorff perfused rat heart beats at its intrinsic rate with no influence imposed by the nervous system.

METHODS

Male Sprague Dawley rats (400-450 g) were prepared as described, their hearts removed and cannulated onto the Langendorff perfusion system where they were perfused with bicarbonate buffer containing 1.5 mM $CaCl_2$ and allowed to stabilize at a constant perfusion pressure of 60 mmHg. Following stabilisation (roughly 5-10 minutes), the interval between contractions at 10 random points was measured in order to determine the variation in basal heart rate in Hz. Hearts were then paced at various frequencies at three different left ventricular end diastolic pressures (0, 5 and 10-15 mmHg) to determine the effect of frequency of stimulation on contractility in the Langendorff perfused rat heart.

RESULTS

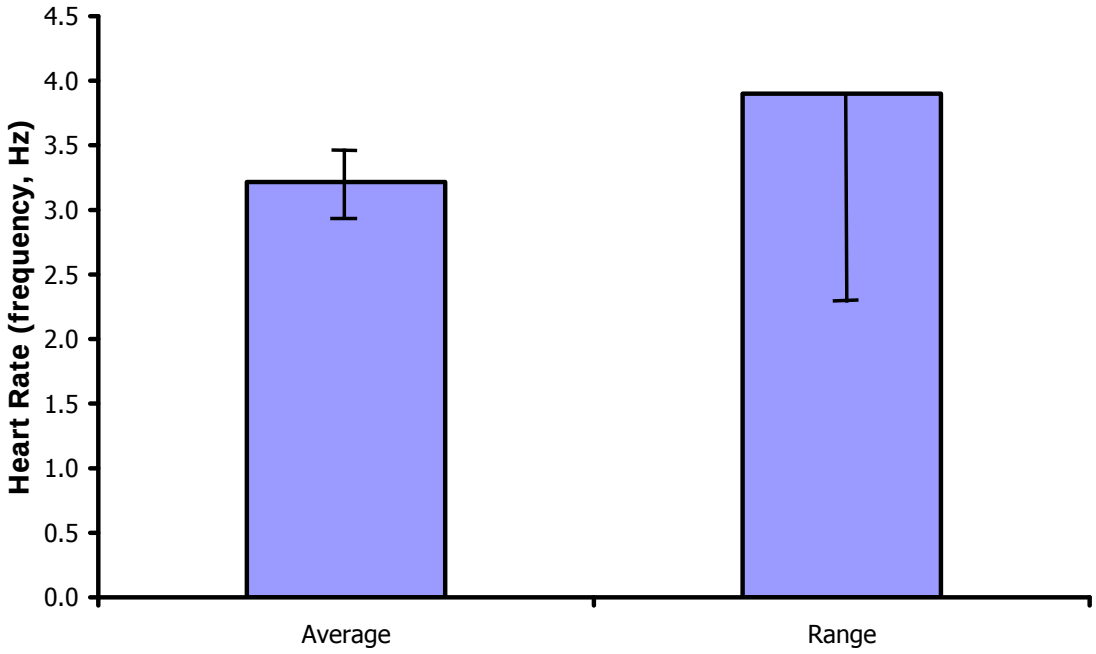

Figure 5: Intrinsic Heart Rate of Isolated Perfused Hearts

Rat hearts were perfused with bicarbonate buffered perfusate and allowed to stabilise in constant perfusion pressure mode, at a perfusion pressure of 60 mmHg without pacing at 36 °C. Error bars indicate ± SEM, n = 5. HR was derived from the time between two consecutive contractions for a series of 20 contractions chosen at random (giving 10 values from which the average was taken for each rat). Average Intrinsic HR under these conditions was 3.3 ± 0.27 Hz (or 198 ± 16.2 beats per minute, BPM). The slowest HR observed was 2.3 Hz (160 BPM) and the highest 3.9 Hz (234 BPM).

Following establishment of the average intrinsic HR, the effect of pacing frequency on contractility was investigated with the aim of optimizing the observation of changes in contractility (and hence action potential morphology) throughout stretch-induced length-tension manipulations in the heart.

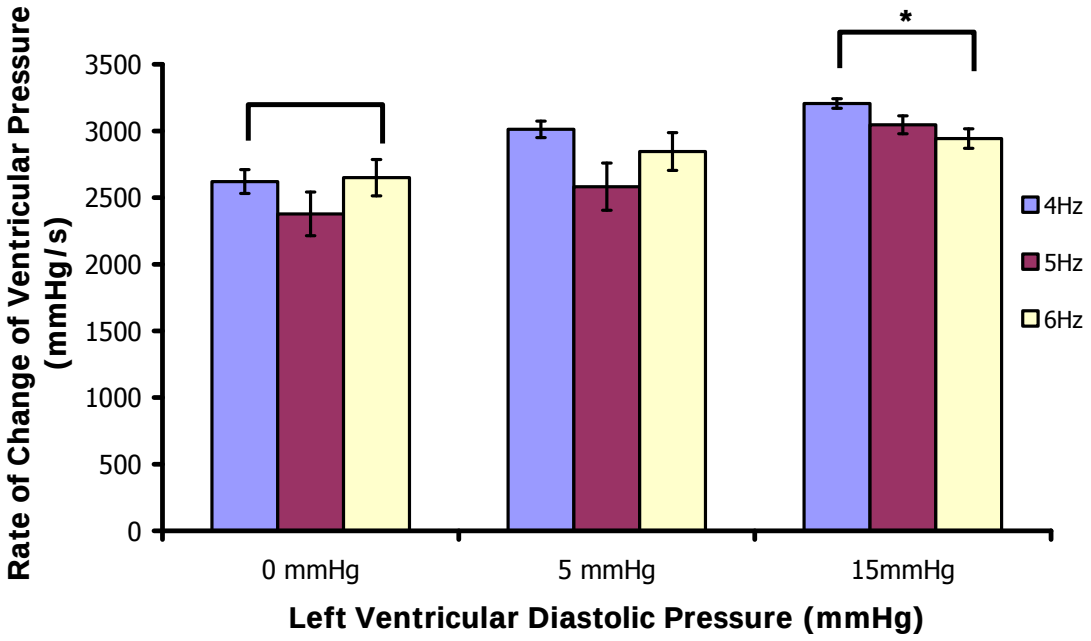

Figure 6: Effect of Frequency of Stimulation on Contractility

Changes in maximal contractility of isolated Langendorff perfused rat hearts. Hearts were perfused with bicarbonate buffered perfusate and allowed to stabilise in constant perfusion pressure mode, at a perfusion pressure of 60 mmHg at various pacing frequencies (blue = 4, Red = 5, Yellow = 6 Hz). Error bars indicate ± SEM, n = 3 hearts (each measurement in each heart is an average of 10 contractions at each level of stretch). The average rate of maximal contractility in the absence of left ventricular stretch was not significantly different between the different pacing frequencies (3621 ± 89, 2378 ± 163 and 2652 ± 136 mmHg/s for 4, 5 and 6 Hz respectively). When compared to basal conditions (0 mmHg), there was a significant increase in contractility observed with left ventricular stretch (left ventricular diastolic pressure of 15 mmHg) regardless of pacing frequency (denotes $p < 0.05$, paired t-test)*

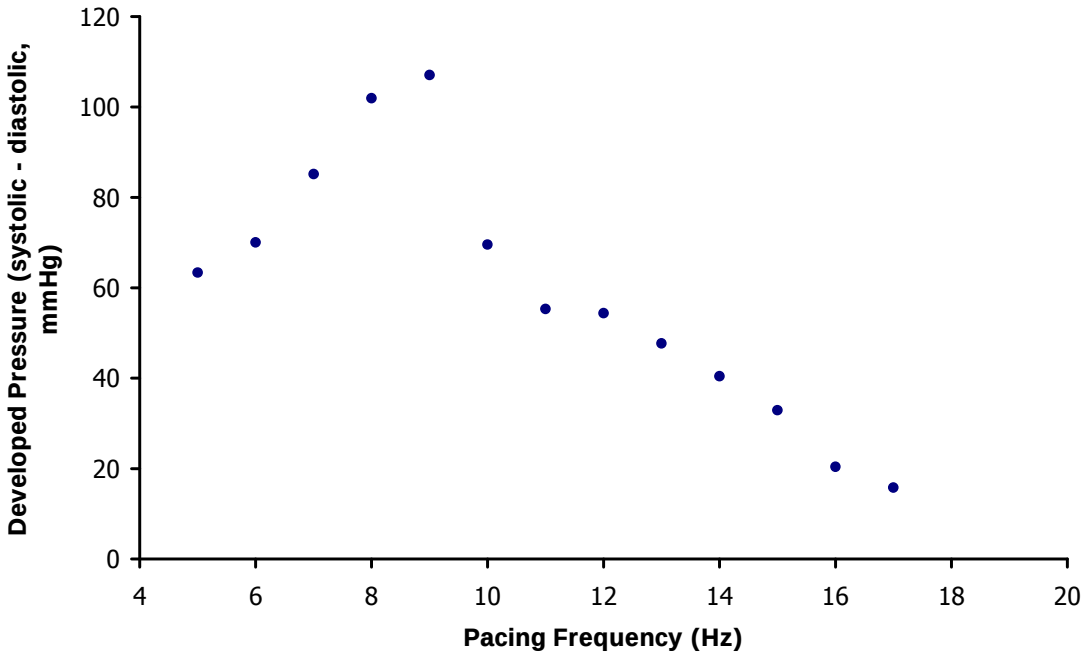

Figure 7: Effect of Frequency of Stimulation on Developed Pressure

Changes in contractility (expressed as developed pressure = systolic pressure – diastolic pressure) of an isolated Langendorff perfused rat heart paced at various frequencies in random order. Here, the rat heart was perfused with bicarbonate buffered perfusate and allowed to stabilise in constant perfusion pressure mode, at a perfusion pressure of 60 mmHg at a pacing frequency of 4 Hz. Diastolic pressure increased from 3 mmHg at 5 Hz to 31 mmHg at 17 Hz pacing frequencies. By contrast, Systolic pressure was 66 mmHg at 5 Hz, peaked at 112 mmHg when paced at 9 Hz before declining steeply to 47 mmHg when paced at 17 Hz.

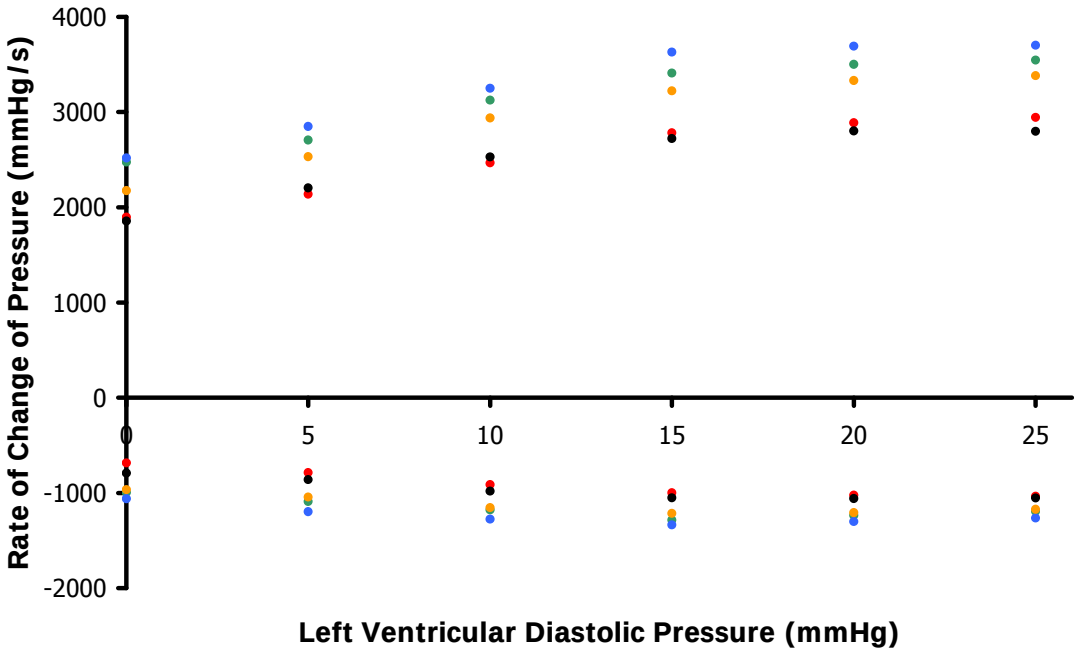

Figure 8: Effect of Perfusion on Contractility

Figure showing effect of coronary perfusion pressure on stretch induced changes in left ventricular contractility (Frank-Starling effect). Black, Red, Orange, Green and blue circles denote perfusion pressures of 40, 50, 60, 70 and 80 mmHg respectively. Coronary flow rates ranged from 6 to 15 ml/min for coronary perfusion pressures of 40 and 80 mmHg respectively.

Lastly, the effect of various coronary perfusion pressures was tested to observe the contribution that perfusion pressure would make to stretch-induced changes in contractility of the heart. This was done to ensure that higher perfusion pressures would not eliminate stretch-induced changes in contractility observed when inflating the left ventricular balloon (Figure 8)

DISCUSSION

The Frank-Starling response is known to be sensitive to ventricular rate (Popovic *et al.*, 2004). Figure 5 shows that HR varied between animals ranging from 160 to 234 beats per minute under the same conditions. Despite these variances, once pacing was initiated, contractility at the various pacing frequencies tested, the various EDLVPs were consistent and did not vary considerably between groups. Stretch induced increases in contractility were more evident at a pacing rate of 4 Hz, increasing from 2621 ± 89.4 to 3206 ± 35.6 mmHg/s with an EDLVP of 15 mmHg (~22 % increase) compared to the 11 % increase to 2943 mmHg/s seen at 6 Hz. Likewise, an increase

in contractility of ~28 % was observed when hearts were paced at 5 Hz, although the response was somewhat more variable. Since increasing pacing frequency diminished the contractile response to stretch, it was chosen to perform all experiments by pacing hearts at 4 or 5 Hz with bi-polar pulses of 10 ms duration. This allowed for the largest possible contractility changes and enhancing the likelihood of related changes in action potential duration to be observed during MEF manipulations. In general, pacing was initiated at 5 Hz, 2-3 times threshold and then reduced to 4 Hz once contractility was stable. This method helped capture the higher intrinsic HR seen in some heart preparations. At these frequencies, and a constant perfusion pressure of 60-70 mmHg the average approximate flow rate of perfusate through the rat heart was 11.9 ± 0.7 ml/min (n=5). Whilst a higher perfusion pressure could have been chosen, initial experiments (not shown) suggested that stretch-induced increases in contractility would be significantly blunted at perfusion pressures greater than 80 mmHg.

CONCLUSION

Results from preliminary experiments suggested that a pacing frequency of 4-5 Hz would be adequate to normalise the physiological and electrophysiological parameters of Langendorff perfused rat hearts. At these frequencies, similar increases in contractility were observed in response to changes in EDLVP and they were high enough to overcome the intrinsic rate of the hearts tested.

Preliminary

Experiment 2: MAP Recording Electrodes

INTRODUCTION

Possible changes in morphology of the cardiac action potential in this dissertation were recorded in the form of monophasic action potentials (MAPs). MAPs (both contact and suction) have been previously demonstrated in isolated heart preparations to closely reflect the time course of membrane repolarisation but limited information regarding resting membrane potential and action potential amplitude (Hoffman *et al.*, 1959; Franz *et al.*, 1986; Franz, 1994; Kondo *et al.*, 2004). In addition, MAP recordings have been used successfully in numerous studies to show electrophysiological changes under a variety of conditions from characterization of various arrhythmic states (Swartz *et al.*, 1993) through to stretch-induced changes in heart electrophysiology (Franz *et al.*, 1992; Zabel *et al.*, 1996; Ravelli & Allessie, 1997; Takagi *et al.*, 1999; Eckardt *et al.*, 2000; Babuty & Lab, 2001; Sung *et al.*, 2003; Chen *et al.*, 2004). As such, MAP recordings have become a widely accepted method of establishing the cellular effects of antiarrhythmic agents, cardiac arrhythmia and stretch-related electrophysiological changes. The observation that MAP recordings closely reflect intracellular recordings made using microelectrode techniques supports their use in the following series of experiments. (Franz *et al.*, 1986; Ino *et al.*, 1988; Kondo *et al.*, 2004). MAP recordings are recorded as the differential voltage between one electrode positioned in the area of interest (the recording electrode) and a 2nd electrode, the differential or reference electrode on the heart surface. Thus the recording is made from what could be considered an active site compared to the other site which is relatively neutral. In the present study, 3 types of MAP electrodes were used, namely the suction and contact or "Franz" electrodes, both being examples of surface electrodes, and the transmural electrode. Suction and contact electrodes were first introduced for use in humans in the late 1960s due to their simplicity and the fact a recording was obtained

without the need for a specific myocardial lesion (Leirner & Cestari, 1999). Later, the suction electrode was replaced with the contact electrode because of the size advantage and reduced possibility of inducing myocardial damage and the possibility of causing air embolisms (Franz, 1983). Whilst this was the case, *in vivo*, the suction MAP retains a useful place in *in vitro* preparations such as when combined with the Langendorff heart. The following series of experiments use both methods to make recordings directly from the epicardial surface of the left ventricular free wall. By contrast, the "transmural" electrode was designed to make recordings from endocardial tissue and sup-epicardial tissue. The two methods differ in that contact electrodes are thought not to damage the cardiac tissue from which they record, whilst transmural electrodes damage the tissue through which they pass in order to establish the MAP recording.

Surface Method

Various surface MAP electrodes were trialled for suitability in Langendorff experiments. Most notably, the more modern "Franz" type contact MAP electrode and original "suction" MAP electrodes were tested. Two different models similar to that described by Knollmann *et al.* (Knollmann *et al.*, 2002) were tested, the 1st being a simple rigid contact electrode and the 2nd a modified spring loaded contact electrode (see Figures 8, 9 & 10 pgs 51-57). In both designs the reference and recording electrodes consisted of a silver ball formed by gradually melting 0.5 mm diameter silver jeweller's wire over a moderate Bunsen flame. Silver ball electrodes of various sizes (all < 1mm diameter) were chloride plated by immersing each individually into a 5 M KCl solution and running 60 V across each for a few seconds until a strong white-grey coating was evident. Springs for the "spring electrode" reference electrode were hand wound using either 0.3 or 0.5 mm diameter silver wire to achieve two different levels of compliance. The spring electrode was mounted protruding from a piece of rigid PVC tubing (inner diameter 4 mm) ensuring freedom of movement. The recording electrode was mounted off centre on the rigid tubing, ensuring that upon contact with the heart, the spring was partially compressed before the recording electrode made contact.

Suction cups for suction electrodes were composed of small light-weight pieces of silicone tubing (4.5 mm ID) measuring no more than 5 mm in length. At one end, a smaller diameter PVC tubing of 5.0 mm OD and smaller ID was used as a female nipple

to reduce the suction cup diameter to that of a much smaller suction line composed of sillastic silicone tubing of 1.5-2 mm OD (Dow Corning, USA). General tightness and matching of tubing diameters/fittings held the small suction unit together, however, clear non-toxic lacquer could be added to increase the rigidity of the nipple. In this "suction electrode", 0.3 mm diameter silver wire and associated chlorinated ball was passed backward through the suction cup between the nipple layers to ensure air-tightness and an uninterrupted suction line. Two different recording electrodes were manufactured, the first being a concentric circle that ran the circumference of the suction electrode tip, the second design consisting of a "floating" indifferent electrode that could be positioned anywhere around the suction/recording electrode. The latter was found to give the most stable recordings as anchorage to the suction cup via a thin piece of wire enabled the recording electrode to maintain constant contact with the beating heart under all conditions.

All electrodes were silver soldered to high quality silver plated wire wrap wire (of length less than 1 m, Kynar – Jaycar Electronics Adelaide) and attached to a bio-amp (AD Instruments, Australia). Two methods of termination were involved, namely balanced pair and shielded pair. In both cases, wires extending from (and including the electrode ball) were matched as closely as possible for length (and thus resistance) to enable differential amplification and hence natural noise/hum cancellation where possible. Of the methods trialed, the suction and Franz electrodes produced the most stable MAP-like recordings.

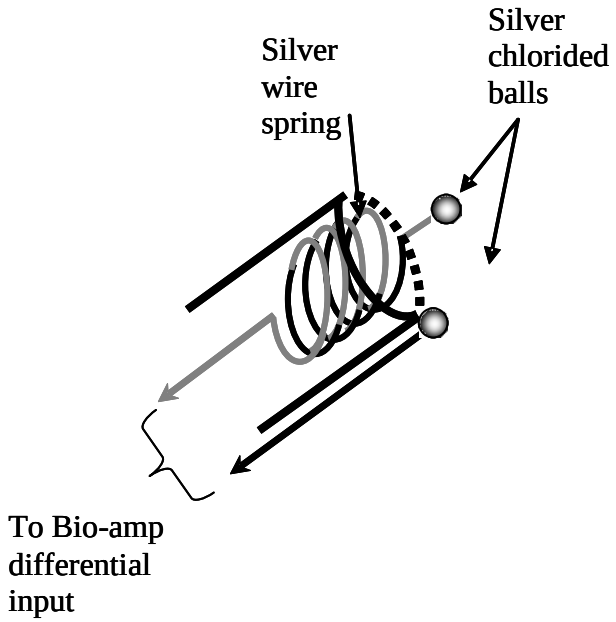

Figure 9: Spring Electrode Design

Composed from silver wire wound into a coil and inserted into a 5 mm diameter polyethylene riser tube, the recordings obtained were extremely noisy and appeared more ECG like than MAP like in nature.

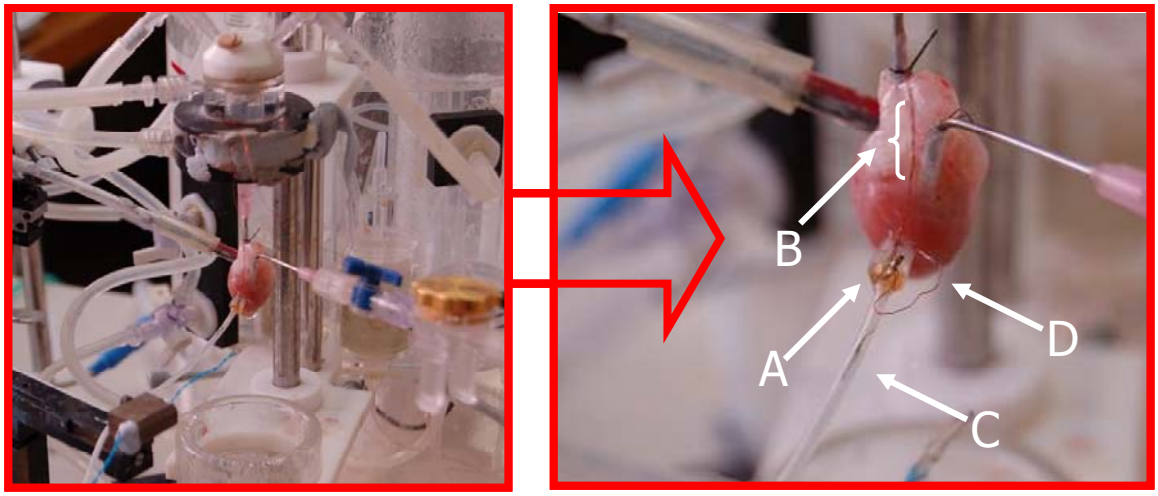

Figure 10: Suction Electrode

Suction style electrode made from fine enameled (0.125 mm ø) wire and chloride coated silver balls. The suction cup that contains the indifferent electrode (A) is suspended from both the copper wire above the heart (B) and the silastic tubing that supplies the suction (C), allowing free movement of the recording electrode that rests in contact with perfusate on the heart surface (D)

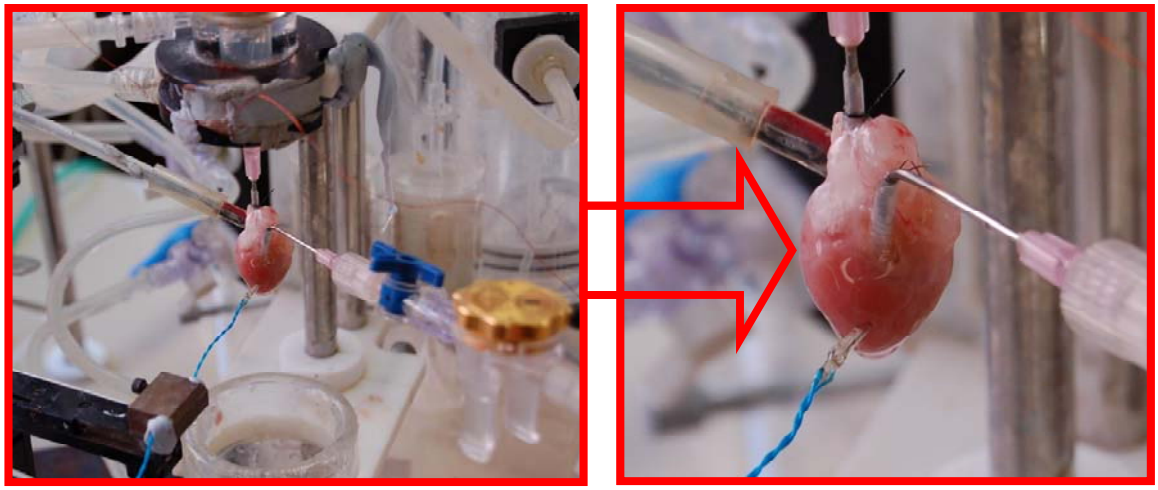

Figure 11: Contact Electrode

Franz electrode pushed into heart at right angles to the surface. The electrode consists of two silver-chlorided balls spaced~ 1 mm apart and connected to a differential amplifier by wires of equal length to minimize noise.

Transmural Method

Measurements of left ventricular free wall thickness at the base, equator and apex were made to determine length and thus depth of transmural recording electrodes. Left ventricular free wall thickness for rats as measured at the equator using metal callipers was found to be 4 mm thick in all cases (n=4). As such, the endocardial electrode was chosen to be 3.5 mm in length, and the epicardial electrode < 0.5 mm in length. Transmural electrodes were made from acupuncture needles 0.18 mm in diameter (Hwato, Australia) coated with fine silicon coatings of sigma coat and a thin layer of Sylgard 184 (Isola Materials, Australia). Silicon coating of the endocardial electrode increased its thickness toward ~ 1 mm depending on the thickness of the sylgard coating. Transmural electrodes were mounted on a coarse xyz micro-manuipulator (Narashigi, Japan) which was used to gently ease the endocardial electrode through the left ventricular wall at the equator. The silguard coating of the endocardial electrode was replaced for each experimental preparation.

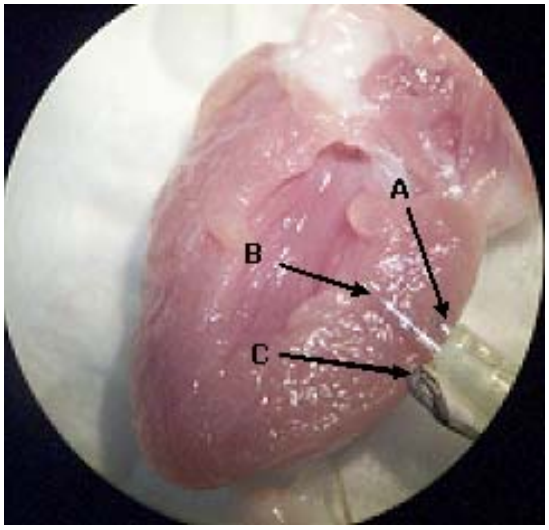

Figure 12: Epicardial-Endocardial MAP electrode

Prototype transmural electrode placed against the cross-section of a rat heart. Arrows A, B and C pointing toward Epicardial, Endocardial and indifferent electrodes respectively. Preliminary experiments (not shown) found little difference between using a silver-chlorided ball or silver wire folded over in a "U" shape and chlorided. MAP recordings were obtained by pushing the transmural electrode into the free LV wall at right angles, with the indifferent electrode contacting only the perfusate on the surface of the heart and not the actual heart tissue.

Obtaining MAP recordings

There are various methods by which a MAP recording can be obtained. In order to maintain reasonable stability in the recording it as little damage as possible was made to the heart by the MAP electrode. For the experiments in this thesis, two types of electrodes were to be used, the contact electrode and transmural electrode. For the contact electrode there were two types, the surface contact type (see Figure 11, pg 57) requiring an external force to push the electrode against the heart surface, and the suction electrode (see Figure 10, pg 57) which required a constant suction to maintain electrode contact with the heart surface. Because experiments require stable recordings of a reasonable duration, it was important to determine which of the electrode types was best capable of producing stable recordings.

METHODS

Male Sprague Dawley rats (400-450 g) were anaesthetised and prepared as outlined above in Chapter 3, their hearts removed and cannulated onto the Langendorff perfusion system where they were perfused with bicarbonate buffer containing 1.5 mM $CaCl_2$ and allowed to stabilize at a constant perfusion pressure of 60 mmHg. EDLVP was held constant at 0-5 mmHg and each electrode was connected to the bio-amp (AD Instruments, Australia) and checked for recording ability.

RESULTS

Of the contact electrodes, the spring type failed to produce a quality recording at all (see Figure 13, pg 60). Recordings obtained were extremely noisy, greatly fluctuated in baseline and were generally unstable and ECG like in nature. Increasing the force applied by the electrode against the heart wall by pinning the heart between the electrode and a finger appeared to help, but no reasonable recording was obtained in any of the hearts. In addition, the pressure applied by the electrode also interfered with the contractility of the heart by increasing diastolic pressure in a variable way depending on the position and force applied by the electrode to the heart wall.

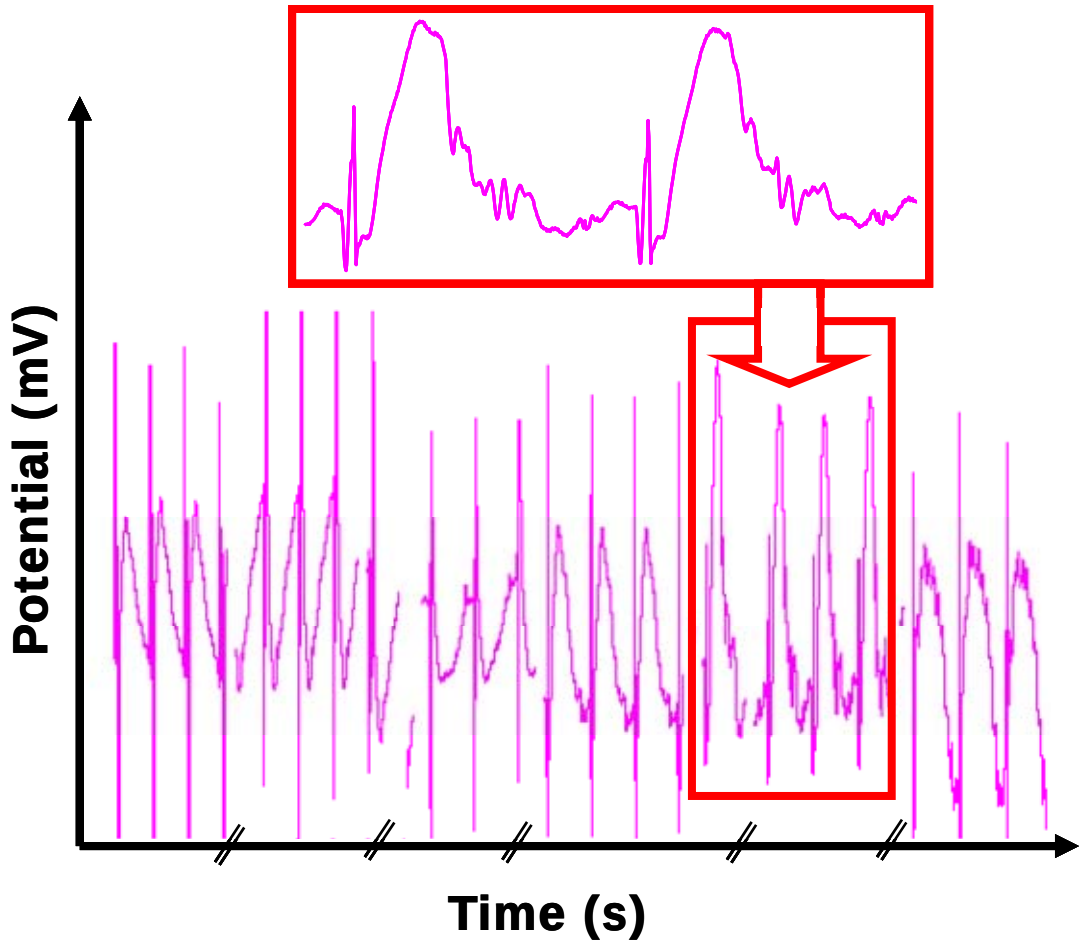

Figure 13: MAPs Obtained With Spring Electrode

Monophasic action potential (MAP) recordings obtained using the spring electrode pictured in Figure 9. Recordings were heavily complexed by ECG interference. Double lines on time axis denote changes in the amount of force applied to the electrode with progressively more force used, resulting in a "MAP-like" signal as pictured in red highlighted panel, although still extremely noisy and of poor quality.

By contrast, suction and Franz electrode recordings worked considerably well regardless of the indifferent electrode type (concentric, floating or fixed). During these experiments it was found that a constant vacuum source of at least 30 mmHg was required to obtain a stable, drift-free MAP recording when using the suction electrode. Lower levels of suction generally resulted in ECG-like recordings or loss in stable diastolic potential (baseline) whilst higher levels could be initially used to initiate MAP recordings before reducing the vacuum to a lower level.

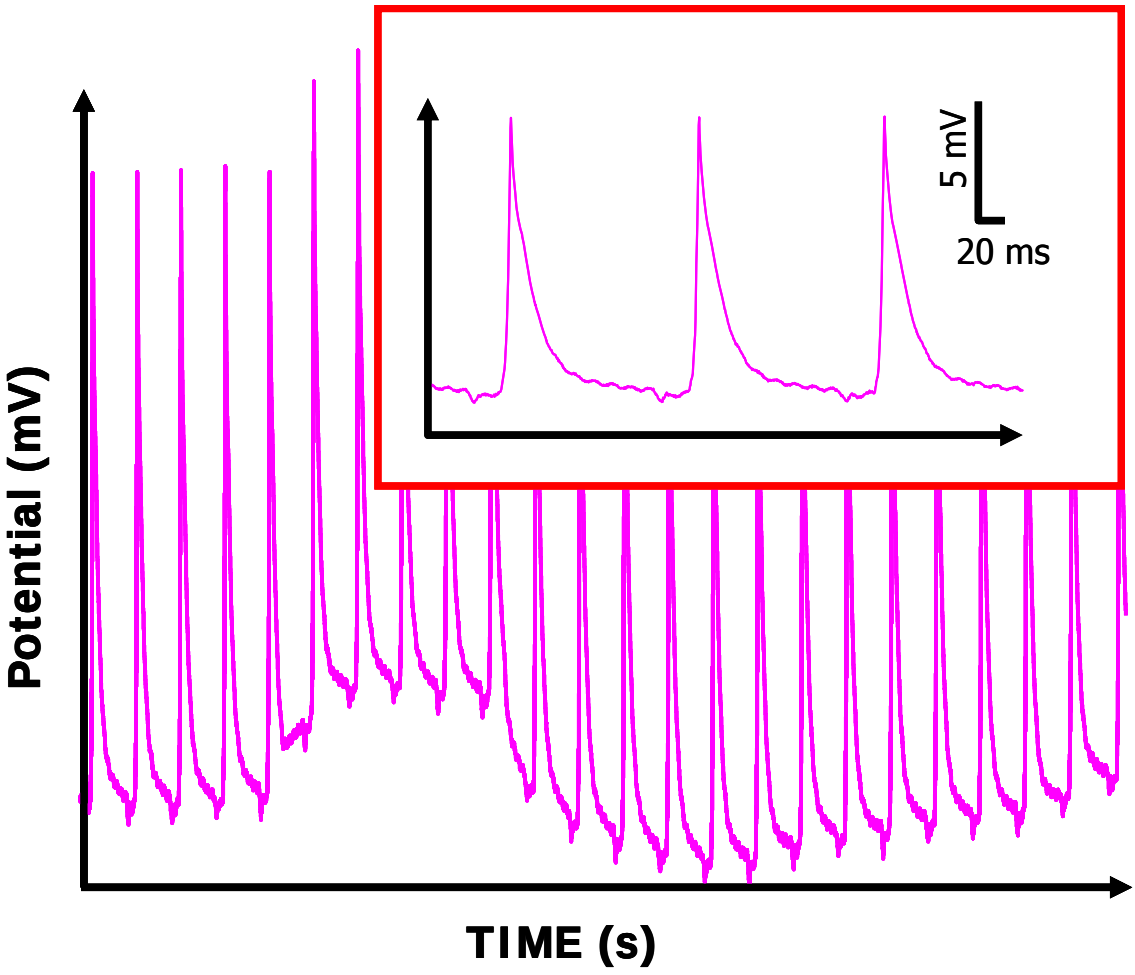

Figure 14: MAPs Obtained With Suction Electrode

Monophasic action potential (MAP) recordings obtained using the suction electrode were reasonably stable except for the occasional loss of diastolic potential (baseline drift) as pictured above in the main panel. Insert showing close up of 3 MAPs from the main panel. Contact (see Figure 11) and transmural (see Figure 12) MAP electrodes produced essentially the same result.

In the case of the Franz electrode, a reasonable amount of pressure had to be applied to obtain a MAP, but once established, the electrode pressure could be reduced and a stable recording was maintained. Stability of the recordings appeared to be site dependent and varied between hearts ranging from several minutes to tens of minutes. In addition, placement of the suction and Franz electrodes did not affect left ventricular pressure recordings greatly.

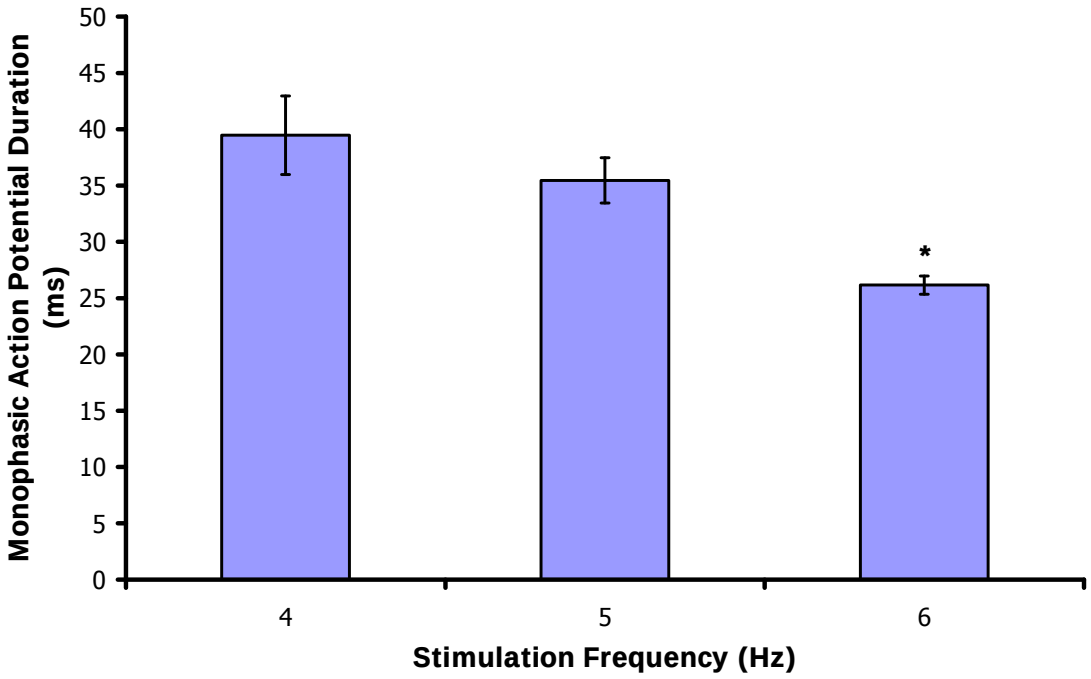

Figure 15: Effect of Stimulation Frequency on MAP durations

Figure showing the effect of pacing frequency on MAP duration at 80 % repolarisation as recorded by surface (Franz) method (n=3). Error bars indicate ± SEM, each measurement in each heart is an average of 10 action potentials at each stimulation frequency. Pacing at 4 or 5 Hz resulted in action potential durations of 39 ± 3.5 and 35 ± 2.0 ms respectively. By contrast, pacing at 6 Hz significantly reduced action potential durations at 80 % repolarisation to 26 ± 0.2 ms and made it more difficult to establish the actual resting diastolic potential (denotes $p < 0.05$, ANOVA GLM).*

Lastly, varying the pacing rate on the MAP signal was tested. As pacing frequency increased, MAP duration (as recorded by contact electrode) decreased. Whilst there was no significant difference in durations between 4, 5 and 6 Hz pacing frequencies, 6 Hz did show a clear drop in MAP APD_{80}. In addition, increasing the frequency of action potentials concurrently decreased the length of diastolic potential available for accurate calculation of MAP baselines used to determine APD.

DISCUSSION

From preliminary experiments it was apparent that suction or Franz – contact electrodes were capable of obtaining a reasonable stable surface MAP recording without affecting left ventricular pressure readings. This factor is important in the later experiments where EDLVP is manipulated independently of the recordings made by the MAP electrode. Reasons for the failure of the spring loaded contact electrode remain unclear, but are likely to be because the silver wire spring was not rigid enough to supply the initial pressure to obtain a MAP recording. Unlike the Franz electrode and transmural electrodes, the suction electrode had the advantage that it floated on the heart's surface (through the suction applied). As such, the floating electrode attached by a thin wire to the suction cup also floated freely during each contraction of the heart maintaining what appeared to be more constant contact with each contraction.

DATA ACQUISITION

All chart recordings were sampled at a rate of 2 kHz, run through an analogue 1 kHz low pass filter (at time of acquisition) and filtered to remove DC voltages. No digital 50 Hz notch or hum filters were applied at time of acquisition or post filtering as these are known to induce "ringing" or harmonics into the final recording to varying degrees. Instead all cyclic noise removal (50 Hz hum interference) was avoided at the source as best as possible. This was done by using the inverted and un-inverted input of a balanced bio-amp amplifier (AD Instruments Australia). With this method, all electrodes were made using wires of equal length such that any noise picked up along the wire would be equal in amplitude along both wires and thus subtracted from one another to result in complete (or near complete) cancellation. With this balanced, "twisted pair" setup for electrodes, the majority of interference was avoided without the aid of grounding the aorta or notch filters.

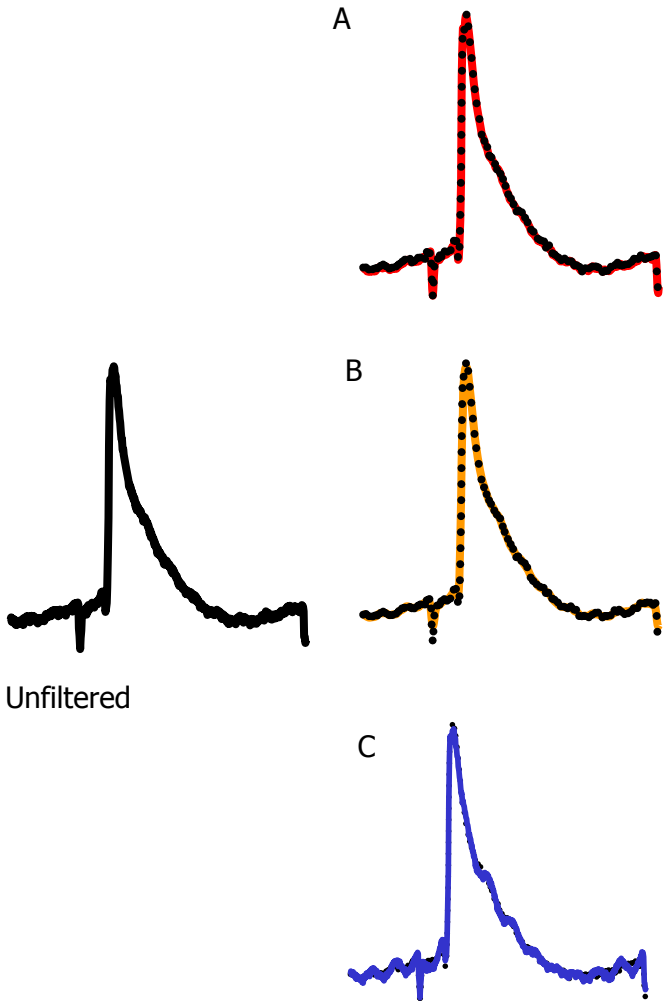

Figure 16: Filtering MAP Signals

Post filtering of data. (A) shows the effect of a 500 Hz low pass post digital filter, (B) a smoothing (moving average) filter set to 7 samples, and (C) a digital 50 Hz notch filter showing ringing in the signal developing. Action potential duration at 80 % repolarisation for all 3 filtering methods was 50.5, 52.0 and 50.5 ms respectively compared to 51.0 ms unfiltered. Action potential amplitude changed from 7.18 mV to 7.16, 6.96 and 7.02 mV for the three methods respectively.

CONCLUSION

From the preliminary data it was decided that all rat Langendorff heart preparations would be paced at either 4 or 5 Hz (depending upon intrinsic HR), and run at perfusion pressures of between 60-80 mmHg to avoid ischaemic conditions developing. In addition, data would be filtered to remove high frequency noise using the smoothing filter for fewer than 7 samples. Hum and other 50 Hz interference were be avoided at the source, wherever possible, rather than post acquisition using digital notch filters that may introduce harmonics or ringing distortions of the MAP signal.

Limitations

In the preliminary experiments, ventricular and coronary perfusion pressures were measured using the MLT0380 reusable blood pressure (BP) transducers (AD Instruments, Australia). This device was initially chosen as the MLT0380 BP transducer is sold as a low-cost pressure sensor system suitable for measuring blood pressure. The transducer is an external sensor coupled to vascular or ventricular pressure via a liquid-filled catheter and a gel-filled diaphragm. In theory, the gel and diaphragm isolate the internal electronic circuitry from the solution. The operating pressure range of the device is described as –80 to +380 mmHg with a sensitivity of 5 μV/mmHg and requires an external power source through a pod device (AD Instruments Australia). These units, however, were found to be extremely unreliable in that the gel/diaphragm on all the transducers used would frequently fail, shorting the internal circuitry of the device. In addition, the units (possibly whilst failing) would produce inaccurate results (poor response to pressure changes) and uncorrectable amounts of DC offset to the pressure signal. As such, much of the preliminary data was inadvertently damped to changes in pressure, blunting many of the responses observed.

As a result, all following experiments that involved measurements of ventricular or coronary perfusion pressures used the MLT844 Physiological Pressure Transducer (AD Instruments, Australia). This device, although significantly more expensive, was highly responsive to contractility and perfusion changes and considerably more robust in design that the other model. This piezo-resistive transducer had an operating range that exceeded the normal physiological range of the preparations and the same sensitivity as the other model of transducer, but lasted 4 years longer!

DRUGS AND CONCENTRATIONS

The contribution of NSACs to MEF-induced changes in cardiac electrophysiology upon manipulating LVEDP was studied by streptomycin-induced inhibition at a concentration of 80 μM. This concentration is double that used to successfully inhibit NSAC related responses by other authors (Gannier *et al.*, 1994; Lamberts *et al.*, 2002b; Belus & White, 2003) and significantly less that that known to inhibit various ionic currents contributing to the normal morphology of the cardiac action potential (Belus & White, 2001, 2002).

Contribution of KSACs or more specifically, TREK-1 was tested using less specific pharmacological agents. Riluzole at a concentration of 30 μM, a concentration likely to double TREK-1 channel activity (Enyeart *et al.*, 2002) was used to activate TREK-1 channel activity (Enyeart *et al.*, 2002; Caley *et al.*, 2005). Riluzole was diluted from stock solutions (10 mM) made in dimethylsulfoxide (DMSO) and added to the perfusate as a 0.03% DMSO solution with 30 μM Riluzole. Chlorpromazine at a concentration of 10 μM was used to inhibit TREK-1 channel activity as previously demonstrated by others (Maingret *et al.*, 2000b; Miller *et al.*, 2003) and was diluted from a stock concentration of 10 mM in milli-Q H_2O. Nickel Chloride ($NiCl_2$) was used to inhibit the Na^+-Ca^{2+} exchanger in rapid ectopic experiments (chapter 8). $NiCl_2$ was prepared as a 1 mM stock solution in mili-Q H_2O and diluted to 5-10 μM as concentrations higher than this induced uncontrollable runs of ectopic and arrhythmic activity. A reasonably high concentration of Verapamil (20 μM) was used to inhibit L-type Ca^{2+} channel activity and relax the heart (Takagi *et al.*, 1999).

EXCLUSIONS

Isolated Langendorff perfused hearts that failed to achieve stable contractile responses with left ventricular developed pressures greater than 60 mmHg at a constant perfusion pressure of 60 mmHg or coronary flow rate of greater than 8 ml/min and an EDLVP of 0-5 mmHg were excluded from further experimentation. In addition, hearts that displayed erratic activity or runs of uncontrollable ectopic activity and/or local discolourations caused by infarcts were also excluded from further experimentation.

CHAPTER 5:

CONTRIBUTION OF MEF TO THE FRANK-STARLING RELATION

Accomplished dance band vocalist : Maurice Elwin
(Sep 30, 1895 – Jan 4, 1975)

Chapter 5 - Table of contents

INTRODUCTION

In the previous chapters, the setup of the Langendorff perfused heart model and construction of monophasic action potential (MAP) recording electrodes was explained and tested. The aim of the present chapter is to demonstrate the contribution of MEF to electrophysiological changes throughout the cardiac cycle thought to take place at physiological and pathological levels of stretch. In this chapter, the MAP electrodes are used to explore the phenomenon of MEF during an experimental approximation of the normal cardiac cycle using Langendorff perfused heart techniques. The first part of the chapter gives a background to the stretch-induced phenomenon of the Frank-Starling or length-tension relationship of the heart (a normal physiological response of the heart to stretch). The next section deals with a series of experiments to determine the contribution of MEF to the stretch-induced increases in cardiac contractility described by the Frank-Starling response. The final part of the chapter provides a detailed discussion of the experimental results as they relate to one another and to the present understanding from past and current literature and a final conclusion.

BACKGROUND

Frank-Starling Response

In 1866 Leipzig Cyon first described the influence of diastolic filling of the isolated perfused heart on cardiac output. These observations, later published by Joseph Coats (1869) demonstrated the negative inotropic effects of decreasing ventricular filling pressure and the positive inotropic effects of increasing ventricular filling pressure on cardiac contractility (Figure 17). Later, (1895-1914), Otto Frank and Ernest Starling demonstrated that greater diastolic volumes of the heart result in an increase in cardiac performance during subsequent contractions (Patterson *et al.*, 1914; Starling & Visscher, 1927; Zimmer, 2002). More recently, this increase in contractility has been shown to be biphasic in nature (Parmley & Chuck, 1973), being composed of both a primary response (within a couple of heart beats) and a secondary, delayed response that develops over several minutes; the Anrepp effect (Calaghan & White, 1999; Zimmer, 2002).

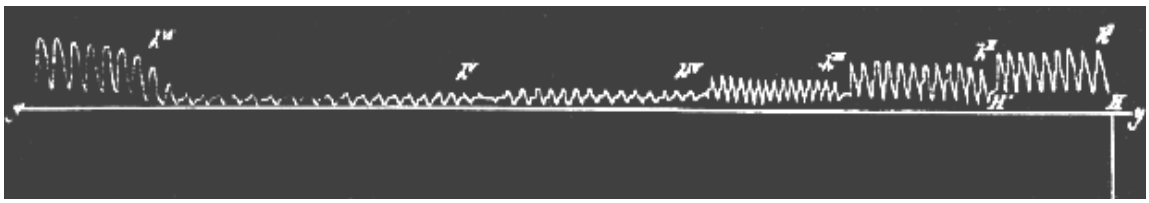

Figure 17: Frank-Starling Response

Effect of lowering filling pressure (from right to left) on diastolic pressure and amplitude of contraction of the isolated frog heart. Restoration of amplitude when original filling pressure was applied (far left) is shown. Recording made by H. P. Bowditch, (reprinted from Zimmer, 2002).

Initially it was thought that, like striated skeletal muscle, the ascending limb of the Frank-Starling curve could be described in terms of decreasing double overlap of the actin myofilaments (Gordon *et al.*, 1966; Allen & Kentish, 1985). However, it has been proposed that the relationship between cardiac distension and contractile force is far too steep to be adequately explained by such a mechanism (Allen & Kentish, 1985; Kentish *et al.*, 1986). The first, rapid increase in contractility has since been related to an increase in the sensitivity of the myofilaments to intracellular calcium ($[Ca^{2+}]_i$) (Allen & Kurihara, 1982; Kentish & Wrzosek, 1998) and more loosely to the length-dependence of intracellular Ca^{2+} release (see Allen & Kentish, 1985 for review).

Changes in lattice spacing and myofilament Ca^{2+} sensitivity

Increases in contractility have also been suggested to result from a reduction in the lattice spacing of the thick and thin filaments and a concurrent increase in myofilament Ca^{2+} sensitivity (Allen & Kurihara, 1982; Kentish *et al.*, 1986; Calaghan & White, 1999; Fuchs & Smith, 2001; Moss & Fitzsimons, 2002). When a muscle is stretched, not only does it lengthen, but its diameter also decreases as the thick and thin filaments are brought closer together. The result is a decrease in filament spacing and, in theory, a corresponding increase in the number of active cycling cross-bridges being formed, resulting in an increase in contractility (Allen & Kentish, 1985; Irving *et al.*, 2000; Wannenburg *et al.*, 2000). However, this model does not account for the observed decrease in contractility on the descending portion of the Frank-Starling curve. Furthermore, Wannenburg *et al* (1997) demonstrated that there was no change in the rate of cross-bridge detachment over the ascending portion of the Frank-Starling relationship, suggesting that there was no change in the rate of active cycling cross-bridges being formed (Wannenburg *et al.*, 1997). More recently, the theory of decreasing filament spacing was further compromised by Konhilas *et al* (2002) by mimicking the reduction in myofilament lattice spacing with 1-1.5 % dextran. Unexpectedly, the associated decrease in myofilament spacing was found not to affect myofilament Ca^{2+} sensitivity (Konhilas *et al.*, 2002b). This is in contrast to earlier studies suggesting that the contractile apparatus is more sensitive to intracellular calcium fluctuations at longer sarcomere lengths (Fabiato & Fabiato, 1978; Hibberd & Jewell, 1979). These observations, among others (Wannenburg *et al.*, 1997; Konhilas *et al.*, 2002a) suggest the Frank-Starling mechanism is complex and likely to be contributed to by more than one factor.

Changes in the intracellular Ca^{2+}

In contrast to the mechanism that initiates the immediate increase in contractility, the gradual increase in myocardial contractility has been attributed to a slow rise in the $[Ca^{2+}]_i$ transient (Allen & Kurihara, 1982; Bustamante *et al.*, 1991; Kentish & Wrzosek, 1998). In addition, biphasic responses have been demonstrated at the level of single cardiomyocytes (White *et al.*, 1995). However, while these studies suggest that changes in the $[Ca^{2+}]_i$ transient are not responsible for the immediate effects of the Frank-Starling response, this inference is questionable for the following reason: The changes in contractility associated with the Frank-Starling response occur over several heartbeats. That is, the steep rise in contractility associated with the Frank-Starling mechanism reflects the effect of stretch observed over several beats rather than the instantaneous effect seen in a single heart beat (Lakatta & Jewell, 1977). In support of this observation, studies conducted in isolated cardiomyocytes from atria and ventricles demonstrate that diastolic $[Ca^{2+}]_i$ changes immediately with stretch (Tavi *et al.*, 1998; Fuchs & Smith, 2001) while the $[Ca^{2+}]_i$ transient changes over a short period (~4 seconds). Therefore, the changes in diastolic $[Ca^{2+}]_i$ and the $[Ca^{2+}]_i$ transient may account for the observed increase in cardiac contractility in Frank-Starling's experiments (Gannier *et al.*, 1996; Tavi *et al.*, 1998). This is likely since it has been shown that stretch increases diastolic $[Ca^{2+}]_i$ through NSACs (Kim, 1993), and that this enhances Ca^{2+} transients most likely through increasing SR Ca^{2+} content (Frampton *et al.*, 1991). In addition, others suggest that increases in diastolic $[Ca^{2+}]_i$ may result from secondary effect on the Na^+-Ca^{2+} exchange (Gannier *et al.*, 1996; Tavi *et al.*, 1998), possibly as a result of diastolic Na^+ influx through NSACs. It should be noted, however, that at least one other study has demonstrated an apparent decrease in SR Ca^{2+} content with stretch in isolated rat papillary muscles (Gamble *et al.*, 1992). Regardless of the mechanism, changes in the Ca^{2+} transient brought about by stretch are likely to result in changes to action potential morphology (duBell *et al.*, 1991). Likewise, changes in the action potential brought about by the involvement of NSACs can be expected to alter the contractile response (Bouchard *et al.*, 1995).

In summary, the Frank-Starling (Length-Tension) relation is thought to be composed of two phases, the first relating to changes in structure, function and cross bridge kinetics, whilst the second phase to changes in calcium handling. However, some debate remains regarding the mechanisms at the molecular level (see review by

Rassier, 2000). Whilst stretch through increasing EDLVP is known to result in an increase in sarcomere length and Ca^{2+} affinity of the contractile apparatus (Fuchs & Smith, 2001), the method by which stretch alters Ca^{2+} affinity remains to be defined (Konhilas *et al.*, 2002b). With the advent of MEF theory and the observation of stretch-induced changes in $[Ca]_i$, there is the possibility that the Frank-Starling response may be brought about, in part, by stretch-generated changes in the membrane potential. These electrophysiological changes could be induced indirectly by NSACs through changes in the reverse mode Na-Ca exchange current. Likewise, the proposed activation of stretch-activated ion channels (of both non-selective and K-selective type) could also contribute directly to the morphology of the action potential throughout the Frank-Starling response.

Whilst the effects of mechanical stretch on the electrophysiology of heart muscle have been studied in isolated cell and tissue preparation, little is known about the changes in electrical behaviour of the whole heart in response to physiological stretch. This may be partly due to the methodological difficulties associated with making intracellular recordings from isolated, perfused Langendorff hearts. As a result, this study was designed to obtain electrophysiological changes associated with stretch by means of MAP recordings which provide a reliable index of the shape of the action potential during the entire repolarisation phase (Hoffman *et al.*, 1959).

METHODS

Male Sprague Dawley rat hearts were prepared as outlined in chapter 3 from rats weighing 400-500 g. Hearts were paced at 4 Hz and perfused with bicarbonate buffer containing 1.5 mM $CaCl_2$ at coronary perfusion pressures of 40 (n=4), 50 (n=5), 60 (n=7), 70 (n=7), 80 (n=7) or 100 (n=3) mmHg in randomised order. Where possible, the same hearts were used to observe the Frank-Starling response and changes in MAP electrophysiology for more than one coronary perfusion pressure. Once contractility had stabilised with the left ventricular balloon in place and the heart perfused in the constant perfusion pressure mode, left ventricular diastolic pressure was increased in 2.5-5 mmHg steps using a calibrated glass screw driven plunger syringe (Henke Germany) to obtain the Frank-Starling effect (Figure 18, pg 75). During this period, a suction MAP electrode supplied with a vacuum pressure of 30-50 mmHg recorded MAP signals from the left ventricular free wall (see Figure 10 pg 57). For analysis, 10 monophasic action potentials were taken from three pressure ranges

to identify changes in MAP morphology. Action potentials were taken in the ranges 0-5 mmHg (control, no stretch), 10-15 mmHg and 20-25 mmHg (at the peak of the Frank-Starling response). Data in each range were then averaged and any changes in MAP duration (as measured by action potential duration, APD, at 20, 50 and 80 % repolarisation (see Figure 2 pg 17) calculated and presented as a % of initial values seen with no stretch (0-5 mmHg) to reduce inter-rat variability.

RESULTS

Coronary flow rate varied from 10 ± 1 ml/min at the lower coronary perfusion pressure of 40 mmHg to 17 ± 3 ml/min at the highest coronary perfusion pressure of 100 mmHg. MAP recordings made at a coronary perfusion pressure of 40 mmHg suffered from diastolic artifacts making calculations of diastolic potential inaccurate and resultant measurements of APD difficult.

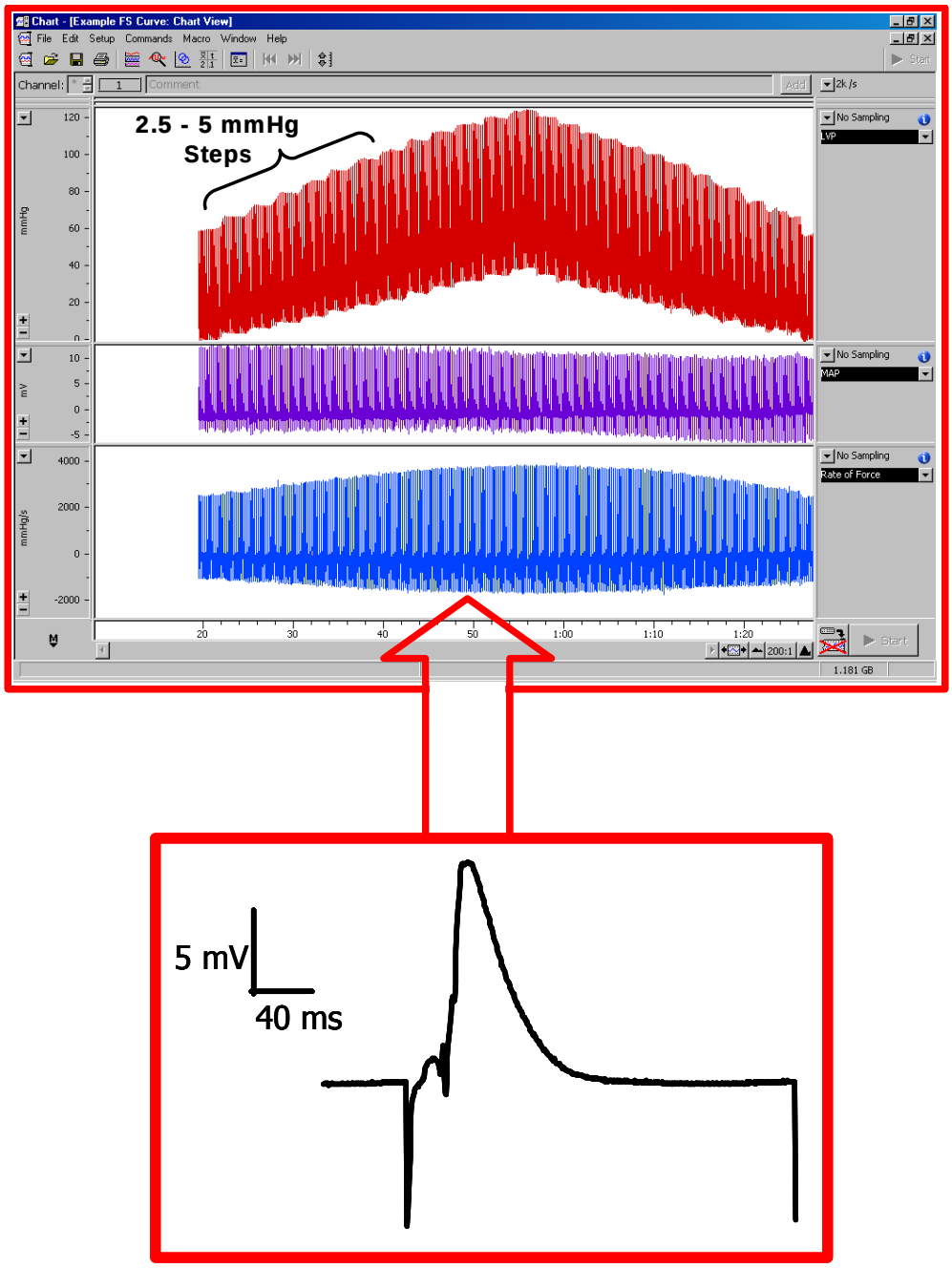

Figure 18: Effect of Stepwise increases in EDLVP on MAP Morphology

Figure showing 2.5 mmHg stepwise increases in left ventricular end diastolic pressure (EDLVP) every 2 seconds from 0 to 30 mmHg. Red (upper) trace shows raw left ventricular pressure as recorded from the intra-ventricular balloon placed in the left ventricle (in mmHg). Purple (middle) trace shows monophasic action potentials (in mV) as recorded using a suction MAP electrode using 30 mmHg vacuum. Blue (lower) trace is the differential of the pressure trace, showing rate of change in intra-ventricular balloon pressure (and hence contractility, in mmHg/s)

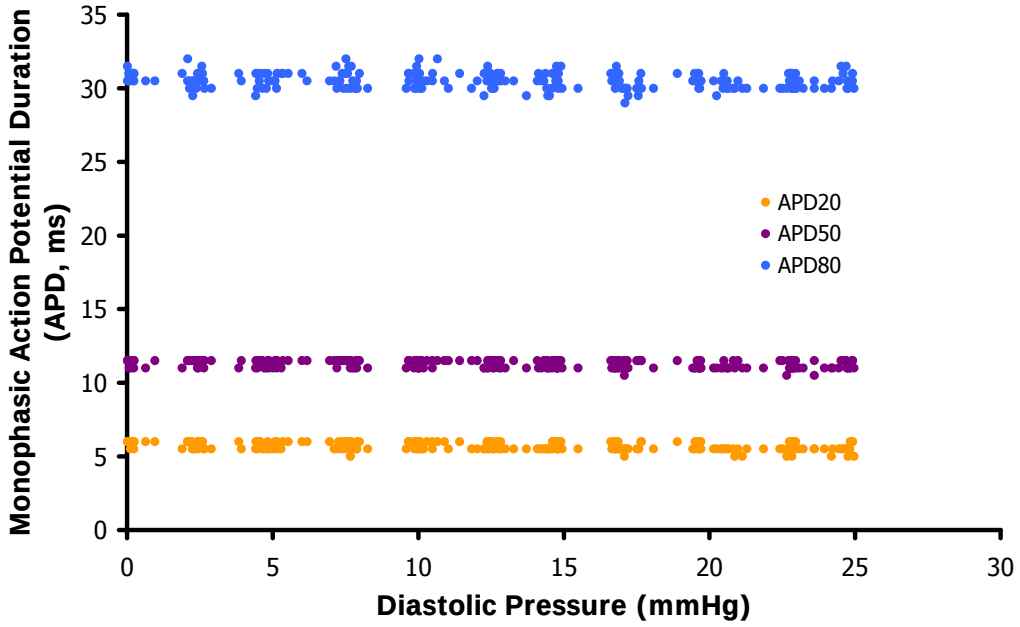

Figure 19: Effect of EDLVP on MAP Durations

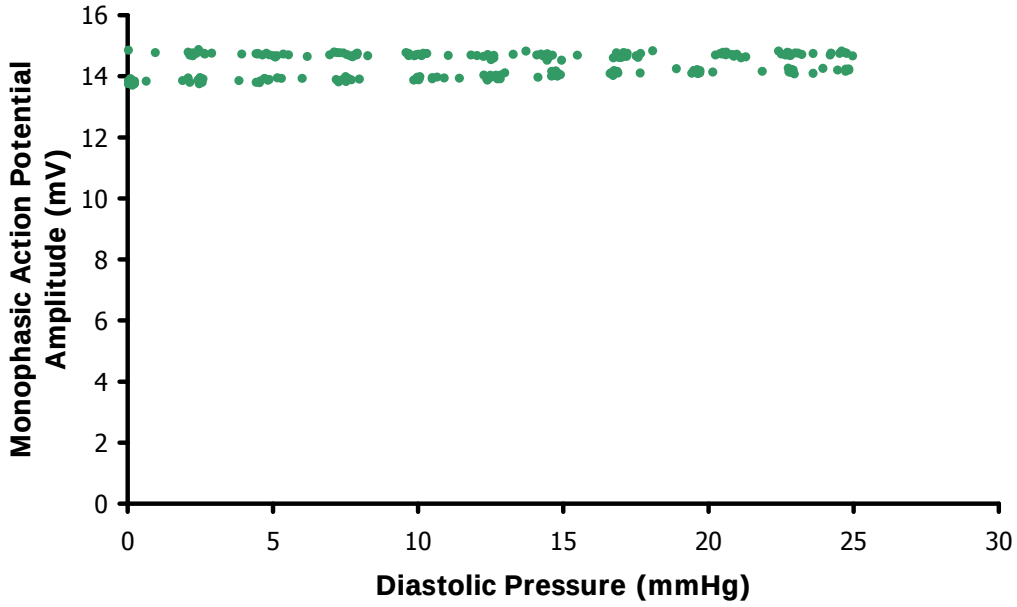

Figure 20: Effect of EDLVP and Time on MAP Amplitude

Figures showing a complete data set from one Frank-Starling curve obtained from one rat heart representative of the data obtained. Figure 19 showing Monophasic action potential changes as measured at 20, 50 and 80 % repolarisation in response to changes in end diastolic left ventricular pressure (EDLVP) in the range 0 to 25 mmHg. This figure shows that although there was a drop in amplitude over time (~1 minute to perform stewpwise increases in EDLVP to 25 mmHg and back down to 0 mmHg), MAP duration remained near constant throughout manipulations in end diastolic left ventricular pressure. It should be noted that MAP durations and amplitudes varied between preparations. As a result, further data are expressed as a percentage of control conditions for each rat and recording/perfusion pressure. MAP durations were calculated using an automated macro to detect diastolic potential, with MAP detection threshold set to 1 %. Durations are calculated from the peak of the MAP recording to the respective % repolarisation point. Figure 20 shows a ~1 mV decrease in monophasic action potential amplitude over the range of the Frank Starling curve.

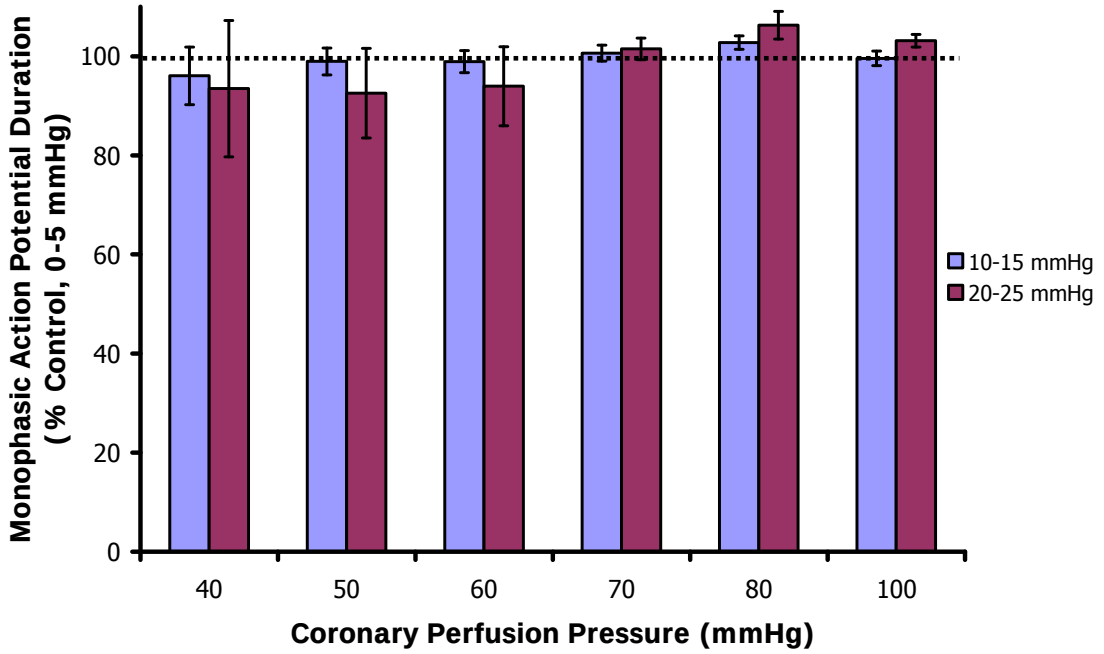

Figure 21: Effect of Stretch on APD_{20} at Different Perfusion Pressures

Figure showing effect of varying coronary perfusion pressures and end diastolic left ventricular pressures on monophasic action potential durations as measured at 20 % repolarisation (APD_{20}). Data shown are for perfusion pressures (in mmHg) of 40 (n=4), 50 (n=5), 60 (n=7), 70 (n=7), 80 (n=7) or 100 (n=3). Whilst monophasic action potentials were recorded continuously over the 0-25 mmHg range of the Frank-Starling response, only 10 action potentials in each of the ranges 0-5, 10-15 and 20-25 mmHg were chosen for analysis, error bars indicate ± SEM. MAPs recorded at 10-15 and 20-25 mmHg are expressed as a percentage of those MAPs obtained in the absence of any left ventricular stretch (0-5 mmHg diastolic pressure, as denoted by the broken line). Analysis using a GLM ANOVA and general linear model showed no significant difference between diastolic pressure groups or from control at any of the specified coronary perfusion pressures.

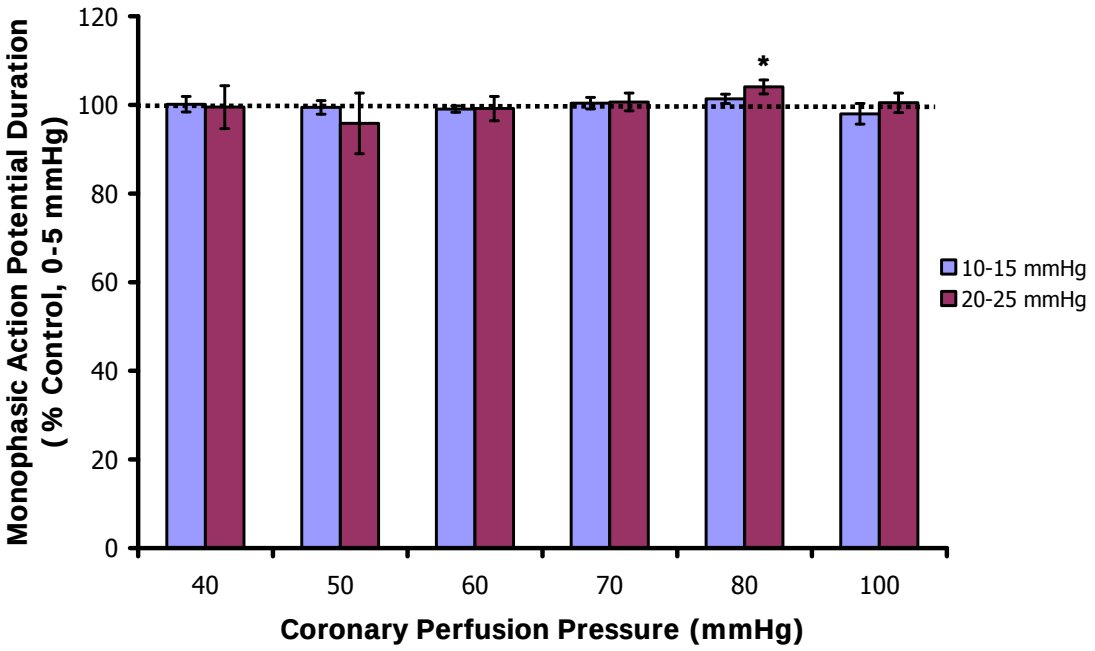

Figure 22: Effect of Coronary Perfusion Pressure on MAP APD_{50}

Figure showing effect of varying coronary perfusion pressures and end diastolic left ventricular pressures on monophasic action potential durations as measured at 50 % repolarisation (APD_{50}). Data shown are for perfusion pressures (in mmHg) of 40 (n=4), 50 (n=5), 60 (n=7), 70 (n=7), 80 (n=7) or 100 (n=3). Whilst monophasic action potentials were recorded continuously over the 0-25 mmHg range of the Frank-Starling response, only 10 action potentials in each of the ranges 0-5, 10-15 and 20-25 mmHg were chosen for analysis, error bars indicate ± SEM. MAPs recorded at 10-15 and 20-25 mmHg are expressed as a percentage of those MAPs obtained in the absence of any left ventricular stretch (0-5 mmHg diastolic pressure, as denoted by the broken line). Analysis using a GLM ANOVA showed a statistically significant increase in MAP APD_{50} when EDLVP was increased to 20-25 mmHg during a static coronary perfusion pressure of 80 mmHg (denotes $p < 0.05$, GLM ANOVA followed post hoc by Tukey's pair wise comparisons). No other significant differences between diastolic pressure groups or from control at any of the specified coronary perfusion pressures were observed.*

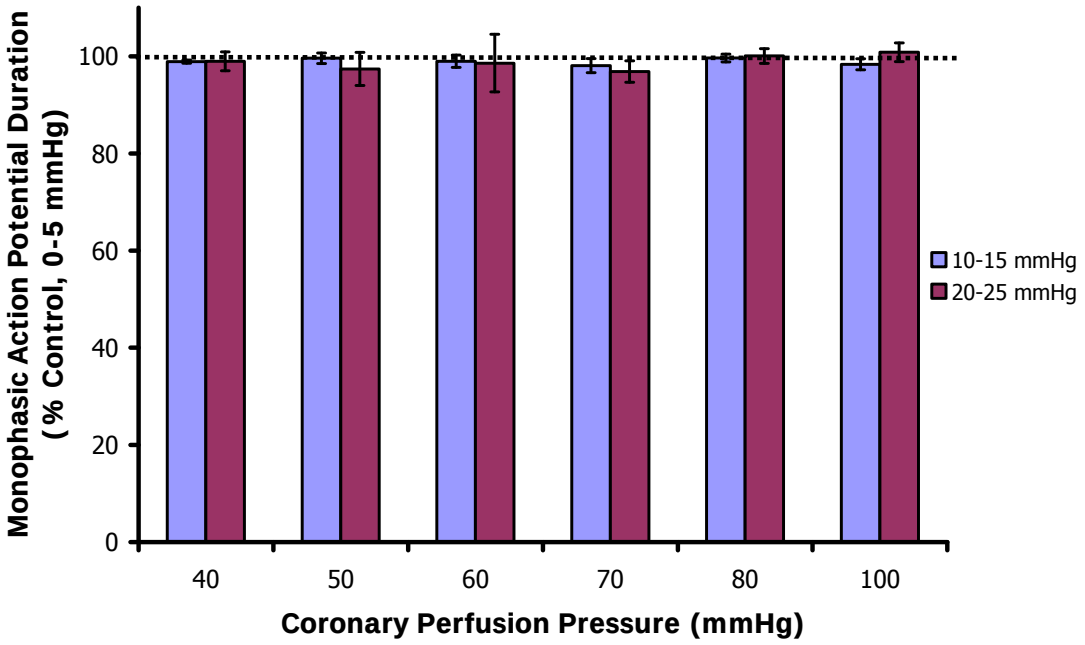

Figure 23: Effect of Coronary Perfusion Pressure on MAP APD_{80}

Figure showing effect of varying coronary perfusion pressures and left ventricular diastolic pressures on monophasic action potential durations as measured at 80 % repolarisation (APD_{80}). Data shown are for perfusion pressures (in mmHg) of 40 (n=4), 50 (n=5), 60 (n=7), 70 (n=7), 80 (n=7) or 100 (n=3). Whilst monophasic action potentials were recorded continuously over the 0-25 mmHg range of the Frank-Starling response, only 10 action potentials in each of the ranges 0-5, 10-15 and 20-25 mmHg were chosen for analysis, error bars indicate ± SEM. MAPs recorded at 10-15 and 20-25 mmHg are expressed as a percentage of those MAPs obtained in the absence of any left ventricular stretch (0-5 mmHg diastolic pressure, as denoted by the broken line). Analysis using a GLM ANOVA showed no significance between diastolic pressure groups or from control at any of the specified coronary perfusion pressures.

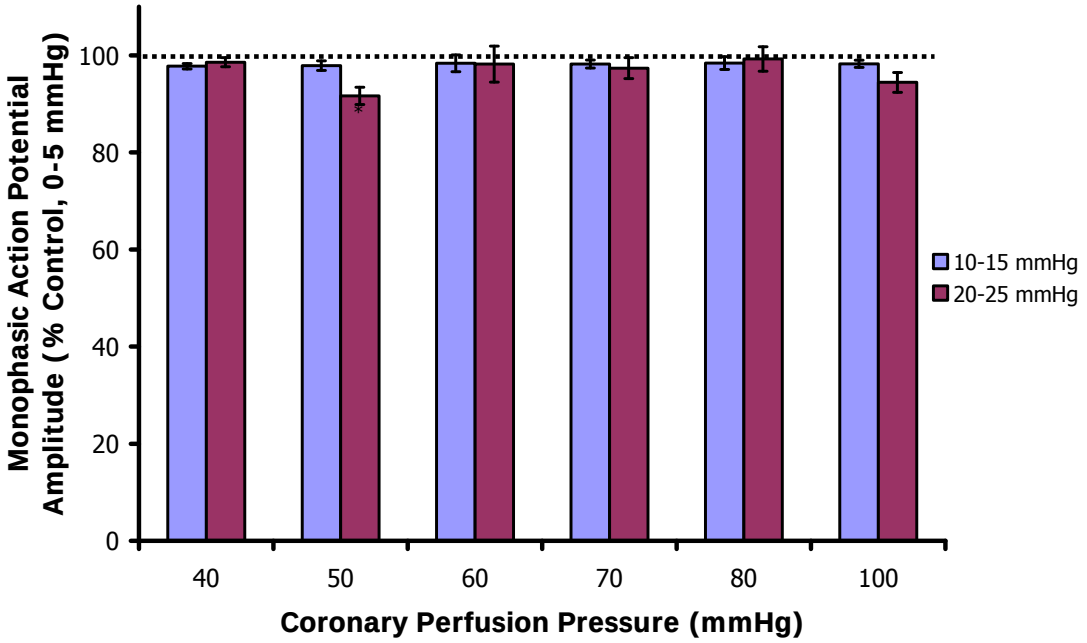

Figure 24: Effect of Coronary Perfusion Pressure on MAP Amplitude

Figure showing effect of varying coronary perfusion pressures and left ventricular diastolic pressures on monophasic action potential amplitude. Data shown are for perfusion pressures (in mmHg) of 40 (n=4), 50 (n=5), 60 (n=7), 70 (n=7), 80 (n=7) or 100 (n=3). Whilst monophasic action potentials were recorded continuously over the 0-25 mmHg range of the Frank-Starling response, only 10 action potentials in each of the ranges 0-5, 10-15 and 20-25 mmHg were chosen for analysis, error bars indicate ± SEM. MAPs recorded at 10-15 and 20-25 mmHg are expressed as a percentage of those MAPs obtained in the absence of any left ventricular stretch (0-5 mmHg diastolic pressure, as denoted by the broken line). Analysis using a GLM ANOVA followed post hoc by Tukey's pair wise comparisons showed that monophasic action potential amplitude decreased (denotes $p < 0.05$, GLM ANOVA followed by Tukey's pairwise comparisons) when the heart was perfused at a coronary pressures of 50 and 100 mmHg and the ventricle stretched with 20-25 mmHg intra-ventricular pressure. This reduction was less than 0.8 mV on average for MAP recordings of amplitude > 10 mV (n=2), and in most cases less than 0.4 mV (n=3).*

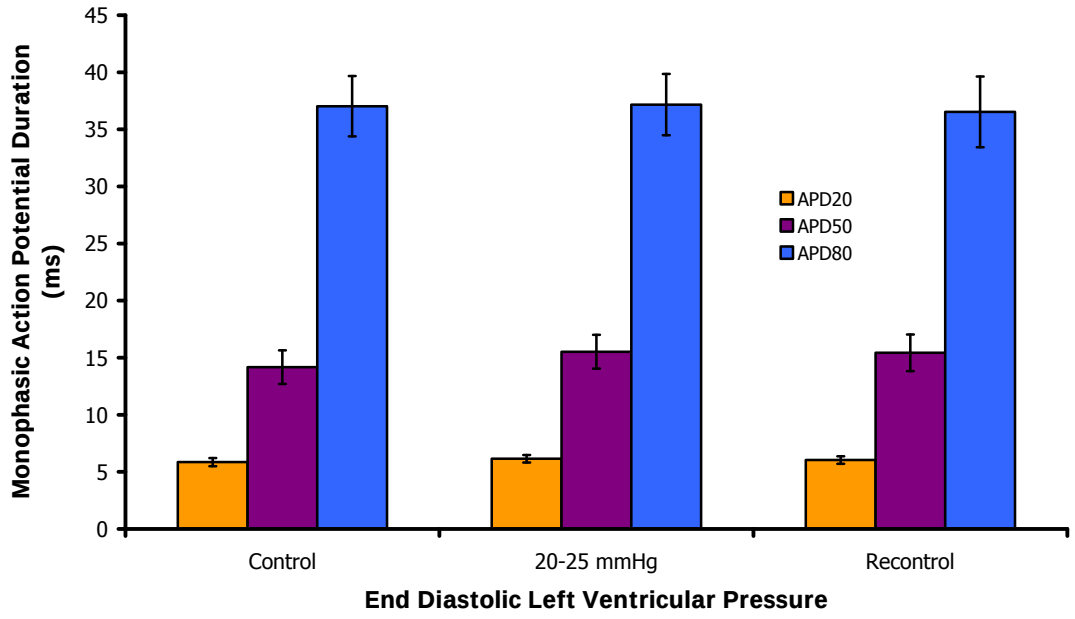

Figure 25: Effect of EDLVP on MAP Duration at Constant Perfusion Pressure

Figure showing effect of stepwise increases in Left Ventricular End Diastolic Pressure on Monophasic action potential durations at different levels of repolarisation in rat hearts perfused at 60 mmHg showing constant MAP amplitudes. MAP durations at 20%, 50% and 80% repolarisation plotted for control (close to zero EDLVP), EDLVP 20-25 mmHg and re-control (EDLVP returned to close to zero). Error bars indicate ± SEM, n = 4 hearts (each measurement in each heart is an average of 10 action potentials at each level of stretch). There are no significant differences in MAP durations between control, 20-25 mmHg and re-control (GLM ANOVA). Note, these rats were chosen for their MAP amplitude stability over the entire Frank-Starling curve. MAP amplitudes were 14.4 ± 1.1 control and 14.6 ± 1.1 mV re-control.

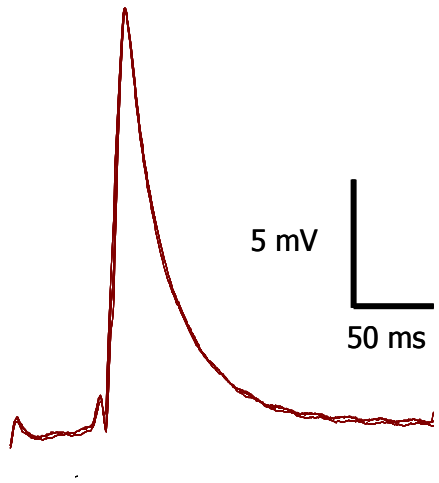

Figure 26: MAP overlay at different EDLVP

Figure showing monophasic action potentials from control, 20-25 mmHg EDLVP and re-control conditions overlaid.

DISCUSSION:

In this section, the electrophysiological response of the rat heart to changes in end diastolic left ventricular pressure (EDLVP) over a range or coronary perfusion pressures is described. One of the greatest difficulties experienced throughout these experiments was in obtaining a MAP recording with a stable amplitude (see Figure 18). However, electrodes were developed from preliminary experiments to give the most stable response throughout experimental manipulations. Figure 18 and Figure 19 show the amplitude and duration of MAPs taken throughout the entire Frank-Starling curve of one rat. Even though in this particular example, MAP amplitude dropped by almost 1 mV (~ 7 %) across the entire Frank-Starling curve (ascending and descending), there was no obvious change in MAP duration. In addition, the figures show the general spread observed in MAP recording durations when analysed using AD Instrument peak parameter macros for automation. Manual analysis of action potentials at random appeared to decrease the variation in APD through more accurate determination of action potential base line or diastolic potential.

Figure 24 shows changes in action potential amplitude for all rats at the various coronary pressures tested. It can be seen that at within the nominal (or optimal) perfusion pressure range of 60-80 mmHg, there was no significant change in MAP amplitude in any of the groups. By contrast, at 50 and 100 mmHg perfusion

pressures, there was a reduction in MAP amplitude associated with the peak of the Frank-Starling curve. However, as noted previously, this reduction was less than 0.8 mV (~ 8 %) for MAP recordings of amplitude > 10mV (n= 2), and in most cases less than 0.4 mV (~ 4 %, n= 3) and did not affect action potential durations. This is well within the changes in MAP amplitude observed over time by others. In the rabbit heart, MAP amplitude reductions of up to 50% over time have been found not to interfere with the respective MAP durations (Hoffman *et al.*, 1959). Since MAP amplitude has long been recognized to detect only a part of the full transmembrane potential (Hoffman *et al.*, 1959; Franz, 1999), it has little bearing on MEF-related changes. However, MAP amplitude serves as a simple measure of MAP stability enabling the detection of a failing recording. As such, it can be concluded that MAP amplitude in the present results did not interfere with MAP duration measurements.

Figure 21 - Figure 23 (pgs 78-80) show changes in APD 20, 50 and 80 expressed as a percentage of the same measurements taken in the absence of stretch at the start of each Frank-Starling curve. As it can be seen, regardless of perfusion pressure and the potential effect on basal MAP durations, there were no obvious changes in MAP morphology induced by the majority of EDLVP manipulations from basal (0-5 mmHg), mid (10-15 mmHg) and at the peak (20-25 mmHg) of the Frank-Starling length tension relationship. There was noted a 4 ± 1.6 % increase in MAP APD_{50} at a coronary perfusion pressure of 80 mmHg amounting to a 0.7 ms increase in action potential duration. Given the variability in the response (almost half the suggested increase), this is an effect that is unlikely to be physiologically significant. Lower perfusion pressures and associated flow rates abbreviated the action potential duration (most likely a result of activation of K_{ATP} channels) as a result of increasingly ischaemic/hypoxic conditions (Hamada *et al.*, 1998). As such, one would expect that if SACs were to increase action potential duration, this would be most obvious at low flow rates and perfusion pressures where larger increases in action potential duration through inward NSAC Na^+ and Ca^{2+} ion currents would be more likely to occur. However, the present results failed to demonstrate any modification in MAP duration during stretch when compared to basal conditions. Likewise, at higher flow rates where ischaemic conditions are unlikely to be present, any action potential shortening through SAC activation should also be obvious since at higher pressures K_{ATP} channels would be expected to contribute less to the overall action potential morphology. The

present results do not support either case suggesting that SACs play little (an undetectable) or no role in the overall Frank-Starling response.

From the above observations of the data it becomes increasingly apparent that static coronary flow rates and hence coronary perfusion pressure had little or no effect on stretch-induced changes in the rat MAP between control (un-stretched) and stretched conditions. Small stepwise increases in EDLVP had no influence over MAP durations throughout experiments, nor MAP amplitude. As such, it would appear that physiological ranges of stretch did not induce any obvious changes in MAP morphology through MEF mechanisms. This is inconsistent with the observation that that stretch can abbreviate action potential duration, induce depolarisations and decrease action potential amplitude at the cellular level (Lab, 1980; Eckardt *et al.*, 2000; Isenberg *et al.*, 2003). By contrast, others have found an increase in action potential duration accompanies stretch (Belus & White, 2003). The main concern with these results at the cellular level is the ability to be accurately extrapolated back to the *in vitro* whole heart and whole animal models. There is no means to reliably compare the forces used to elicit these electrophysiological changes with those used in the current study.

The present findings are consistent with the observation in isolated cat papillary muscles where moderate levels of stretch induced no change in action potential morphology (Lab, 1980). Moreover, the present study is not the first to demonstrate inconsistent or little change in cardiac action potentials as observed by MAP recordings (Babuty & Lab, 2001) or by other methods (Riemer & Tung, 2000, 2003). Inconsistent effects of stretch and streptomycin (80 μM), on MAP APD_{50} and 80 have also been reported in the guinea pig atria during acute stretch (Babuty & Lab, 2001). Equally interesting was the observation that increasing end-diastolic volume in canine hearts by up to 51 ± 6 ml resulted in a very small (< 1.0 %) shortening of MAP duration that had no effect on overall MAP contour (Calkins *et al.*, 1989). As a result the authors of this experiment concluded that alterations in volume load are of little electrophysiological or arrhythmogenic importance in normal canine ventricles under physiologically loaded conditions similar to that demonstrated here. In addition, the results reflect those observed using intracellular recordings in isolated cat papillary muscle preparations subject to stretch (Lab, 1980; Hennekes *et al.*, 1981) and the inconsistent effects seen at the cellular level (White *et al.*, 1995; Hongo *et al.*, 1996).

Unsurprisingly, when looking at 4 amplitude stable MAP recordings (refer Figure 25 and Figure 26) where there was no change in MAP amplitude over time or as a result of stretch, there was no statistical difference in MAP durations before, during or after left ventricular stretch. It should be noted that these changes in left ventricular stretch occurred over a reasonable length of time (> 1 min) since they were conducted in 2.5 mmHg steps across the entire length-tension relationship. In addition, the coronary perfusion pressures were static throughout EDLVP manipulations and should not be confused with other phenomena such as the Gregg effect discussed later. If SACs are able to adapt as has been suggested (Hu & Sachs, 1996; Bett & Sachs, 2000a; Niu & Sachs, 2003), then this could explain the present result. In the present experiments, the stepwise increases in EDLVP may have allowed adequate time for SACs to adapt to the ventricular stretch hence terminating any visible MEF response. Likewise, since previous attempts at demonstrating MEF in cardiomyocytes and in the whole heart have demonstrated action potential shortening to be the major response, it is possible that the rat heart is not a good model for demonstrating MEF-induced decreases in action potential duration due to the brief nature of the rat cardiac action potential. This is a likely factor since many cellular studies of MEF are performed at room temperature (Akay & Craelius, 1993; Ruknudin *et al.*, 1993; Gannier *et al.*, 1994; Gannier *et al.*, 1996; Tavi *et al.*, 1998; Kawakubo *et al.*, 1999; Kamkin *et al.*, 2000a; Zeng *et al.*, 2000; Zhang *et al.*, 2000; Isenberg *et al.*, 2003; Kamkin *et al.*, 2003b; Riemer & Tung, 2003) where the cellular action potential is prolonged due to altered cellular kinetics involved with the substantial temperature change. Such experiments with longer action potential durations are theoretically likely to show greater action potential changes – especially if a reduction in duration is expected.

Despite the general consensus that stretch induces changes in the morphology of the action potential (Franz *et al.*, 1989; Nazir & Lab, 1996; Rice *et al.*, 1998; Tavi *et al.*, 1998; Takagi *et al.*, 1999; Eckardt *et al.*, 2000; Kamkin *et al.*, 2000a; Zeng *et al.*, 2000; Zhang *et al.*, 2000; Parker *et al.*, 2001; Belus & White, 2003; Isenberg *et al.*, 2003; Riemer & Tung, 2003), the present study finds that physiological levels of stretch are insufficient to induce changes in rat ventricular electrophysiology be it influxes of Na^+ and Ca^{2+} through NSACs or K^+ through KSACs. This is somewhat surprising given cellular studies suggesting that these currents are maximally activated at low pipette vacuum pressures of 20 mmHg for NSACs and even less for KSACs (~1.5 mmHg) (Ruknudin *et al.*, 1993). However, this supports concerns regarding the

extrapolation and physiological relevance of such methods back to the whole heart scenario. This is especially important considering the limited number of studies showing any stretch-induced electrophysiological changes over physiological ranges such as those experienced in the Frank-Starling response.

Others have suggested that the diastolic $[Ca^{2+}]_i$ does not change with stretch (Hongo *et al.*, 1996; Tavi *et al.*, 1998) and that this is likely to be because of the Ca^{2+} buffering power of the cell increases as indicated by faster decay rates of Ca^{2+} transients during stretch (Tavi *et al.*, 1998). Moreover, NSACs may not directly modulate the function and electrophysiology of cardiomyocytes but may instead increase SR Ca^{2+} stores and resultant release of Ca^{2+} during systole (Tavi *et al.*, 1998). As such, the increased Ca^{2+} current would induce a larger inward Na^+-Ca^{2+} exchange Na^+ current depolarising the cell and increasing action potential duration. An effect and observation not demonstrated in the present study.

Thus, the present experiments suggest that NSACs play little role in the increase in cardiac contractility mediated by modest changes in EDLVP as described by the Frank-Starling phenomenon. Moreover, this mechanism does not involve NSACs or stretch-induced increases in $[Ca^{2+}]_i$ which would be expected to alter the action potential either directly or indirectly via the Na^+-Ca^{2+} exchange mechanism. This is a conclusion supported by (Tavi *et al.*, 1998) who suggested that SACs do not, during moderate stretch, directly modulate action potentials. Instead, any effects are mediated by the SR through increased SR Ca^{2+} release during systole (Tavi *et al.*, 1998) or other secondary mechanisms (von Lewinski *et al.*, 2003). In addition, the present results reflect the observation that previous-beat contraction history is not influenced by NSAC blockade with either 100 or 500 μM streptomycin (Slinker & Tobias, 1996).

The theory that suction MAPs induce irreversible damage to the myocardium can be somewhat disputed due to the present experiments and those performed by other experimentalists (Leirner & Cestari, 1999). The suction MAP electrode used here was a variant of that described by (Leirner & Cestari, 1999), the main difference being the location of the reference electrode in the present study, outside of the suction field. By past definition, suction induces regions of ischaemia and hence "a sink" or area of inexcitability. The assumption that suction induces myocardial death must be inaccurate since both the exploring and reference electrodes observed in the design by (Leirner & Cestari, 1999) are contained within the suction field. Recent literature

suggests the requirement of both inactive and active fields of cells to obtain a MAP recording, thus, the tissue within the suction field must not be completely inactivated for these electrodes to work (Franz, 1983, 1999; Kadish, 2004; Kondo *et al.*, 2004; Tranquillo *et al.*, 2004; Franz, 2005). As such, one could conclude that the damage induced by suction electrodes is not as severe as has been previously suggested. Certainly, in the present study, no permanent physical damage to the heart was observed following removal of the suction electrode. Any discolouration seen generally disappeared after several minutes of undisturbed perfusion.

Considerations

With the current study, it is possible that the suction MAP electrode used throughout these experiments was unsuitable for picking up small APD changes in MAP signals, and hence MEF in the rat model. The MAP signal is a summation of extracellular currents from a number of cells under the electrode (Franz, 1994, 1999, 2005). As such, MAP electrode size has a significant influence on the recorded signal. With the current electrode configuration, suction is applied to a relatively large area of the heart surface and the resultant signal generated from the difference in potential between the cells under the area of suction and the floating electrode. As such, it is possible that the present surface/suction electrode configuration is too large and hence any small changes in action potential morphology may have been lost in the summation of signals across cells undergoing heterogeneous changes in electrophysiology. Since MEF changes may be distributed across the heart wall or surface and are likely to be small in the rat heart given the short action potential duration, the size of the recording electrode could have a significant affect on our ability to observe stretch-induced changes in MAP morphology. In addition, the heterogeneous nature of the action potential across the heart wall and its propagation through the cells could make the electrode less likely to pick up small MEF events if it is recording from a considerably large population of cells.

With MAP electrodes, the active recording area remains the most likely reason since the recording electrode is in contact only with the surrounding solution on the surface of the heart and hence records electrophysiological activity less specific to the local activity of the electrode (Kondo *et al.*, 2004; Okamoto *et al.*, 2006). This, however, does not explain why others have observed MEF-induced changes in MAP signals

(Zabel *et al.*, 1996), although the longer duration of the rabbit MAP may alleviate some of these constraints.

Another distinct possibility for the present results is that the static volume/pressure loading used in the present series of experiments may have allowed adequate time for the myocardium to adapt to the load increase. A number of cellular studies suggest that SACs can inactivate during sustained stretch (Hu & Sachs, 1996; Bett & Sachs, 2000a) which may explain the present results. This, however, seems unlikely since others have found that NSACS remain in the open state for long periods of time during stretch without any sign of adaptation (Craelius *et al.*, 1988; Zeng *et al.*, 2000), and few cells actually display inactivation with sustained stretch (Hu & Sachs, 1996).

Of particular interest in the present study, and those that utilise MAP recordings for observing MEF responses is the suggestion that MAPs themselves are generated through the presence of stretch-activated ion channels (Knollmann *et al.*, 2002; Tranquillo *et al.*, 2004). Recent models propose that stretch or suction of the myocardial tissue activates NSACs allowing local tissue depolarisation, generating a potential boundary critical to the generation of the MAP (Knollmann *et al.*, 2002; Tranquillo *et al.*, 2004). Indeed , the application of MAP electrodes causes a maximum depolarisation of around -23 mV (Franz, 1999) which corresponds with the reversal potential of NSACs (Isenberg *et al.*, 2003) (Sasaki *et al.*, 1992; Hu & Sachs, 1996; Kamkin *et al.*, 2000a; Zeng *et al.*, 2000; Zhang *et al.*, 2000). Although depolarisation of tissue underlying the MAP signal is not a new theory (Franz, 1999; Knollmann *et al.*, 2002), if activation of NSACs are a requirement for generating a MAP, then use of pharmacological modulators such as streptomycin and gadolinium should, in theory, abolish the signal or at least significantly attenuate or distort it. This, however, is not the case nor has it ever been reported. More specifically, if NSACs do play a role in generation of the MAP signal then one would have to question the suitability of using MAP electrodes to monitor and demonstrate MEF in the heart. The only other possibilities are that MAP electrodes induce a localised ischemia due to the external application of pressure and its effects on coronary flow or much more localised tissue damage, the later suggesting that all MAPs are indeed injury potentials.

One of the major limitations with the suction electrode used in the present study was that deflections similar to QRS waves superimposed in the MAP signal were difficult to avoid (although it was possible with careful electrode placement). This is suggested to

result from the fact that any pair of electrodes placed on the heart will detect the potential difference associated with the activation of the entire heart (Hoffman *et al.*, 1959). By contrast, contact electrodes or paired electrodes where the recording and indifferent electrodes are positioned in close proximity to one another will suffer little from this problem since global variations in membrane potential will reach them closer in time and thus subtract from the final signal more efficiently. The suction electrode was chosen because the suction enables the electrode to maintain a constant contact angle perpendicular to the heart surface as well as constant contact of the recording electrode. This was ensured by suspending the electrode from its fine copper wire such that electrode weight was minimised and had negligible drag on the heart surface. This type of electrode has also been demonstrated to be relatively free of artifactual changes in MAP morphology (Babuty & Lab, 2001) and to correlate extremely well with transcellular recordings (Hoffman *et al.*, 1959). However, the suction cup itself limits the minimum distance between the two electrodes and thus enables a small amount of ECG-like artefact to appear in the MAP signal.

Summary

The only limitation of the present series of experiments is the cardiac surface area from which the suction electrode establishes a recording. It is possible that the sensitivity of the electrode was jeopardised by its size, resulting in the summation of electrical activity from a larger population of cells. This has the potential of "blurring" small changes in action potential repolarisation between cells from different areas of the epicardial surface and thus could mask any small MEF-induced changes in cardiac electrophysiology of the rat heart. However, the possibility that the suction electrode used could undermine the present results can be addressed by testing pharmacological agents known to alter the cardiac action potential by changing the rate of cardiomyocyte repolarisation. The main reason for using the suction electrode was the elimination of movement artefacts since the suction electrode and associated electrode were free to move with the epicardial surface in that it was light weight and suspended from the Langendorff setup.

CONCLUSION

In this chapter the effect of step-wise increases in end diastolic left ventricular volume over the range of 0 – 30 mmHg on MEF-induced changes to cardiac electrophysiology were examined in the rat heart. The first section described changes in the action potential (as recorded by the suction MAP electrode) as a result of both changes in coronary perfusion pressure and end diastolic volume. These changes in durations were recorded at three different levels of repolarisation (20, 50 and 80% repolarisation) and plotted against the respective end diastolic left ventricular pressure that provided the stretch. The results showed that contrary to popular belief, there were no significant differences in MAP durations between control and modest levels of left ventricular stretch (10-15 mmHg and 20-25 mmHg EDLVP) across a wide range of static coronary perfusion pressures. Equally as important was that variations in MAP amplitude had little or no effect on MAP durations, an observation supported by past research (Hoffman *et al.*, 1959).

The second section examined, briefly, the responses of four hearts to changes in EDLVP at a coronary perfusion pressure of 60 mmHg before and after stretch. It can be seen from the examples in this chapter that MEF appears to play little or no role in the normal physiological or functional range of the heart. Whilst this is more than possible with the advent of several plausible mechanisms having already been attributed to the explanation of the Frank-Starling response, several areas require further investigation. The suitability of the rat heart as a model for MEF induced reductions in APD as well as the ability of the suction MAP electrode to record these changes should be addressed. In addition, the possibility of adaptation of SACs thought responsible for MEF should be taken into account before concluding the absence of MEF under near-physiological conditions during the normal cardiac cycle.

CHAPTER 6: FURTHER INVESTIGATIONS OF MEF

American singer & dancer: Ginger Rogers
(July 16, 1911 – April 25, 1995)

Chapter 6 - Table of contents

INTRODUCTION

In this chapter some of the possible problems highlighted in the previous section are addressed. Structurally, this chapter is divided into two major components. The first component looks at addressing issues previously raised in the same animal model through the use of other techniques and pharmacological agents. The second component investigates the same Frank-Starling response in a different mammal, the guinea pig addressing the suitability of the rat as an appropriate model for demonstrating the contribution of MEF to normal cardiac physiology. The results in this chapter are consequently divided into two sections, those relating to MEF in the rat model, and those observations in the guinea pig. At the end of the chapter, the issues presented in the previous chapter are addressed and a further discussion of the current implementation of MEF in the mammalian heart discussed.

BACKGROUND

Although still not unequivocal, the ability of SACs to adapt to situations of stretch has been demonstrated (Hu & Sachs, 1996; Bett & Sachs, 2000a; Niu & Sachs, 2003). In addition to this observation, others have found that rapid changes in ventricular distension can modify MAP morphology (Stacy *et al.*, 1992; Zabel *et al.*, 1996) with the possibility of forming ectopic contractions suggesting SACs are more responsive to rapid changes in stretch (Franz *et al.*, 1992). However, it should be noted that the later was demonstrated in the rabbit and canine models and not the rat. With the observations of the previous chapter in mind, the experiments presented herein are aimed at addressing some of the valid experimental concerns that may have inhibited visualisation of MEF in the previous model. The first set of experiments focus on the ability of SACs to adapt to the slow stepwise increases in EDLVP used in the previous chapter. This issue was addressed by using a single step from control conditions (0 mmHg) to a final EDLVP of 20-25 mmHg instead of using small, individual steps. The second series of experiments address the suitability of the suction MAP electrode for measuring small changes in action potential duration in the rat heart. This was achieved through the used of 4-aminopyridine (4-AP), a potassium channel blocker know to delay repolarisation of the mammalian cardiac action potential (Wollmer *et al.*, 1981). Lastly, the third set of experiments deal with MEF in the guinea pig model. Since guinea pigs have a significantly longer action potential duration compared to the

rat, it was expected that small changes in APD would be detected and more easily measured.

METHODS

In the first set of experiments, 4 rat hearts were perfused as previously discussed in chapter 3 at a constant perfusion pressure of 60 mmHg with bicarbonate buffered solution containing 1.5 mM $CaCl_2$. In this set of experiments, however, unlike in the previous chapter, EDLVP was stepped between control (0-5 mmHg) and 20-25 mmHg in a single step whilst a suction MAP electrode monitored any electrophysiological changes (refer Figure 10 pg 57). In a separate series of experiments, rat hearts were paced at 5 Hz, EDLVP was kept constant at 0-5 mmHg, and the rat hearts perfused with the potassium channel blocker 4-amino pyridine (4-AP). 4-AP was prepared in a stock solution (100 mM) dissolved in milli-Q water before being added to the perfusate to form a final concentration of 10 μM. Hearts were paced slightly faster than previously as a lengthening in APD was the expected outcome.

Guinea pig hearts were prepared using the same protocols as used for rats with the exception that MEF induced changes in MAP morphology were tested for at only two coronary perfusion pressures (60 and 80 mmHg). In every other respect, the hearts were prepared as in chapter 3.

RESULTS

The first set of experiments addressed the possibility that SACs that underlie the MEF response may be able to adapt to slow changes in EDLVP. As such, the effect of a more rapid, single step in EDLVP from 0-5 mmHg to 20-25 mmHg was tested in three rat hearts perfused at a constant coronary perfusion pressure of 60 mmHg (see Figure 27, pg 97). Every other aspect of the experiments was the same as presented in the previous chapter.

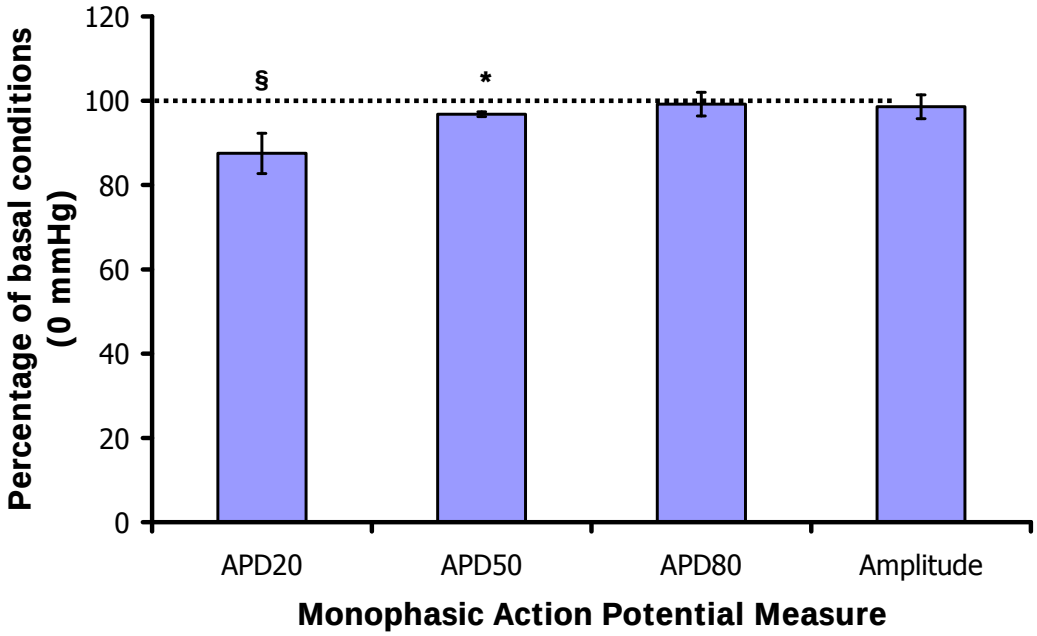

Figure 27: Effect of a Single Step in EDLVP on MAP Morphology

Figure showing the immediate effect of a single step increase in EDLVP on monophasic action potential durations at different levels of repolarisation and MAP amplitude in rat hearts. MAP durations at 20, 50and 80 % repolarisation at EDLVP 20-25 mmHg plotted as a percentage of basal conditions (close to zero EDLVP). Error bars indicate ± SEM, n = 4 hearts (each measurement in each heart is an average of 10 action potentials at each level of stretch). Under these conditions, APD_{20}, 80 and AMP amplitude did not change as a result of left ventricular stretch ($p > 0.05$). By contrast, APD_{50} decreased by 3 % upon stretch (denotes $p < 0.05$, § denotes $p = 0.059$, ANOVA GLM), this however, only accounted for at most a 0.6 ms decrease in action potential duration which was 20.3 ± 2.2 ms pre-stretch.*

To validate the MAP electrodes used throught experiment, the effect of exposing paced rat hearts to the potassium channel blocker 4-amino pyridine (4-AP) can be seen in Figure 28, pg 98. Here it can be seen that a low concentration of 4-AP promotoed a measureable reduction in MAP duration suggesting the MAP electrode is capable of detecting small electrophysiological changes associated with MEF.

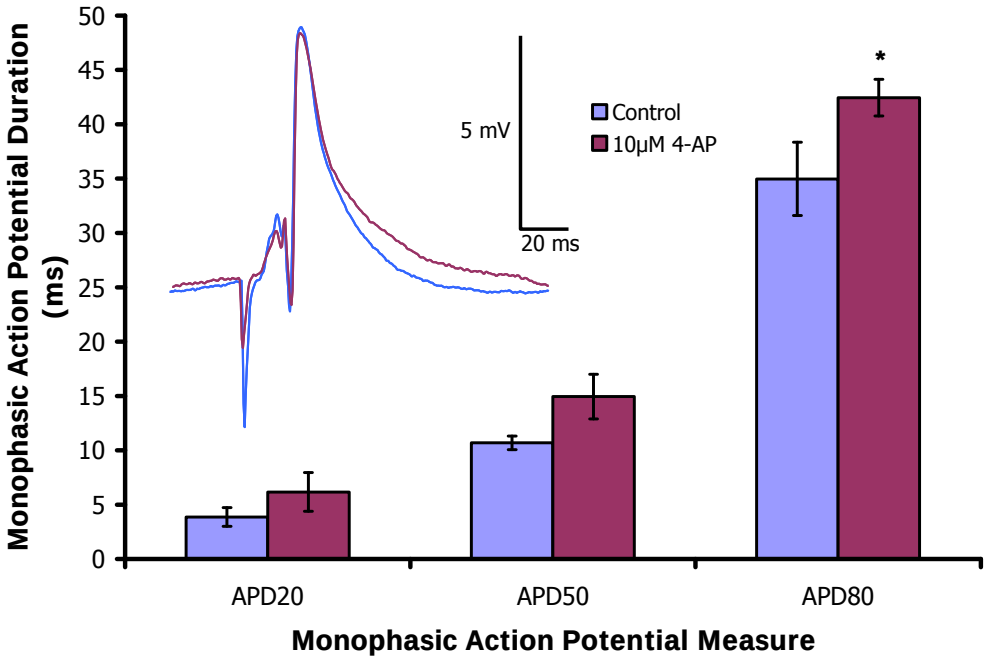

Figure 28: Effect of 4-AP on MAP Morphology

*Figure showing the ability of the suction MAP electrode to detect changes in action potential duration induced by 4-aminopyridine (4-AP, n= 4). Main Panel: Action potential duration measured by monophasic action potential recording in an isolated rat heart paced at 5 Hz at 20, 50 and 80 % repolarisation in control (blue bars) and in the presence of 10 μM 4-AP (purple bars). * indicates significant difference as determined by paired t-test ($p < 0.05$), error bars indicate ± SEM. Inset shows typical MAP traces superimposed for control and in the presence of 10 μM 4-AP. 4-AP significantly increased APD_{80} duration from 35 ± 3.4 ms under control conditions to 42 ± 1.7 ms.*

DISCUSSION

The above results provided little evidence as to the involvement of MEF in the length-tension relationship. Whist in the majority of cases MAP morphology did not change, the more rapid increases in EDLVP suggest that stretch effects may exist and be time dependent. Due to the variability in the amplitude between hearts and large variability at 20 % repolarisation, the decrease although becoming apparent, was not statistically significant. By contrast there was a small but statistically significant reduction in the APD at 50 % repolarisation (relating to ~0.6 ms, see Figure 27). Thus, the present results suggest that more rapid increases in EDLVP may result in action potential shortening. With this in mind, however, the physiological consequence of a 0.6 ms decrease in action potential duration is not known and would have to be tested. A

more comprehensive study would need to be conducted utilizing a larger number of animals to test the validity of the observed reduction.

In the second series of experiments involving the rat and the largest of the electrodes, the suction electrode, was used to observe 4-Aminopyridine (4-AP) induced changes in action potential duration in the isolated, Langendorff perfused rat heard. 4-AP is a known K^+ channel blocker and hence also blocks the repolarising current required for termination of the action potential. Previously others have used modest amounts of 4-AP (3 μM) to improve myocardial function following ischaemia with great success (Perchenet & Kreher, 1995). A concentration of 10 μM was used as concentrations above 20 μM generally resulted in unstoppable arrhythmias The results (Figure 28) clearly show that even with this low dose of 4-AP that there was a significant and detectable increase in MAP duration at 80 % repolarisation. This increase equated to an increase of APD_{80} by ~ 7 ms.

The 4-AP data support the previous use of the suction electrode and suggest several possibilities regarding SACs and the role of MEF in the normal cardiac cycle: (1) MEF is not strictly observable over physiological (10-15 mmHg) and sub pathological ranges of stretch tested in the present chapters (20-25 mmHg). Although this is a distinct possibility, the results presented earlier suggest that the duration and rate of stretch may also play an important role in MEF (2) The rat may not be an adequate model to study the involvement of MEF in the beat to beat regulation of contractility due to the brief nature of the rat action potential. This is quite possible, especially since the MAP electrodes used in the study were able to pick up electrophysiological changes associated with small doses of 4-AP. Lastly, (3) MEF may be small and variable in nature, thus not easily detected using the current techniques and model. Since little can be done about the brief nature of the rat action potential, the next series of experiments were based on the guinea pig Langendorff perfused heart which has an action potential many times longer than the rat. Due to the significantly different shape and duration of the guinea pig action potential compared to that of the rat, it is also likely that any MEF induced changes in the MAP should be significantly larger and more likely to outweigh random variations in the MEF-MAP response.

CONCLUSION

The present results suggest two distinct possibilities regarding earlier experiments into the function of MEF during the normal cardiac cycle and Frank-Starling relation. Firstly, the suction electrode used is more than capable of detecting small changes in action potential duration that may/may not occur with MEF responses induced by changes in EDLVP. Secondly, faster steps (and possibly larger steps) in EDLVP appear to induce a reduction in APD although this reduction was small (~0.6 ms at APD_{50}) or statistically insignificant but apparent. It remains possible that a cardiac model with longer action potential duration may provide a positive, measurable result.

MEF IN THE GUINEA PIG HEART

INTRODUCTION

This next section addresses the possibility that MEF only results in small changes to action potential duration that may not be clearly evident in the rat heart model due to the already brief nature of the action potential. MEF investigations in the guinea pig model have previously shown a reduction in APD_{50} and development of after depolarisations or ectopic beats in the atria (Nakagawa *et al.*, 1988; Nazir & Lab, 1996) or even hyperpolarisation of resting membrane potential with extreme stretch (Nakagawa *et al.*, 1988). Of the various methods used, stretch of guinea-pig cardiac tissue reduces action potential duration (White *et al.*, 1993; Wang *et al.*, 1996) whilst stretch of isolated guinea pig myocytes presented inconsistent results (Nakagawa *et al.*, 1988; White *et al.*, 1995; Belus & White, 2003).

As such, the present experiments are intended to investigate these responses using the same MAP electrodes used in the rat model.

METHODS (GUINEA PIGS)

Hearts from 8 guinea pigs of weight 500-680g (prepared as per chapter 3), paced at 4 Hz and underwent stretch via manipulations in EDLVP similar to that in rat experiments at one of two coronary perfusion pressures (60 and 80 mmHg). MAP recordings (although significantly more difficult to obtain) were analysed for changes in duration (APD 20, 50 and 80 % from repolarisation) upon stretch of the left ventricle. 10 action potentials were chosen for each EDLVP at random and the average calculated. Data presented are expressed as % basal conditions (0 mmHg) ± SEM to account for inter-guinea pig variability. Note that in experiments not involving SAC modulators that control and basal conditions are one of the same thing. Herein, however, control conditions will refer to an EDLVP of 0-5 mmHg in the absence of pharmacological modulators whilst basal conditions refer to an EDLVP of 0-5 mmHg in the presence of any drug.

RESULTS

Hearts perfused at a coronary perfusion pressure of 60 mmHg had a coronary flow rate of ~ 15 ml/min whilst those perfused at 80 mmHg displayed variable flow rates of 44 ± 7 ml/min (likely to reflect system inefficiencies rather than cannulation leakage around the aorta). Moreover, due to the indirect measurement of flow rate (based on pump speed/voltage as opposed to a true measure) this number is likely an over-estimation of the actual coronary flow. Due to the larger size and intra-ventricular volumes of the hearts, EDVLP was stepped up to the various final pressures in 5 mmHg steps (ie, more than a single step). Consistent with similar experiments in the Langendorff rat heart, little or no change in MAP duration was observed despite manipulation of EDLVP (see Figure 29 Figure 32).

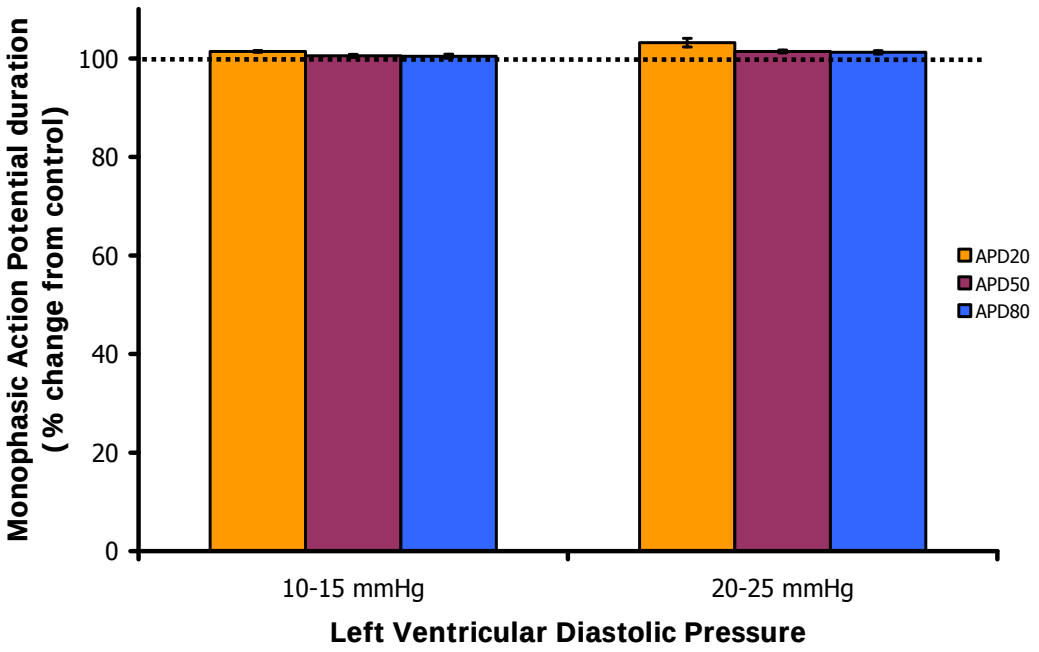

Figure 29: Effect of Changes in EDLVP on MAP Morphology in the Guinea Pig Isolated Perfused Heart (CP = 60 mmHg)

Figure showing the effect of stepwise increases in End Diastolic Left Ventricular Pressure (EDLVP) on Monophasic action potential durations at different levels of repolarisation in guinea pig hearts perfused at 60 mmHg MAP durations at 20, 50 and 80% repolarisation at EDLVP 20-25 mmHg plotted as a percentage of basal conditions (close to zero EDLVP). Error bars indicate ± SEM, n = 4 hearts (each measurement in each heart is an average of 10 action potentials at each level of stretch). Under these conditions, APD_{20}, 50 and 80 changed by less than 1 % from basal conditions. By contrast, increasing EDLVP to 20-25 mmHg significantly decreased MAP amplitude ($p < 0.05$, ANOVA GLM).

At a static coronary perfusion pressure of 60 mmHg, increasing EDLVP to 10-15 and 20-25 mmHg significantly reduced MAP amplitude by 8 ±2.8 and 11 ±3.4 % respectively (a 0.7 mV reduction). Changes in APD 20 and 50 were < 1 % from control. These effects were not observed at the higher coronary perfusion pressure of 80 mmHg.

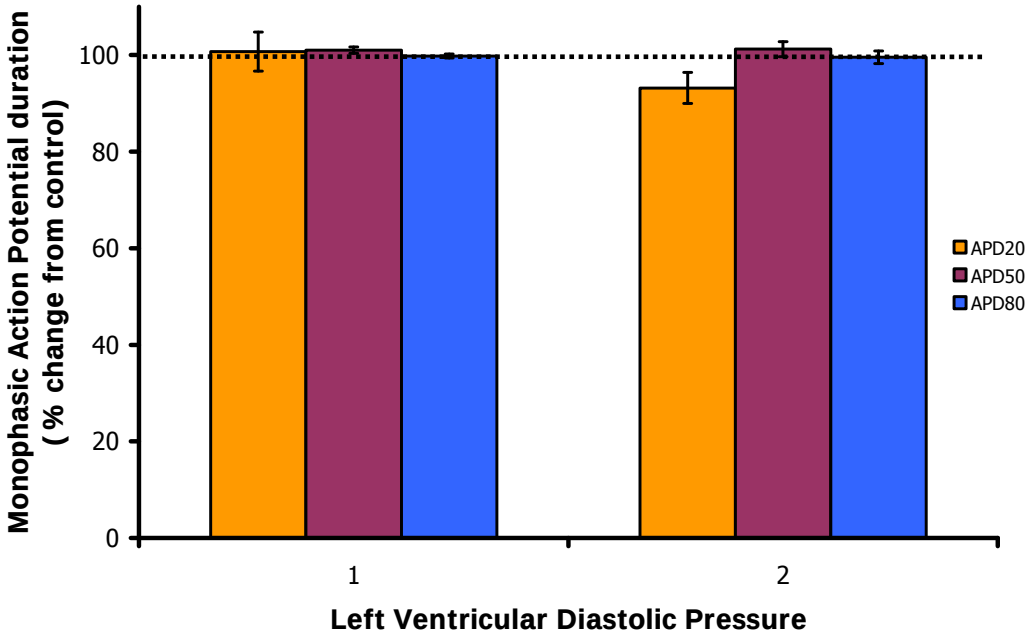

Figure 30: Effect of Changes in EDLVP on MAP Morphology in the Guinea Pig Isolated Perfused Heart (CP = 80 mmHg)

Figure showing the effect of stepwise increases in End Diastolic Left Ventricular Pressure (EDLVP) on Monophasic action potential durations at different levels of repolarisation in guinea pig hearts perfused at 80 mmHg. MAP durations at 20, 50 and 80% repolarisation plotted for basal (close to zero EDLVP), EDLVP = 10-15 mmHg and 20-25 mmHg. Error bars indicate ± SEM, n = 4 hearts (each measurement in each heart is an average of at least 10 action potentials at each level of stretch). Changes in EDLVP did not significantly alter MAP durations at any of the levels of repolarisation tested (p > 0.05, ANOVA GLM). Data presented as % of basal conditions (EDLVP = 0-5 mmHg, no stretch). There are no significant differences in MAP durations between basal conditions and any level of stretch.

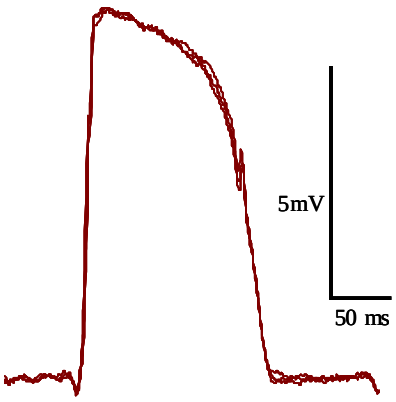

Figure 31 shows Guinea pig MAPs recorded at end diastolic left ventricular pressures (EDLVP) of 0, 25 and 0 (re-control) mmHg shown superimposed. Heart was perfused at a constant coronary pressure.

Figure 31: Guinea Pig MAPs superimposed

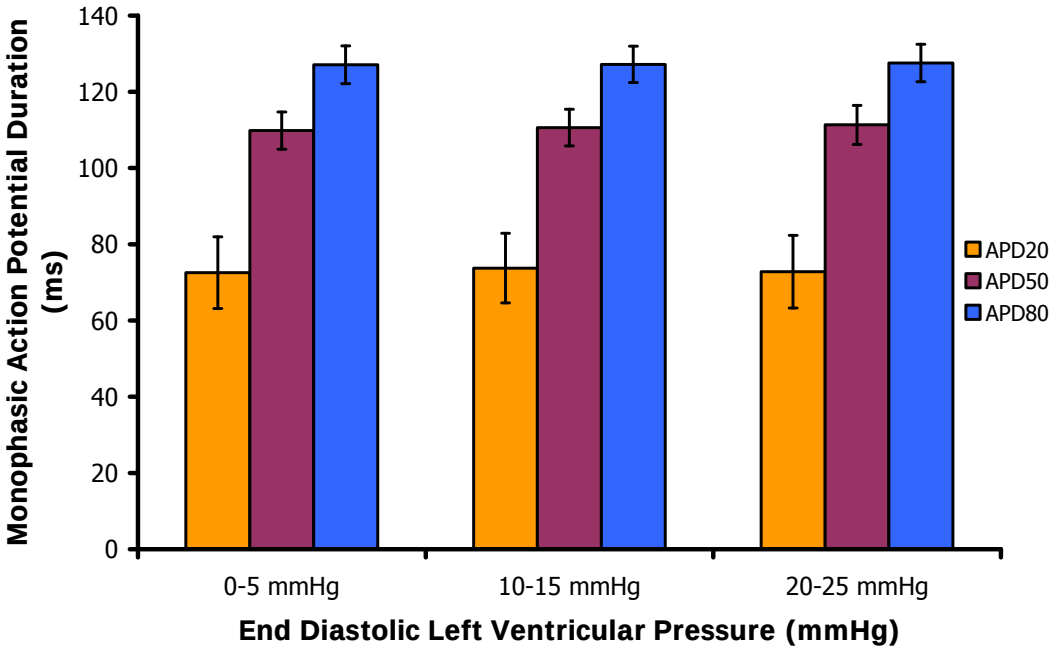

Figure 32: Effect of Changes in EDLVP on Guinea Pig MAP Morphology

Combined results - effect of increasing End Diastolic Left Ventricular Pressure (EDLVP) on monophasic action potential durations at different levels of repolarisation in guinea pig hearts. MAP durations at 20, 50 and 80 % repolarisation plotted for basal (close to zero EDLVP), EDLVP 10-15 mmHg and 20-25 mmHg. Error bars indicate ± SEM, n = 8 hearts (each measurement in each heart is an average of at least 10 action potentials at each level of stretch). There are no significant differences in MAP durations between basal and stretch conditions ($p > 0.05$, ANOVA GLM).

Effective Refractory periods were also tested using a Ventritex high voltage stimulator (Ventritex HVS, USA) for several of the 8 guinea pigs to ensure that the absence of change in action potential duration were not artificial or masked by inadequacies of the MAP electrode. However, difficulty was encountered as the device (intended for human use) was unsuitable for determining ERPs in the guinea pig heart because it was incapable of directly pacing the heart at a reasonable basal rate (3-5 Hz) between ERP tests to maintain consistency with previous experimental conditions. As a result, hearts were paced independently by a second stimulator and ectopic activity was often evoked at the start of ERP runs resulting in unstable/inconsistent ERP values (not presented). Only one heart paced at 3 Hz gave consistent results when the programmable stimulator of the defibrillator was allowed to assume control during ERP testing. Under control conditions, the refractory period of the guinea pig when stimulated with a run of 6 beats at 200 ms intervals (S1) was 145 ms with a threshold

of 1.8 V Stretching the Left Ventricle to 20-25 mmHg increased the threshold for pacing to 3.0 V whilst the effective refractory period remained at 145 ms (see Figure 33).

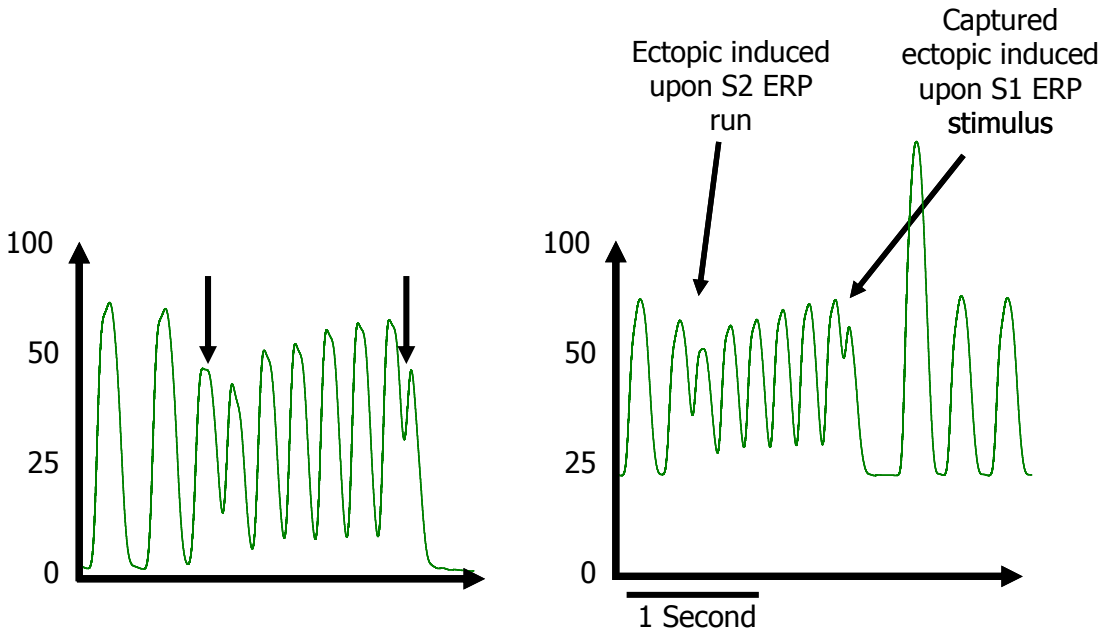

Figure 33: Testing Refractory Periods During Stretch

Testing of effective refractory periods in Langendorff perfused guinea pig heart. ERP testing was initiated by a train of pulses (S1) 200 ms apart followed by an early beat (145 ms, S2) before, during and after the heart was stretched to 20-25 mmHg. Effective refractory period did not change during or following stretch. Stretch increased pacing threshold from 1.8 to 3.0 volts. The heart was paced at 3 Hz between bursts (see text, note, 200 ms = 5 Hz). Y Axis shows left ventricular pressure, x-axis shows time

DISCUSSION

General consensus in the literature suggests that stretch of myocardial tissue causes cellular depolarisation, a reduction in action potential amplitude and a shortening of the duration (Franz, 1996; Taggart, 1996; Hu & Sachs, 1997). By contrast, other observations have been made namely crossover of repolarisation combined with action potential lengthening (Nilius & Boldt, 1980; Tavi *et al.*, 1998).

With this in mind, it might be expected that similar results would be obtained in an animal model with longer action potential durations if not in the rat. However, the

results presented above (refer to Figure 29 - Figure 32, pgs 102-104) clearly demonstrate the absence of any significant stretch-induced effect along EDLVPs associated with the Frank-Starling mechanism. In addition, even if the chosen electrodes were unable to detect the proposed shortening as suggested earlier, a decrease in the ERPs should have still been evident since a reduction in refractory period is associated with reductions in APD. In fact, no obvious reduction was observed although it is accepted that this could reflect technical issues. At the same time, it was observed that more rapid changes in EDLVP failed to adequately produce observable MEF affects in the Langendorff perfused rat heart. This is reflected by the observation that APD_{50} reduced by only 0.6 ms whilst no other measurable change was present in APD_{20} or 80 as would be expected from the literature. In fact, further investigation of similar conditions in the guinea pig heart failed to reveal obvious, measurable MEF changes in Langendorff perfused heart electrophysiology as detected by MAP electrodes.

Initially with the discovery of SACs in the heart it was proposed that these stretch-modulated ion channels would be involved in the beat-to-beat regulation of cardiac contractility and hence may be involved in inotropic effects such as those described by the Frank-Starling relationship (Lab, 1996; Vemuri *et al.*, 1999). However, it would appear unlikely that either rapid changes in the intracellular environment through activation of SACs or changes in calcium handling are likely to explain the changes in contractility under the semi-physiological conditions explored here. This suggests that the dominant mechanism involved in the Frank-Starling mechanism is mechanical and not electro-chemical or electro-mechanical in nature.

Whilst the present results give no definitive mechanism to the absence of MEF in the present studies, there remain only a few possibilities to explain the present results. Firstly and foremost, as mentioned, is that the Frank-Starling effect is primarily a mechanical response of the contractile apparatus to stretch and independent of any biochemical and electrochemical changes (Fuchs & Wang, 1996; Kentish & Wrzosek, 1998; Irving *et al.*, 2000; Fukuda *et al.*, 2001). This is supported by studies that fail to show alterations in the Ca^{2+}-transient immediately upon stretch (White *et al.*, 1993; White *et al.*, 1995; Hongo *et al.*, 1996) and in the present study, the absence of any obvious electrophysiological changes not to mention the delay in activation of SAC currents described elsewhere (Bett & Sachs, 2000b). These changes in contractility are likely to involve alterations in cellular kinetics including those involving the

elastomer protein Titin (Fukuda *et al.*, 2001; Fukuda *et al.*, 2003; Fukuda & Granzier, 2004).

Other reasons for the absence of MEF may involve time dependent rundown of SAC currents, although this seems unlikely given the lack of response in rat hearts exposed to faster changes in EDLVP. It is also possible that SACs are unequally distributed across the heart as was demonstrated for TREK-1 where by greater expression of TREK-1 stretch sensitive channels was observed in the endocardium of the rat heart (Tan *et al.*, 2004). In addition, typical current sizes reported to pass through SACs are considerably small and thus any rundown or adaptation is likely to mask or counteract any possibility of observing MEF induced changes in cardiac electrophysiology in the present model. It remains possible (although untested for NSACs) that there is a heterogenous distribution of NSACs channels across the ventricular wall representative of differences in myocardial stress or that stretch-induced APD shortening is actually due to the presence of TREK-1 channels (KSACs) and not NSACs as reported. As such, MEF may also be heterogeneous across the left ventricular wall. According to (Tan *et al.*, 2004), TREK-1 KSACs were expressed in greater quantities in the endocardial tissue of the rat than in the epicardial regions. This could explain the present results since the methods utilised involved surface recordings from the epicardial tissue with light levels of suction applied by the MAP electrode. This, however, does not explain why others have successfully observed MEF unless greater forces were applied during MAP recordings inducing deeper depolarisation and access to endocardial currents (Hansen, 1993; Zabel *et al.*, 1996; Takagi *et al.*, 1999; Eckardt *et al.*, 2000; Babuty & Lab, 2001). It remains possible that other electrode combinations (transmural, contact) may allow better observation of MEF through access to sup-epicardial or endocardial electrophysiological changes deeper in the tissue.

CONCLUSION

With the above results in mind, it remains to be explained why others have been able to demonstrate stretch induced ectopic beats in the rabbit and canine models (Franz *et al.*, 1992; Stacy *et al.*, 1992; Zabel *et al.*, 1996) or changes in action potential durations both *in vitro* (Nilius & Boldt, 1980; Stacy *et al.*, 1992; Ravelli & Allessie, 1997; Salmon *et al.*, 1997; Kiseleva *et al.*, 2000), *in vivo* (Levine *et al.*, 1988; Eckardt *et al.*, 2001; Garan *et al.*, 2005) and at the cellular level (Akay & Craelius, 1993; Craelius, 1993; White *et al.*, 1993; Suchyna *et al.*, 2000; Kamkin *et al.*, 2003c). No

obvious changes in guinea pig MAP were observed during stretch despite the fact that the MAP electrode used was able to pick up small changes in MAP duration in the rat induced by 4-AP. Moreover, it would appear that rapid stretch may be able to invoke MAP changes, although this requires further investigation. Others have demonstrated on a consistent basis that stretch *in vivo* also induces alterations in electrophysiology (Taggart & Sutton, 1999; Garan *et al.*, 2005), and rapid timed stretches can induce diastolic depolarisations and ectopic activity (Zabel *et al.*, 1996). As such, a final attempt was made to demonstrate the presence of MEF in the rat heart through rapidly induced inflations of the rat ventricle. It should be noted with great interest at this point that MEF in the whole heart is regularly observed when physically preparing the Langendorff heart. As reported by others, cardiac MEF is observed as a side-effect of handling Langendorff hearts during cannulation procedures, often stopping or restarting rhythmic contractions upon touch (Kohl *et al.*, 2006).

MEF IN THE RAT HEART

INTRODUCTION

Others have successfully shown that rapid stretch can elicit ectopic activity in the heart (Franz *et al.*, 1992; Stacy *et al.*, 1992; Zabel *et al.*, 1996), indeed, manipulations of the heart during setup of the Langendorff parameters readily induces ectopic activity as (see Figure 3 pg 42). Thus, some form of MEF must exist. The last section of this chapter is dedicated to demonstrating the ability of rapid stretch to induce ectopic beats in the rat heart through MEF mechanisms.

METHODS

In the last series of experiments to complete this chapter, rapid changes in EDLVP were applied to Langendorff perfused rat hearts using a very course means of a 1 ml fluid filled disposable syringe connected to the intra-left ventricular balloon and pressure transducer setup (described earlier) by means of a 3 way tap. At random intervals and for short or long durations, EDLVP was rapidly increased from 0-5 mmHg to values of between 20-100 mmHg (see Figure 34, Figure 35). Because of the crude setup, it was not possible to time the point of inflation with the cardiac cycle nor the EDLVP reached as a result of the rapid inflation.

RESULTS

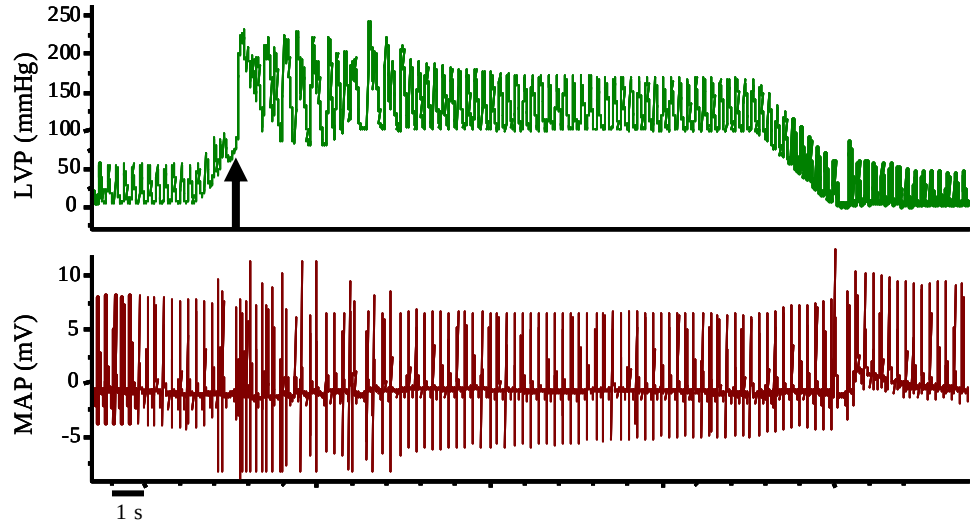

Figure 34: Rapid Stretch Induced Ectopic Beats

Figure shows ectopic beats evoked in isolated rat heart by rapid stretch. Top trace; intraventricular pressure, lower trace; MAP recorded from the epicardial surface. Heart paced at 4 Hz. Increase in EDLVP from near 0 to ≈100 mmHg over 2 to 3 seconds produced a series of ectopic beats.

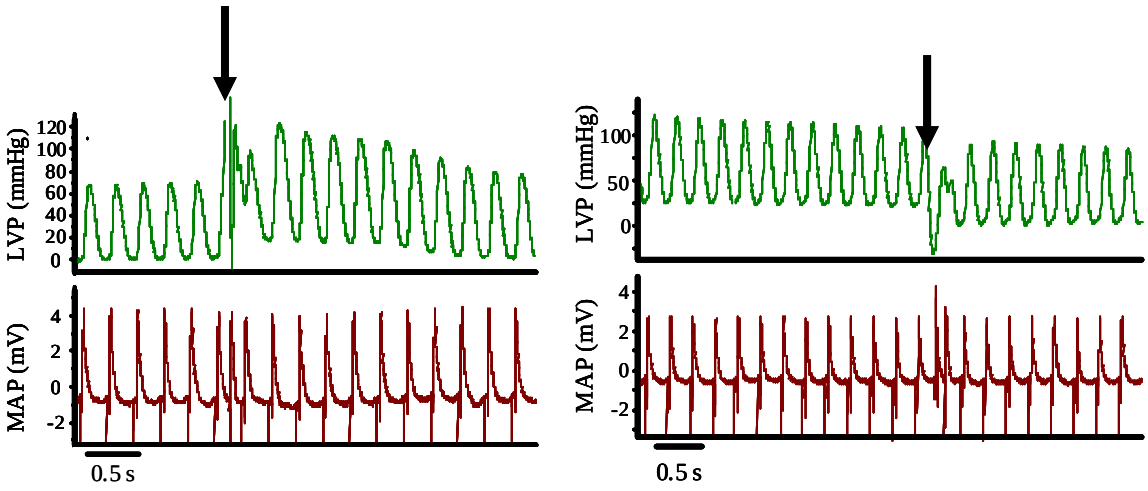

Figure 35: Rapid Inflation and Deflation Induced Ectopic Beats

Panel A: effect of a sudden increase in EDLVP pressure from near zero too 20 mmHg. Upper trace; ventricular pressure, lower trace is MAP recording from surface electrode. Pressure was increased and at the arrow. Panel B: Effect of sudden release of stretch (at arrow) from an EDLVP of 25 mmHg to near 0 mmHg also resulting in an ectopic beat.

DISCUSSION

In isolated rat hearts using the coarse method outline above, MEF in the form of stretch-induced ectopic beats was readily demonstrated through rapid increases in EDLVP via the 1 ml fluid filled syringe attached to the left ventricular balloon. Inflating the balloon from 0-5 mmHg to 100 mmHg or more within seconds would result in reproducible runs of ectopic beats lasting up to 10 seconds (see Figure 34), after which the rhythm returned to normal. By contrast, a sudden increase in EDLVP from 0-5 mmHg to ~20-25 mmHg within a beat or two (randomly in the cardiac cycle) would induce in most cases a single ectopic beat (see Figure 35, left panel). Similarly, a sudden decrease in diastolic pressure from 20-25 mmHg to near zero also resulted in an observable ectopic (see Figure 35, right panel). However, smaller increases in pressure, regardless of how sudden, consistently failed to elicit ectopic beats.

The present results show that in the isolated Langendorff perfused heart it is difficult to convincingly demonstrate MEF using slow or modest changes in EDLVP comparable to those normally encountered in the myocardium. In many of the cases presented, moderate levels of stretch over a reasonably short period induced little or no change in the action potential as depicted by the MAP recording from either rat or guinea pig isolated perfused hearts. This result, although negative, closely reflects many of the inconsistencies presented in the literature; with some studies presenting a lengthening of action potential with stretch whilst others report a shortening or no MEF effect (Akay & Craelius, 1993; Franz, 1996; Hu & Sachs, 1996; Lab *et al.*, 1996; Hu & Sachs, 1997; Bett & Sachs, 2000b, a; Franz, 2000; Zeng *et al.*, 2000; Kohl & Sachs, 2001) (see section titled: Non-Selective Stretch-Activated Cation Channels (NSACs) Pg 17)

The lack of reproducible MEF at what can be considered "physiological" levels of stretch is a confounding result, since there is no doubt from rapid stretch experiments (past and present) that changes in cardiac electrophysiology in the form of ectopic beats and action potentials are indeed produced. Moreover, it is widely accepted that stretch activated channels are present in the membrane of both ventricular and atrial cardiac myocytes (Sackin, 1995b, a; Lab, 1996; Hu & Sachs, 1997; Lab, 1999; Tan *et al.*, 2004) (although not yet demonstrated at the single channel level in the adult rat ventricular myocyte). This failure to demonstrate MEF in our experiments may be explained by the observation that it appears to be necessary to rapidly stretch the myocardium in order to elicit obvious changes in the action potential or to produce

ectopic beats (see Figure 34 and Figure 35 Pg 109). This suggests that the rate of stretch is an important determinant factor in MEF responses, and likely related to the visco-elastic properties of the myocardium, or perhaps due to MEF being elicited by sheer stress between planes of muscle fibres (Legrice *et al.*, 1997). More intriguingly, ectopic beats can be elicited by rapid release of the myocardium from stretch (see Figure 35). This may be related to the dissociation of calcium from the myofilaments, due to the tension-dependence of the affinity of troponin for calcium (McDonald *et al.*, 1995; Tavi *et al.*, 1998; Wang & Kerrick, 2002), the consequent rise in intracellular calcium accelerating sodium-calcium exchange or other electrogenic calcium dependent processes which in turn result in after depolarisations (Lab, 1999). If this change in calcium handling is indeed responsible for generating ectopic beats, it is not prominent enough at physiological levels of stretch to alter action potential morphology.

SUMMARY

In this chapter, the possible role of MEF in the normal cardiac cycle in the rat model was further investigated. Initially several issues that were raised from the previous chapters were addressed and the ability to induce ectopic beats was informally examined. The chapter began with an examination of the method of increasing EDLVP used. It was found that even single, larger and hence, faster steps in EDLVP failed to reproducibly induce MEF changes in the MAP. The same electrode was then tested for its ability to detect changes in cardiac electrophysiology by altering the cardiac action potential using the K^+-channel blocker 4-AP.

It was shown that modest increases in action potential duration induced by 4-AP were observable with the suction MAP electrode. The next section then demonstrated that the notable absence of MEF at "physiological" levels of stretch was not solely limited to the rat Langendorff perfused hear model. Finally, the chapter briefly examined the ability to induce ectopic beats with a single rapid increase in EDLVP.

CONCLUSION

The present results fail to demonstrate MEF in the physiological range of EDLVPs tested during the normal cardiac cycle. This may be because the stimulus used (moderate, relatively slow stretch) is neither appropriate nor strong enough or rather MEF responds to the rate of change of myocardial stretch and not the degree of stretch *per se*, as suggested by others (Calkins *et al.*, 1989). In addition, as

mentioned previously, it still remains possible that MEF induced effects may be heterogeneous across the ventricular wall, a likely result of heterogeneous expression of KSACs (Tan *et al.*, 2004). Another problem may be that our current electrode configuration detects a too larger area and dispersion of repolarisation may be reducing our ability to detect modest changes in APD induced by MEF. Likewise, the method used is only able to detect changes in action potentials from the epicardial surface of the heart, where MEF may be greatest in the endocardial tissues.

CHAPTER 7: MEF IN SUB-EPICARDIAL AND ENDOCARDIAL MYOCARDIAL LAYERS

WWII British singer & comedian: Gracie Fields
(Jan 9, 1898– Sept 27, 1979)

Chapter 7

Chapter 7 - Table of contents

INTRODUCTION

In this chapter, the Langendorff perfused rat heart model and the implementation of the epicrdial-endocardial transmural MAP electrode are examined. The first section provides a general overview to the experiments and describes the possible involvement of the various SACs types in MEF namely NSACs and KSACs such as TREK-1. The second section is composed of the experiments used to investigate the contribution of SACs to MEF. The third section applies similar techniques to the Langendorff perfused guinea pig heart. The final section examines each approach and examines the outcomes. This chapter will form the basis of examining the contribution of the various SACs to MEF in the rat heart and looks for a common mechanism or justification of the outcomes based on present SAC theory.

BACKGROUND

Stretch activated ion channels were first demonstrated in neonatal ventricular myocytes in 1988 (Craelius *et al.*, 1988) and explain mechanically-induced electrical responses (MEF) in isolated cardiomyocytes as well as in the whole heart. The open probability/current of these channels in membrane patches directly reflects changes in membrane stretch (Sigurdson *et al.*, 1992; Kawakubo *et al.*, 1999). As mentioned previously, two types are known to exist in the rat heart namely non-selective (NSACs) and K^+ selective (KSACs) stretch activated channels.

KSACs are likely due to the presence of TREK-1 channels found in cardiomyocytes. Of interest is the fact that these channels are expressed heterogeneously across the ventricular wall (Tan *et al.*, 2004; Stones *et al.*, 2007) with more channels found in the endocardium. As a result, variations in wall stress and strain combined with differences in TREK-1 channel expression may be responsible for different MEF responses across the heart wall (Takagi *et al.*, 1999). It is therefore hypothesised that stretch may induce electrophysiological responses more readily in endocardial layers than in epicardial layers due to the possibility of a greater presence of TREK-1 channels (Tan *et al.*, 2004). Although based on the assumption that mRNA levels reflect channel density, from single channel recording experiments conducted on endocardial and epicardial cells in the lab previously, TREK-1 channels were always more readily found in cells isolated from the left ventricular endocardium than epicardium suggesting a greater density (not shown).

The involvement of SACs in Beat-to-Beat Regulation

Due to the different effects of SAC activation by stretch throughout the cardiac cycle, it remains anticipated that MEF plays an important role in the beat-to-beat regulation of the heart (Lab, 1999) (although this has been largely discounted in the previous chapters). During the cardiac cycle, normal contraction of the intact ventricle is made possible by individual cardiomyocytes contracting in an almost synchronous manner. SACs may play a role in coordinating the cardiomyocytes of the ventricle to contract at the appropriate time and evenly distribute ventricular wall stress (Lab, 1999). Since TREK-1 channels and their respective ion current have been shown to be heterogeneously expressed across the heart wall (Tan *et al.*, 2004), the present series of experiments investigates the possibility of differential MEF responses between endocardial and epicardial cells across the heart wall. In addition, since previous experiments have shown that MEF plays little role at near-physiological EDLVP in epicardial cells, this series of experiments uses a wider range of pressures (up to 50-55 mmHg EDLVP) and the use of a transmural MAP electrode capable of detecting endocardial MAPs to further investigate MEF in the mammalian heart. Of considerable importance in this series of experiments is the understanding that transmural MAP electrodes are more accurate recordings of local activity than the suction electrode, and thus may be better suited to establishing MEF effects (Kondo *et al.*, 2004). In addition, the present chapter will also present data regarding possible modulation of MEF responses through the use of SAC modulators.

METHODS

Rat hearts were prepared as per chapter 3. Hearts were paced at 5 Hz and perfused with bicarbonate buffer containing 1.5 mM $CaCl_2$. Drugs were prepared as stock solutions and aliquoted out to obtain the final concentrations. Streptomycin was used at a concentration of 80 μM as an inhibitor of NSACs as discussed in chapter 2 previously. Riluzole (30 μM) was used as an activator of TREK-1 channels (Duprat *et al.*, 2000) although it is known to inhibit PKC at low concentrations in cortical cells (Noh *et al.*, 2000) and facilitate activation of neuronal SK channels by Ca^{2+} in HEK293 cells (Cao *et al.*, 2002). Chlorpromazine (10 μM) was used as an inhibitor (Patel *et al.*, 1998), whilst in later follow-up experiments, glibenclamide (10 μM) was used to inhibit K_{ATP} channel activation (Han *et al.*, 1996). These concentrations were based on

preliminary experinents demonstrating little or no effect of each respective drug on basal contractility (data not shown) in conjunction with the cited material.

Streptomycin sulphate (ICN) and chlorpromazine (sigma) were prepared in milli-Q water at stock concentrations of 100 and 10 mM respectively. Riluzole (sigma) and glibenclamide (ICN) were prepared in DMSO at a concentration of 100 and 10 mM. Drugs were added to the perfusate solution following control conditions so as not to induce variation from slight differences in perfusate buffer composition. The final DMSO concentrations in perfusate solutions (less than 0.03%) were below that which produces any observable effects.

RESULTS

Effect of stretch on sub-epicardial and endocardial MAPs

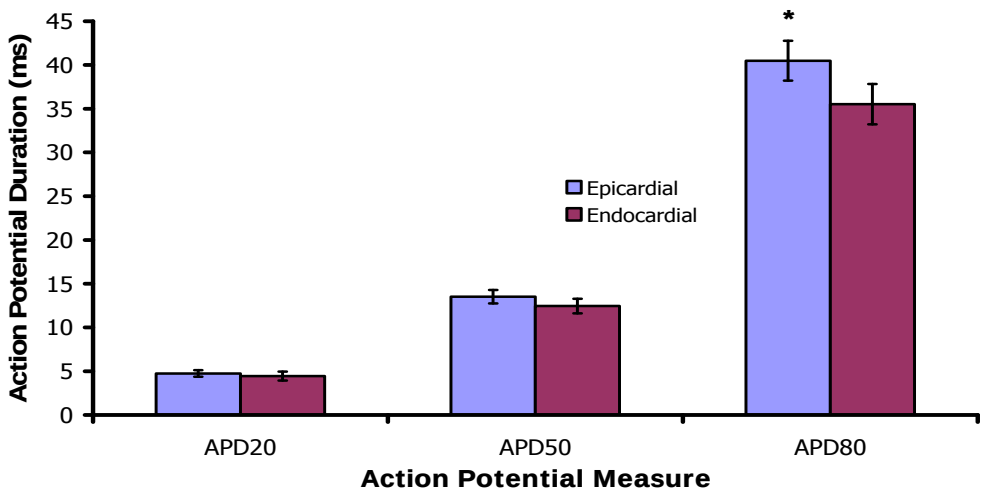

Figure 36: Average Endocardial and Sub-epicardial MAP Durations

Figure showing average sub-epicardial and endocardial monophasic action potential (MAP) durations at 20, 50 and 80% repolarisation recorded under basal conditions (End Diastolic Left Ventricular Pressure, EDLVP < 5 mmHg, for n = 10 hearts). Error bars indicate ± SEM, n = 10 hearts (each measurement in each heart is an average of 10 action potentials). In all cases, sub-epicardial monophasic action potentials at 80% repolarisation were found to be statistically longer than endocardial action potentials (denotes p < 0.05, ANOVA, GLM). By contrast, durations at 20 and 50 % repolarisation were no different between sub-epicardial and endocardial MAPs.*

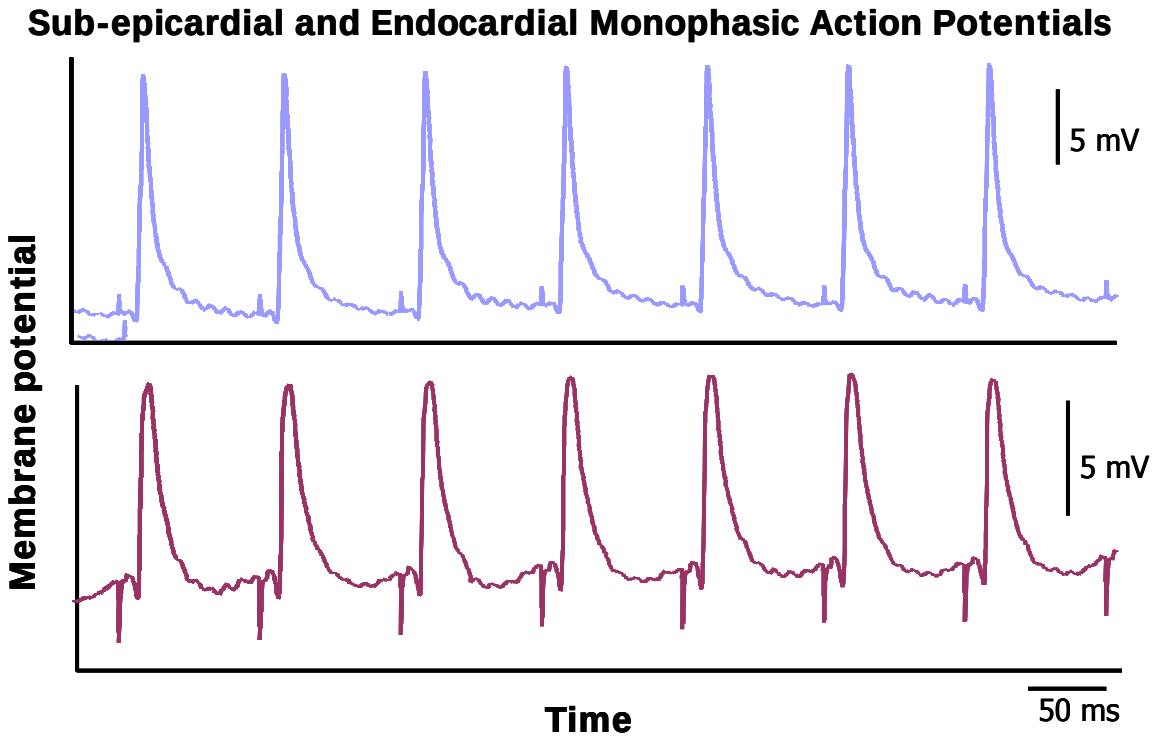

Figure 37: Endocardial and Sub-epicardial MAP Recordings

Raw data recorded by transmural electrode showing endocardial (blue) and sub-epicardial (burgundy) monophasic action potentials at an end diastolic left ventricular pressure of < 5 mmHg (control conditions).

MAPs recorded using the transmural method generally had much higher amplitudes compared with the other MAP electrode techniques. Sub-epicardial MAP amplitudes in control experiments were in the order of 16 ± 2.2 mV under basal conditions (no stretch) whilst endocardial MAPs were on average 17 ± 2.1 mV in amplitude (not statistically different).

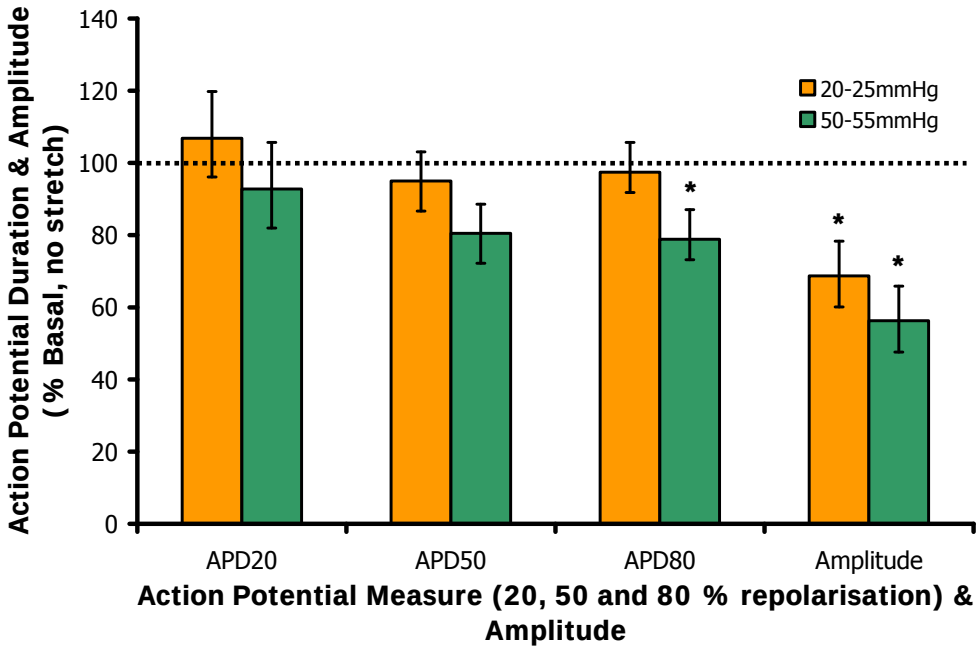

Figure 38: Effect of EDLVP on Sub-epicardial MAP Morphology

Figure showing the effect of increasing Left Ventricular End Diastolic Pressure on Sub-epicardial Monophasic action potential durations at 20, 50 and 80 % repolarisation in rat hearts (n= 10). MAP durations at various points of repolarisation are plotted for EDLVP = 20-25 mmHg and 50-55 mmHg (expressed as a percentage of results obtained for basal, 0-5 mmHg EDLVP). Error bars indicate ± SEM, n = 10 hearts (each measurement in each heart is an average of at least 10 action potentials at each level of stretch). There were no significant differences in MAP durations between basal and 20-25 mmHg EDLVP for sub-epicardial MAPs at 20 or 50 % repolarisation. By contrast, extreme stretch (EDLVP = 50-55 mmHg) significantly reduced epicardial MAP durations to 79 ± 5.6 % at 80% repolarisation. In addition, the period during stretch was associated with a significant reduction in MAP amplitude (denotes $p < 0.05$ relative to basal conditions, ANOVA GLM followed with Tukey's pair wise analysis).*

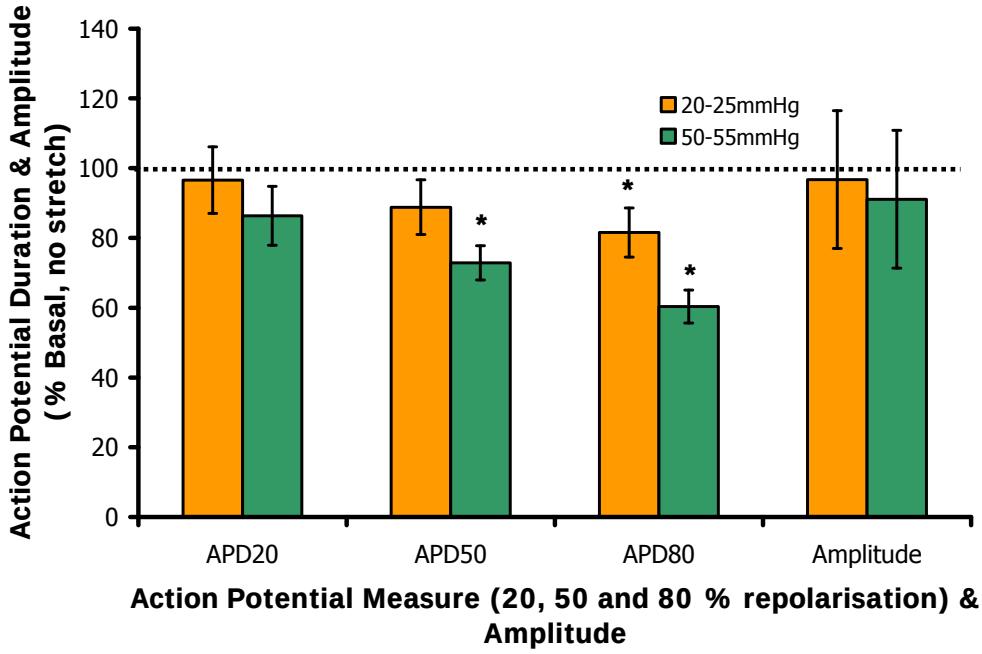

Figure 39: Effect of EDLVP on Endocardial MAP Morphology

Figure showing the effect of increasing Left Ventricular End Diastolic Pressure (EDLVP) on Endocardial Monophasic action potential durations at 20, 50 and 80 % repolarisation in rat hearts (n= 10). MAP durations at various points of repolarisation are plotted for EDLVP = 20-25 mmHg and 50-55 mmHg (expressed as a percentage of results obtained for basal, 0-5 mmHg EDLVP). Error bars indicate ± SEM, n = 10 hearts (each measurement in each heart is an average of at least 10 action potentials at each level of stretch). There were no significant differences in MAP durations between basal and moderate stretch (20-25 mmHg EDLVP) for Endocardial MAPs at 20 and 50% repolarisation, whilst APD_{80} was significantly shortened. By Contrast, extreme stretch (EDLVP = 50-55 mmHg) significantly reduced endocardial MAP at all measures of repolarisation and to a greater extent than moderate stretch for APD_{80}. MAP amplitude remained unchanged (although there was significant variability between results). Endocardial MAPs at 80 % repolarisation decreased to 82 ± 7.1 % of basal conditions (no stretch) with moderate stretch and to 60 ± 4.7 % with extreme stretch. (denotes $p < 0.05$ relative to basal conditions, ANOVA GLM followed with Tukey's pair wise analysis).*

In general, moderate stretch (EDLVP = 20-25 mmHg) reduced MAP APD_{80} from 36 ± 2.3 to 30 ± 3.7 in endocardial layers whilst extreme stretch (EDLVP = 50-55 mmHg) reduced MAP APD_{80} further to 22 ± 2.3 for endocardial ($p < 0.05$, ANOVA GLM with Tukey's post hoc test) and from 40 ± 2.3 ms to 32 ± 3.1 ms in sup-epicardial layers. Thus, extreme stretch decreased endocardial APD_{80} by ~39 % (14 ms) compared to the 20 % (8 ms) reduction seen in sub-epicardial layers (Figure 36 -Figure 39).

Following experiments exposed Langendorff perfused rat hearts to the SAC modulators Chlorpromazine (TREK-1/KSAC inhibitor, n= 6), Riluzole (TREK-1/KSAC activator, n = 7) or Streptomycin (NSAC inhibitor, n = 5) during manipulations of EDLVP.

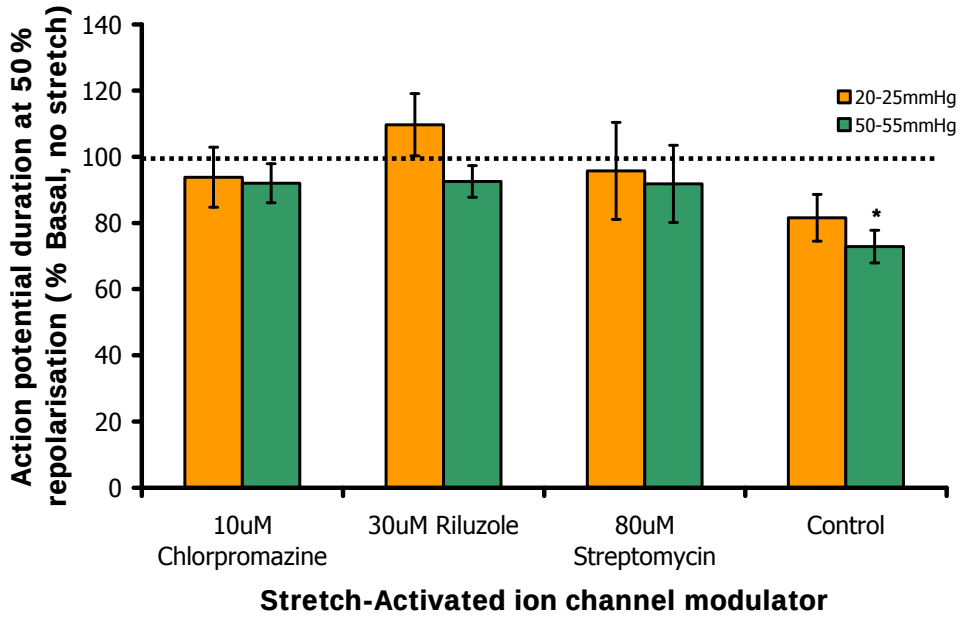

Figure 40: Effect of Stretch on Endocardial MAP APD_{50}

Figure showing effect of SAC modulators (Chlorpromazine TREK-1 inhibitor (n= 6), Riluzole TREK-1 activator (n= 7) and streptomycin NSAC inhibitor (n= 5)) on endocardial monophasic action potential durations at 50 % repolarisation in rat hearts compared to control. MAP durations at EDLVP = 20-25 and 50-55 mmHg plotted as a percentage of control conditions (close to zero EDLVP). Error bars indicate ± SEM (each measurement in each heart is an average of 10 action potentials at each level of stretch). Stretch induced shortening of endocardial MAP APD_{50} at extreme levels of left ventricular stretch (EDLVP = 50-55 mmHg) were abolished in the presence of SAC modulators. Statistical differences were calculated compared to control conditions (no stretch, EDLVP = 0-5 mmHg) using a ANOVA GLM ($p > 0.05$).

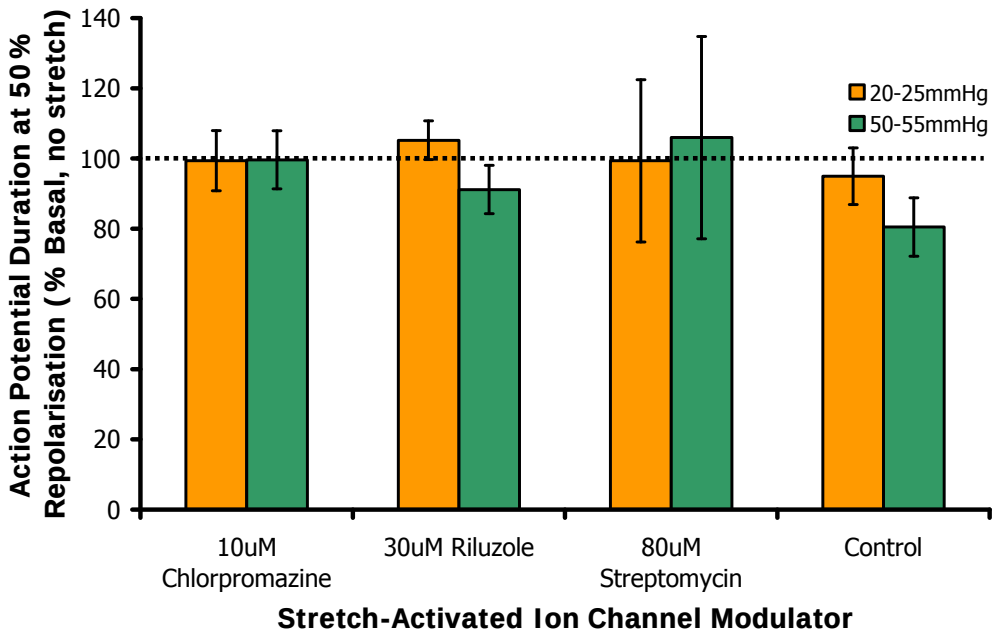

Figure 41: Effect of Stretch on Sub-epicardial MAP APD_{50}

Figure showing effect of SAC modulators (Chlorpromazine TREK-1 inhibitor (n= 6), Riluzole TREK-1 activator (n= 7) and streptomycin NSAC inhibitor (n= 5)) on sub-epicardial monophasic action potential durations at 50 % repolarisation in rat hearts compared to control. MAP durations at EDLVP = 20-25 and 50-55 mmHg plotted as a percentage of control conditions (close to zero EDLVP). Error bars indicate ± SEM (each measurement in each heart is an average of 10 action potentials at each level of stretch). Stretch induced shortening of sub-epicardial MAP APD_{50} at extreme levels of left ventricular stretch (EDLVP = 50-55 mmHg) were abolished in the presence of SAC modulators. Statistical differences were calculated compared to control conditions (no stretch, EDLVP = 0-5 mmHg) using a ANOVA GLM ($p > 0.05$).

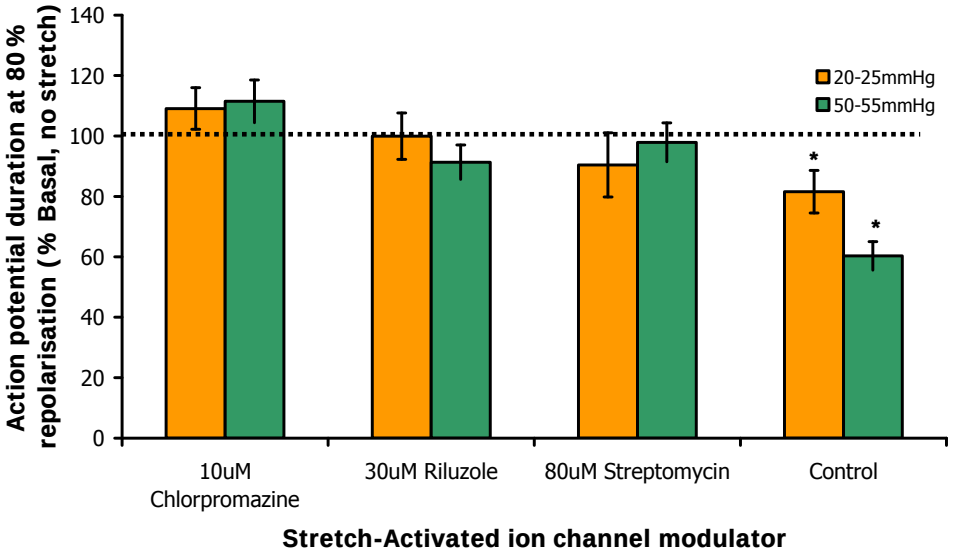

Figure 42: Effect of Stretch on Endocardial MAP APD_{80}

Figure showing effect of SAC modulators (Chlorpromazine TREK-1 inhibitor (n= 6), Riluzole TREK-1 activator (n= 7) and streptomycin NSAC inhibitor (n= 5)) on endocardial monophasic action potential durations at 80 % repolarisation in rat hearts compared to control. MAP durations at EDLVP = 20-25 and 50-55 mmHg plotted as a percentage of control conditions (close to zero EDLVP). Error bars indicate ± SEM (each measurement in each heart is an average of 10 action potentials at each level of stretch). Stretch induced shortening of endocardial MAP APD_{50} at extreme levels of left ventricular stretch (EDLVP = 50-55 mmHg) were abolished in the presence of SAC modulators. Statistical differences were calculated compared to control conditions (no stretch, EDLVP = 0-5 mmHg) using a ANOVA GLM ($p > 0.05$).

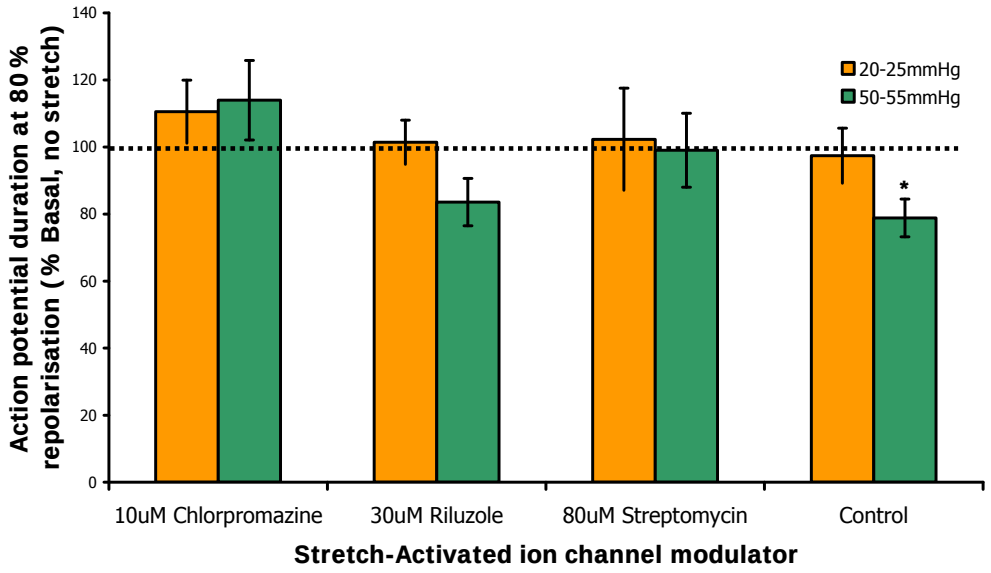

Figure 43: Effect of Stretch on Sub-epicardial MAP APD_{80}

Figure showing effect of SAC modulators (Chlorpromazine TREK-1 inhibitor (n= 6), Riluzole TREK-1 activator (n= 7) and streptomycin NSAC inhibitor (n= 5)) on sub-epicardial monophasic action potential durations at 80 % repolarisation in rat hearts compared to control. MAP durations at EDLVP = 20-25 and 50-55 mmHg plotted as a percentage of control conditions (close to zero EDLVP). Error bars indicate ± SEM (each measurement in each heart is an average of 10 action potentials at each level of stretch). Stretch induced shortening of sub-epicardial MAP APD_{50} at extreme levels of left ventricular stretch (EDLVP = 50-55 mmHg) were abolished in the presence of SAC modulators. Statistical differences were calculated compared to control conditions (no stretch, EDLVP = 0-5 mmHg) using a ANOVA GLM ($p > 0.05$).

Of equal interest was that all three modulators of SACs abolished changes in MAP amplitude with stretch. This was seen across both sub-epicardial and endocardial layers, although variability between rats was considerable (see Figure 40, Figure 43).

DISCUSSION

Effect of Stretch on Sub-epicardial and Endocardial MAPs

In this section, the electrophysiological response of the rat heart to changes in EDLVP were studied in both sub-epicardial and endocardial muscle layers in the presence and absence of various SAC modulators.

Of the sub-epicardial and endocardial MAPs made in the current series of experiments, in the majority of cases, sub-epicardial MAPs were of greater duration than Endocardial MAP recordings (see Figure 36, pg 117). Whilst the reasons behind this observation are not understood, it may have been reflected by changes in the direction of repolarisation across the myocardial wall (Antzelevitch, 2001). No ECG was recorded, however, to confirm this and to see if the T-wave was indeed inverted or if the results were due to some sort of mechanical/recording artefact. It is also possible that instead of recording from a sub-epicardial population of cells that the epicardial electrode was instead recording from the proposed "M-cell" region which has been shown previously in canine wedge preparations to have a much longer action potential duration than both endocardial and epicardial cell populations (Yan & Antzelevitch, 1998), although other research has cast doubt over the presence of this region within the ventricular wall (Vos & Jungschleger, 2001). It is also possible that the anaesthetic used prior to removal of the heart may have influenced repolarisation (Antzelevitch, 2001), but one would expect this to rapidly revert to normal in the isolated Langendorff perfused heart model since the perfusate was not recirculated.

The present repolarisation differences between endocardial and sub-epicardial MAPs were consistent between rats, others have found similar results under hypokalaemic conditions (3 mM external [K^+]) (Sabir *et al.*, 2007) which could suggest K^+ inadequacies in the solutions used in the present study. Of particular interest is that some anaesthetics may induce repolarisation differences in the epi-endocardium (Antzelevitch, 2001). However, in a small number of experiments, animals were anaesthetised by another means (CO_2 induced semi-consciousness followed by swift decapitation) and similar results for sub-epicardial and endocardial MAP durations were

found (data not shown). The present differences in endocardial and sub-epicardial APDs are also unlikely to reflect ischaemic conditions since this results in a greater shortening of epicardial than endocardial action potential duration (Lukas & Antzelevitch, 1993). However, with this in mind, other studies utilising transmural MAP recordings have found Endocardial MAP durations in general to be longer than Epicardial MAP durations (Kongstad *et al.*, 2005) or not obviously different (Franz *et al.*, 1992). This observation is made more puzzling by microelectrode studies supporting the current data. Specifically, no difference or slightly longer epicardial durations have been observed in microelectrode studies in canine cardiac tissue with M-cells having the longest duration (Anyukhovsky *et al.*, 1996) and reviewed by (Taggart *et al.*, 2003). Consistent with this, at least one other study utilising transmural MAP recording methods observed no difference in APD_{90} between endocardial and epicardial recordings from dogs (Zhou *et al.*, 2002). Although not reported, similar results are depicted in figure 3 by Franz *et al* (1992) in Langendorff perfused rabbit heart studies (Franz *et al.*, 1992). As such, it may be concluded that since MAPs only reflect the "time-course" of membrane repolarisation (Hoffman *et al.*, 1959; Levine *et al.*, 1986; Franz, 1999, 2005), it may be possible that the actual MAP duration reflects the number of cells from which the electrode records rather than the true transcellular equivalent. This would appear to be the case since MAP recordings using the suction or Franz-contact techniques repeatedly produced MAP durations at 80% repolarisation shorter than that observed for sub-epicardial MAPs in the present study (refer to Figure 36). The most likely reason for the endocardial-epicardial duration discrepancy is that the presence of surface electrodes makes it physically difficult to keep the epicardial surface of the heart warm by means of warming chambers etc. As such, the surface or sub-epicardial layers may be at a considerably lower temperature than endocardial layers giving rise to longer action potential durations as depicted in Figure 46 & Figure 47 (pg 138). Despite this observation, with increasing EDLVP, Endocardial MAP durations decreased more than their sub-epicardial equivalents. This observation is consistent with the finding that KSACs are more frequently observed and are expressed at greater levels in endocardial tissue of the rat heart (Tan *et al.*, 2004). More importantly, the effect of EDLVP on endocardial MAP duration was proportional with greater EDLVPs resulting in a greater shortening of the endocardial MAP. This result also concurs with TREK-1 distribution theory and the gating properties of SACs in general (Sigurdson *et al.*, 1992; Kawakubo *et al.*, 1999).

Effect of Pharmacological Modulation of SACs

One of the most interesting findings of the present study was that regardless of the SAC modulator used, the MEF response was abolished at both levels of stretch tested (moderate and extreme where the EDLVP used was 20-25 and 50-55 mmHg respectively, see Figure 40 - Figure 43). This interesting observation brings about a number of distinct possibilities:

- Firstly, all SACs (both NSAC and KSAC) may be involved in the modulation of the cardiac action potential with extreme levels of stretch. Due to the poor selectivity of the available modulators cross-inhibition of the different SAC groups may be occurring at the chosen drug concentrations. Indeed, such cross-inhibitory activity has been reported, an example being that Gd^{3+} blocks TRAAK currents (another KSAC) (Maingret *et al.*, 1999a). Given both Gd^{3+} and streptomycin inhibit SACs (Tavi *et al.*, 1996), then it is likely that streptomycin will also have some ability to inhibit K-selective SAC currents, although not yet reported. Thus, it is possible that streptomycin may have inhibited the stretch-induced shortening in the current experiments through a primary action on NSACs and a secondary action on smaller KSAC currents like TRAAK. However, the effects of both streptomycin and Gd^{3+} on other KASC currents like TREK-1 have not been reported or published to date.

- Secondly, the present results only report stretch-induced shortening of action potentials, and this may be due to activation of K^+ efflux only – either through TREK-1 channels or NSACs. It is possible that activation of KSACs by Riluzole or their inhibition by Chlorpromazine was going to completely abolish the small MEF response observed. This would appear a likely reason given the already high open probability of these channels at physiological temperatures (Maingret *et al.*, 2000a; Kang *et al.*, 2005). However, the greater reduction in Endocardial MAP duration with stretch in the present experiments (see Figure 38 and Figure 39, pg 119) would tend to contradict the limited involvement of TREK-1 channels assuming KSAC activity is responsible.

- Thirdly, it remains possible that these stretch-induced effects are due to the presence of other channels altogether. As pointed out from earlier (see: Potassium-Selective Stretch-Activated Cation Channels (KSACs), Pg 21), a number of other channels including K^+_{ATP} channels (Van Wagoner, 1993) have

been reported as stretch sensitive and their involvement in stretch-induced responses shown (Link *et al.*, 1999) Thus, the current series of experiments may be targeting the wrong channels altogether.

- Lastly, it may be that given the very un-physiological nature of the stretch required to elicit an observable MEF response, that SACs are not the key factor. Instead, it may be that at as EDLVP approaches the coronary perfusion pressure, coronary flow is being reduced to an extent that prevailing ischaemic/hypoxic conditions are eliciting the reductions in APD observed in the present experiments (Hamada *et al.*, 1998).

SUMMARY

In this chapter the effect of step-wise increases in EDLVP over the range of 0 to 50-55 mmHg were used to demonstrate an observable, repeatable reduction in action potential duration. This reduction was greatest in endocardial myocardial layers compared to the sub-epicardium. Results were primarily consistent with previous chapters showing little or no change in MAP durations with stretch imposed by EDLVP of equal to or less than 20-25 mmHg in epicardial layers. By contrast, robust responses to stretch were observed in the endocardium which was not addressed previously. More interesting was the observation that all SAC modulators tested abolished these responses and thus were not successfully used to distinguish contributions of NSACs and KSACs to the MEF response

CONCLUSION

Extreme stretch whereby EDLVP was 50-55 mmHg (approaching the chosen coronary perfusion pressure of 60-70 mmHg) resulted in a robust, repeatable reduction in MAP durations of both epicardial and endocardial MAP recordings. Endocardial MAP durations decreased by almost double that of sub-epicardial recordings suggesting a strong involvement for TREK-1 channels in the response. This observation was compounded by the fact the effect was abolished in the presence of TREK-1 activator Riluzole, inhibitor Chlorpromazine and the NSAC inhibitor Streptomycin. Whilst this observation could be attributable to the non-specific nature of the SAC modulators involved, the concentrations chosen were well below the IC_{50} values of other ion channels that may have contributed to the present result (refer to *literature review* Pgs 17-21). As such, other experiments need to be performed to be able to attribute this response to a specific mechanism which currently remains undefined.

ACKNOWLEDGEMENTS

I would like to give my sincere gratitude to Dr Lorraine Mackenzie for developing and applying the concept of transmural MEF in the Langendorff perfused heart. Following many negative outcomes using surface electrode types and modest EDLVP changes, her guidance, theories and suggestions including her direct participation in the above experiments resulted in the present and following chapter contents.

CHAPTER 8: FURTHER INVESTIGATIONS INTO TRANSMURAL MEF

American comedian & Singer : Sophie Tucker
(Jan 13, 1884 – Feb 9, 1966)

Chapter 8 - Table of contents

TEMPERATURE MODULATION OF MEF

INTRODUCTION

This chapter addresses some of the issues that arose from observations of stretch-induced responses in sub-epicardial and endocardial layers of the rat heart. Previous experiments showed that extreme stretch of the left ventricle (EDLVP of 50-55 mmHg) caused a greater reduction in endocardial MAPs than in epicardial MAPs and that this response was both robust and repeatable. The fact that ostensibly selective SAC modulators for KSACs and NSACs abolished the response altogether made it impossible to attribute this response to any one channel type specifically. As such, another method of separating the responses is required. This chapter outlines some of the differences between what is known about KSACs and NSACs and how they may be used to help dissect out the contribution of these channels to the stretch-induced shortening of sub-epicardial and endocardial MAPs seen in the previous chapters.

Temperature modulation of SAC response

Whilst Grammostola toxin (GsMTx-4) is thought to be a selective blocker of NSACs (Suchyna *et al.*, 2000; Oswald *et al.*, 2002), to date, no such compounds have been described that selectively block or activate TREK-1 making the definitive identification of its role in MEF difficult. However, recent literature demonstrates that TREK-1 channels are very sensitive to changes in temperature (Maingret *et al.*, 2000a; Kang *et al.*, 2005). Hence, it can be speculated that temperature changes could be used as another means of clarifying the role of TREK-1 in MEF in mammalian heart. TREK-1 channels are open at all membrane potentials and likely contribute to the resting membrane potential but are also stretch activated (Patel *et al.*, 1998; Maingret *et al.*, 1999a; Maingret *et al.*, 1999b).

The next set of experiments uses the approach of lowering Langendorff perfused heart temperature with the aim of evaluating the contribution of TREK-1 stretch-activated

channels to changes in the rat cardiac action potential duration during stretch by taking advantage of the temperature dependent nature of the TREK-1 K^+ channel that has been demonstrated by others like Kang D *et al* 2005 (Maingret *et al.*, 2000a; Kang *et al.*, 2005).

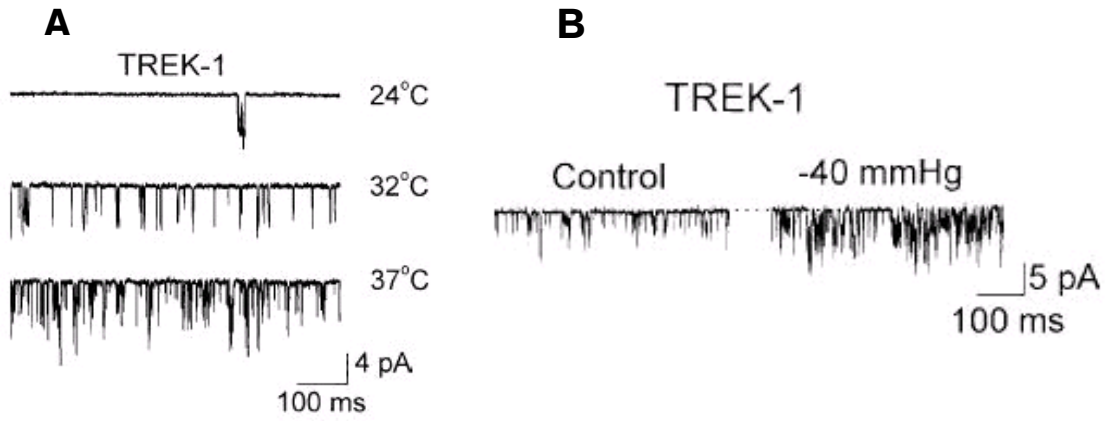

Figure 44: Effect of Temperature on TREK-1 Activity

Effect of temperature (A) and negative pipette pressure (B) on TREK-1 single channel recordings from COS-7 cell line. Data taken from Kang D, Choe C, Kim D. Thermosensitivity of the two-pore domain K^+ channels TREK-2 and TRAAK. J Physiol. 2005 Apr 1;564(Pt 1):103-16.

TREK-1 channels have a small probability of being open (open probability, Po) at lower temperatures (such as at room temperature) unless a small amount of stretch (usually in the form of pipette suction) is applied to the cell (see Figure 44). With increasing temperature, TREK-1 Po significantly increases as is the case for increasing degrees of suction/membrane patch stretch. As a result, it is likely that the changes in Po and hence the TREK-1 whole cell current contribution to the action potential as a result of temperature may mask those changes induced by membrane stretch. In theory, reducing basal channel activity of TREK-1 channels by lowering myocardial temperature will lead to the ability to exaggerate possible stretch-induced increases in TREK-1 channel activity and thus resultant effects of MEF on the cardiac action potential.

METHODS

6 male Sprague Dawley rats (450-490 g) were heparinised and killed after 5 min under CO_2 anaesthesia by cervical dislocation and exsanguination (this step was used to address previous concerns regarding the possible effects of the anaesthetic agent on sub-epicardial and endocardial MAP durations). Hearts were then retrogradely perfused on a Langendorff system (AD Instruments, Australia) as described in chapter 3. Hearts were paced at 4 Hz, twice threshold using bi-polar pulses and allowed to stabilise at a constant coronary perfusion pressure of 60 mmHg with Bicarbonate buffer solution at 37 ^{o}C. Following stabilisation, hearts were subjected to three consecutive diastolic pressures (control: 0-5, 20-25 and 50-55 mmHg) at two randomised perfusion temperatures (32 ^{o}C or 37 ^{o}C). During manipulation of EDLVP, endocardial and epicardial monophasic action potentials (MAP) were recorded and their durations at 20, 50 and 80 % repolarisation (APD_{20-80}) calculated. The perfusate temperature was then changed to the corresponding control or experimental group and the MAP recordings repeated. This protocol enabled MAP recordings to be made from each heart at 32 and 37 ^{o}C in a random order. Whilst larger temperature differentials were tested in preliminary experiments (data not shown), the heart would stop contracting regularly at temperatures below 32 ^{o}C and fail to respond to pacing. As such, 32 ^{o}C was the lowest temperature at which the heart could be paced at 4 Hz in order for the MAP recordings to be comparable with those obtained at 37 ^{o}C (also paced at 4 Hz).

RESULTS

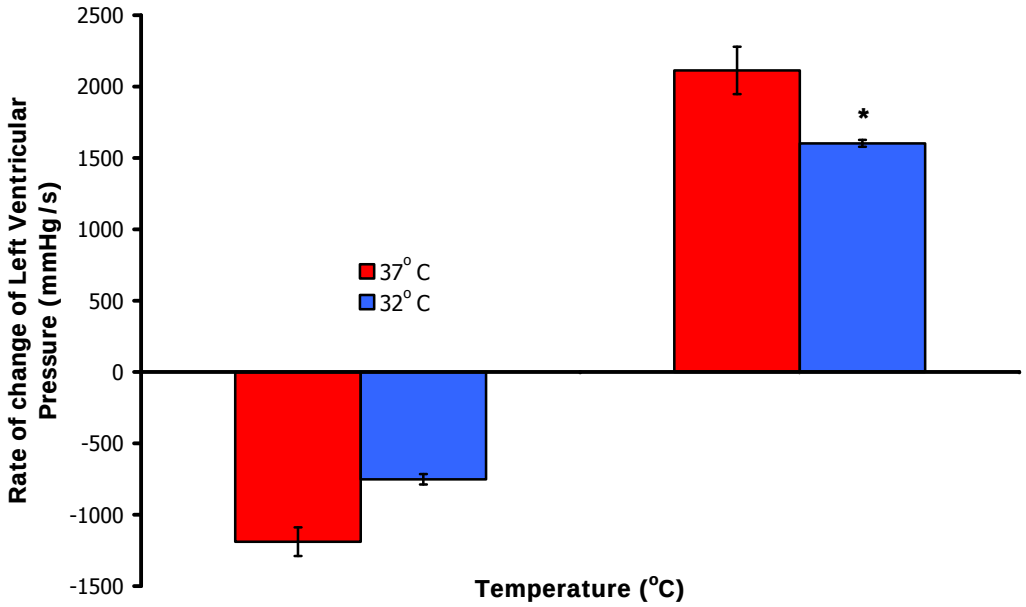

Figure 45: Effect of Temperature on Contractility

Figure showing effect of perfusate temperature on contractibility under control conditions (EDLVP < 5 mmHg). Error bars indicate ± SEM, n = 6 hearts (each measurement in each heart is an average of 10 contractions at each temperature). Uper columns denote positive dLVP/dt whilst downward facing columns denote –dLVP/dt. Reducing perfusate temperature by 5 °C (blue bars), rate of cardiac contraction and relaxation fell by 24.2 % and 36.8 % respectively (denotes p < 0.05, paired t-test).*

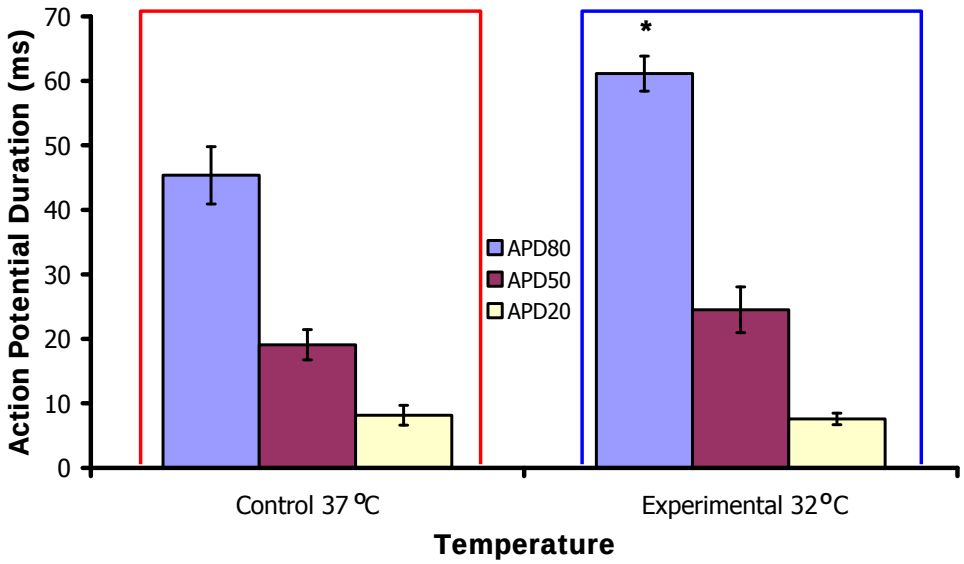

Figure 46: Effect of Temperature on Sub-Epicardial MAP Morphology

Figure showing sub-epicardial MAP durations at 80 % repolarisation increased from 45 ± 4.4 to 61 ± 2.7 ms. By contrast, no statistically significant changes were observed in APD_{20} or APD_{50} (denotes $p < 0.05$, ANOVA GLM). MAP durations under control conditions as calculated in the absence of stretch, EDLVP = 0-5 mmHg. Error bars indicate ± SEM, n = 6 hearts (each measurement in each heart is an average of 10 action potentials).*

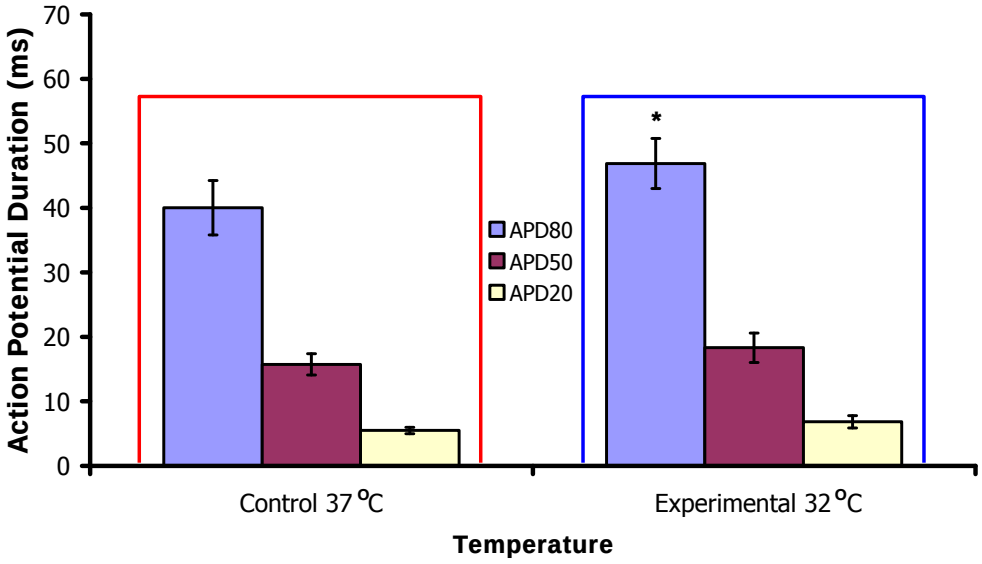

Figure 47: Effect of Temperature on Endocardial MAP Morphology

Figure showing under control conditions endocardial action potential durations at 20, 50 repolarisation did not significantly differ when perfused at either 32 or 37 °C. By contrast, MAP durations at 80 % repolarisation increased from 40 ± 4.6 to 47 ± 3.9 ms (denotes $p < 0.05$, ANOVA GLM). MAP durations under control conditions as calculated in the absence of stretch, EDLVP = 0-5 mmHg. Error bars indicate ± SEM, n = 6 hearts (each measurement in each heart is an average of 10 action potentials at each level of stretch).*

Decreasing perfusion temperature from 37 to 32 °C increased Endocardial APD_{80} from 40 ± 4.6 to 47 ± 3.9 ms and sub-epicardial durations from 45 ± 4.9 to 61 ± 3.0 ms. Likewise, endocardial APD_{50} rose from 16 ± 1.8 to 18 ± 2.3 ms and sub-epicardial durations from 19 ± 2.6 to 25 ± 3.6 ms (see Figure 46, Figure 47).

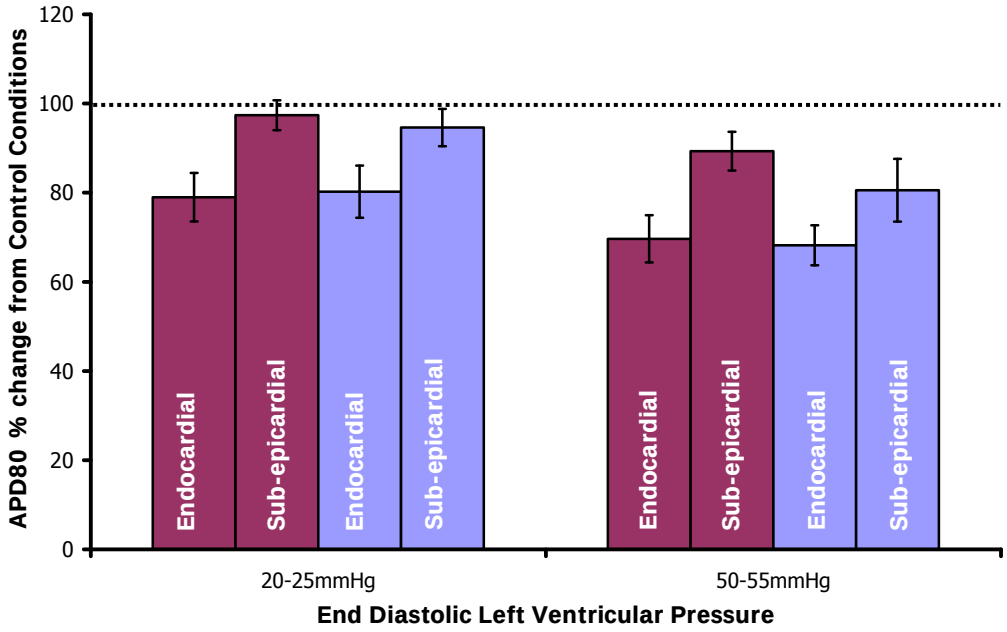

Figure 48: Effect of Temperature on MEF in the Rat Heart (APD_{80})

Figure showing effect of temperature and stretch on sub-epicardial and endocardial MAP APD_{80} relative to control conditions (no stretch, EDLVP = 0-5 mmHg). Whilst decreasing perfusion temperature to 32 °C (blue columns) significantly increased sub-epicardial and endocardial APD_{80} durations, it had no effect on stretch-induced changes at 20-25 or 50-55 mmHg EDLVP ($p > 0.05$, ANOVA GLM, $n = 6$). Red columns denote normal physiological Langendorff temperature (37 °C).

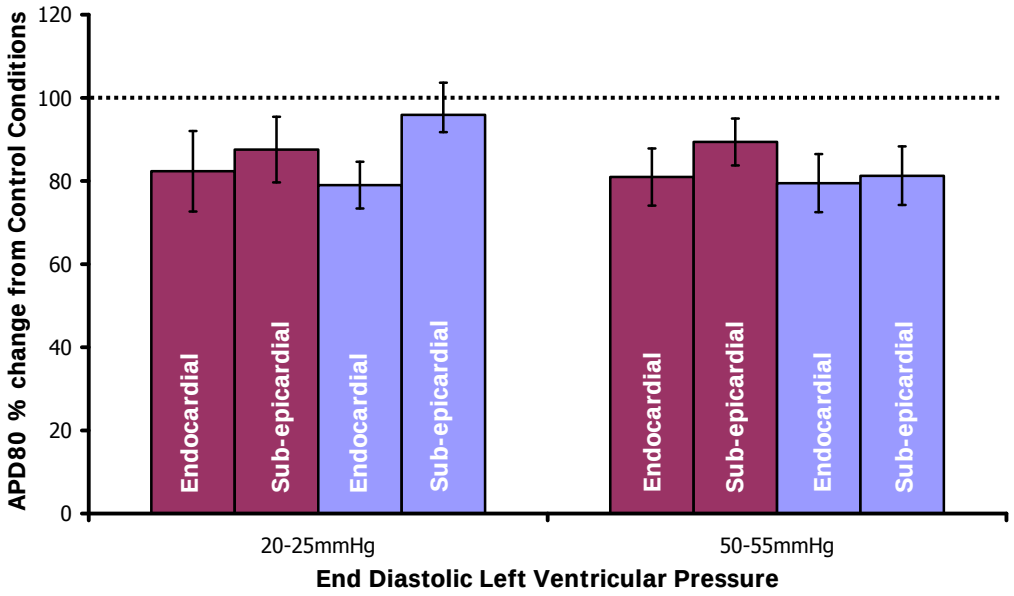

Figure 49: Effect of Temperature on MEF in the Rat Heart (APD_{50})

Figure showing effect of temperature and stretch on sub-epicardial and endocardial MAP APD_{50} relative to control conditions (no stretch, EDLVP = 0-5 mmHg). Decreasing perfusion temperature to 32 °C (blue columns) had no effect on stretch-induced changes in MAP durations at 50 % repolarisation at 20-25 or 50-55 mmHg EDLVPs ($p > 0.05$, ANOVA GLM, n= 6). Red columns denote normal physiological Langendorff temperature (37 °C).

Despite the significant prolongation of action potential duration with lower myocardial temperatures, stretch-induced shortening of action potential duration induced by EDLVP of 50-55 mmHg was no different between myocardial temperature groups (see Figure 48, Figure 50) and summary (Table 1).

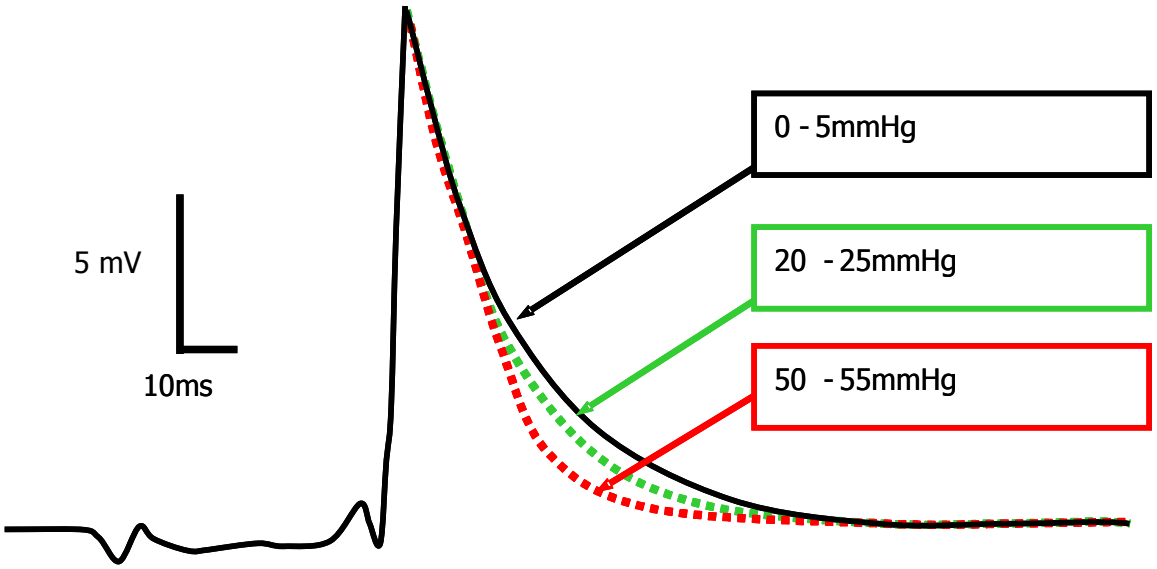

Figure 50: Effect of Stretch on Rat Endocardial MAP

Figure constructed from endocardial MAP data showing observed shortening with stretch under normal conditions. Extreme left ventricular stretch (50-55 mmHg) significantly reduced the APD_{80} in both epicardial and endocardial recordings at 37 °C and 32 °C by the same extent. Epicardial APD_{80} reduced by 10.7 ±4.3% and 19.4 ±7.0 % relative to control conditions at 37 °C and 32 °C respectively. Similarly, endocardial APD_{80} decreased by 30.4 ±5.3 % and 31.8 ±4.5 % at 37 °C and 32 °C respectively.

SUMMARY

Results of altering myocardial temperature and EDLVP on MAP durations in sub-epicardial and endocardial layers are summarised in Table 1 below.

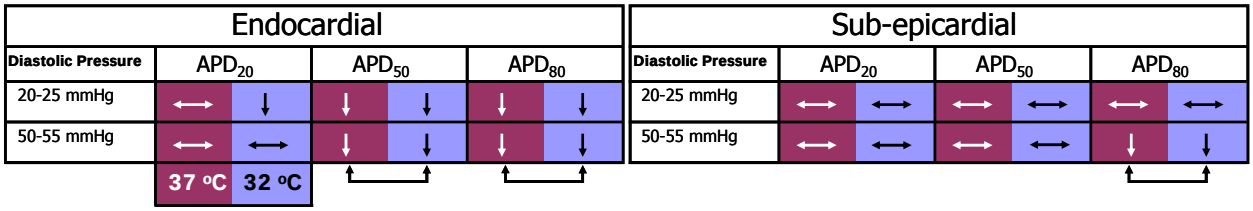

Table 1: Summary of Endocardial and Sub-Epicardial MAP Changes in Response to Stretch at Physiological and Sub-Physiological Temperatures.

Summary of statistically significant changes in Endocardial and Epicardial MAP recordings made at 37 and 32 °C during left ventricular diastolic pressure manipulations (n=6). Downward arrows denote a decrease ($p<0.05$) in action potential duration (APD) and horizontal arrows denote no significant change. Arrows connecting 37 and 32 °C columns denote statistically similar reductions in APD. (significant changes accepted as $p < 0.05$, ANOVA GLM, $n = 6$). Red panels denote normal physiological Langendorff temperature (37 °C) whilst blue panels denote 32 °C.

DISCUSSION

These experiments were prompted by previous demonstrations of MEF in isolated Langendorff rat heart preparations, observed as a reduction in action potential duration. Both TREK-1 (KSAC) and NSACs are likely to contribute to MEF in the heart. Since no compounds have been described that selectively block or activate TREK-1, in an attempt to better understand the ionic basis of MEF and the contribution by TREK-1, MAPs were recorded in isolated rat hearts during EDLVP manipulations at two different temperatures. This was based on the understanding that TREK-1 channels are inactivated at lower temperatures, but remain responsive to stretch (Maingret *et al.*, 2000a; Kang *et al.*, 2005). The present efforts failed to convincingly demonstrate that stretch-induced changes in the action potential, even at extreme tensions, were influenced by temperature (see Figure 48, Figure 49). As with the previous chapter, increases in EDLVP resulted in larger changes to endocardial MAP durations when compared to sub-epicardial durations. However, there was no statistical significance

between the reduction in action potential duration for either myocardial layer at either myocardial temperature. Whilst an equivalent cellular study is yet to have been conducted, it remains a reasonable theory to deduce from the published data of Kang *et al* (2005) and Maingret *et al* (2000) that if both temperature and stretch affect open probability of TREK-1 channels, then the two are likely to interact with each other and limit the range or response for increases in Po available to the other. Despite this hypothesis and the present observations, it remains an intriguing result that Endocardial tissue (as observed through MAP durations) retained its ability to shorten more than sub-epicardial recordings in the presence of the temperature change. Two possibilities encompass this result:

- Firstly, stretch-induced shortening of the action potential is a result of NSACs as suggested by experimental outcomes presented by other authors (Takagi *et al.*, 1999; Eckardt *et al.*, 2000).

- Secondly, a different mechanism, other than SACs, is responsible for the stretch-induced shortening of the cardiac action potential. Moreover, this mechanism is independent of stretch and temperature changes which appear to go hand in hand (Maingret *et al.*, 2000a; Kang *et al.*, 2005) and is the result of ion channels not necessarily unevenly distributed across endocardial and sub-epicardial myocardial layers. A potential component to this may be the K_{ATP} channel which is activated under ischaemic conditions by low ATP and high adenosine concentrations within the cell (Miyoshi *et al.*, 1996; Wirth *et al.*, 2000). However, experiments on ischaemia, pharmacological modulation and histological location of these channels within the heart suggests their greater presence in the epicardial layers than the endocardium (Morrissey *et al.*, 2005). The debate here is whether their activation is due to EDLVP stretch-induced ischaemia within the left ventricle or their activation by stretch (Van Wagoner, 1993; Link *et al.*, 1999)

CONCLUSION

It was concluded that a change in cardiac temperature did not affect the magnitude of reduction in action potential duration (as measured by APD at 20, 50 or 80 % repolarisation) following moderate (20-25 mmHg) or extreme stretch (50-55 mmHg) for either endocardial or sub-epicardial recordings when compared to their respective controls (EDLVP = 0-5 mmHg). Since TREK-1 channels are temperature sensitive,

(inactivating at lower temperatures), these results suggest that non-selective stretch-sensitive cation channels or a third, un-identified mechanism may be more important in modifying action potential duration during stretch than TREK-1 in the rat heart.

STREPTOMYCIN MODULATION OF SAC RESPONSE IN GUINEA PIG

INTRODUCTION

Whilst previous chapters showed there was little or no epicardial response to moderate levels of stretch in the rat heart, MEF was observed at more elevated levels of stretch (50-55 mmHg) and by using sup-epicardial MAP recordings. Similarly, suction electrodes failed to pick up any reasonable change in electrophysiology in the guinea pig heart with moderate stretch associated with the peak of the Frank-Starling relation. As such, similar methods employing the transmural electrode were applied to the Langendorff perfused guinea pig heart to see if there was a differential effect of MEF across sub-epicardial and endocardial layers. In addition, the extreme level of EDLVP stretch was used.

In addition to the aim of observing differences in myocardial MEF, the present experiments attempt to modulate the NSAC family of ion channels with a concentration of streptomycin known to inhibit stretch-activated non-selective currents, and to observe is TREK-1 channels are involved in the MEF response in guinea pig hearts. Follow-up experiments were aimed to determine if there is a differential involvement of TREK-1 channels across the left ventricular wall that could contribute to a differential MEF response from sub-epicardial to endocardial layers.

METHODS

Five male guinea pigs (427 ± 82 g) were anaesthetised with ketamine and domitor as described previously. Hearts were excised and cannulated on an Langendorff setup as described in chapter 3 and retrogradely perfused at a constant coronary perfusion pressure of 70 mmHg with bicarbonate buffered physiological solution containing 1.5

mM $CaCl_2$. Hearts were paced through the right atrium at twice threshold (5 Hz) with 10 ms bipolar pulses. End diastolic left ventricular pressures were obtained as described previously (Chapter 3) and allowed to stabilise. Following stabilisation of baseline recordings, the EDLVP was increased from baseline (0-5 mmHg) until the desired pressures were obtained (20-25 mmHg, moderate stretch, and 50-55 mmHg, elevated stretch). Following control conditions, 80μM streptomycin was introduced into the perfusate and following stabilisation of baseline, the stretch protocol increasing EDLVP was repeated.

MAP recordings were made using a transmural sub-epicardial, endocardial electrode as previously described.

RESULTS

Action potential amplitudes and durations at 20, 50 and 80 % repolarisation were calculated (see Figure 51 - Figure 54) and tested statistically using general linear model analysis of variance (GLM-ANOVA) followed post hoc by Tukey's pair wise comparisons.

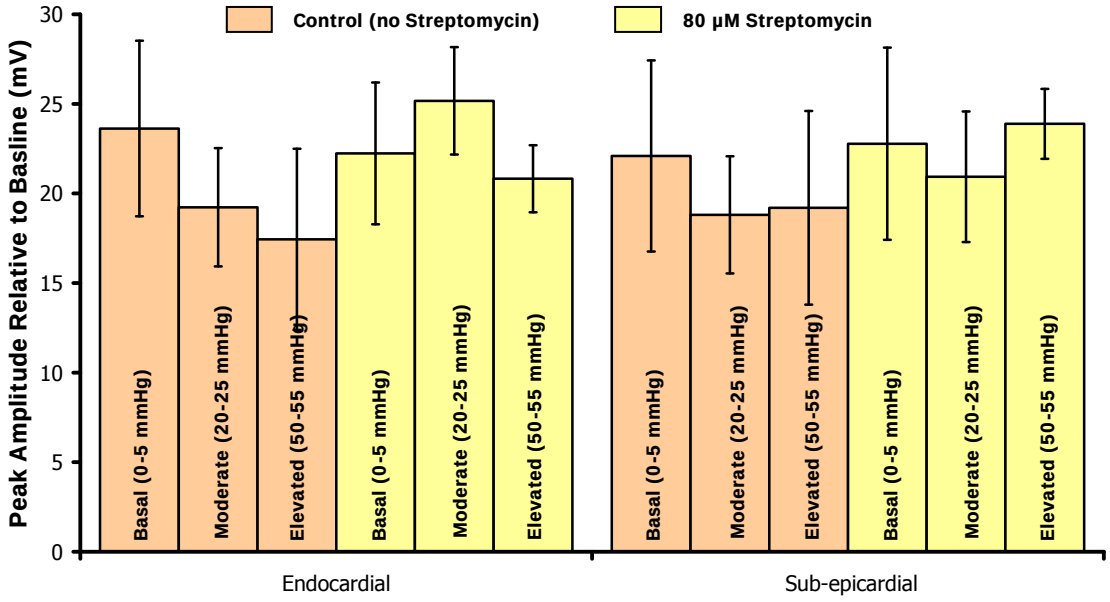

Figure 51: Endocardial and Sub-epicardial MAP Amplitude in the Guinea pig

Under control conditions (EDLVP = 0-5 mmHg) following stabilisation, MAP amplitudes were 24± 4.9 and 22 ± 5.3 mV (measured from peak relative to baseline) for endocardial and sub-epicardial recordings respectively. Addition of Streptomycin (80 µM) or application of moderate and extreme stretch had no significant outcome on MAP amplitudes using the transmural method for either endocardial or sub-epicardial recordings. (p > 0.05, ANOVA GLM, n = 5)

Of interest from the present results was that there were no observable or statistical differences between sub-epicardial and endocardial MAP durations in the guinea pig hearts ($p > 0.05$ for APD_{20}, 50 and 80). Sub-epicardial MAP duration at 80 % repolarisation was 86 ± 3.3 ms whilst Endocardial MAP APD_{80} was 87 ± 3 ms.

Sub-Epicardial Effect of Stretch

Following increases in EDLVP there were significant decreases in action potential durations as measured at 20, 50 and 80 % repolarisation ($p < 0.05$) in all cases. Surprisingly, the changes were statistically different between moderate and elevated stretch in at all levels of repolarisation ($p < 0.05$, see Figure 52 - Figure 54 below). Following inhibition of NSACs with 80 μM streptomycin, increases in EDLVP continued to induce significant decreases in monophasic action potential durations at 20, 50 and 80 % repolarisation. Moreover, the abbreviation of the sub-epicardial MAP recording seen with stretch was not different to that observed in the presence of streptomycin ($p > 0.05$ in all cases).

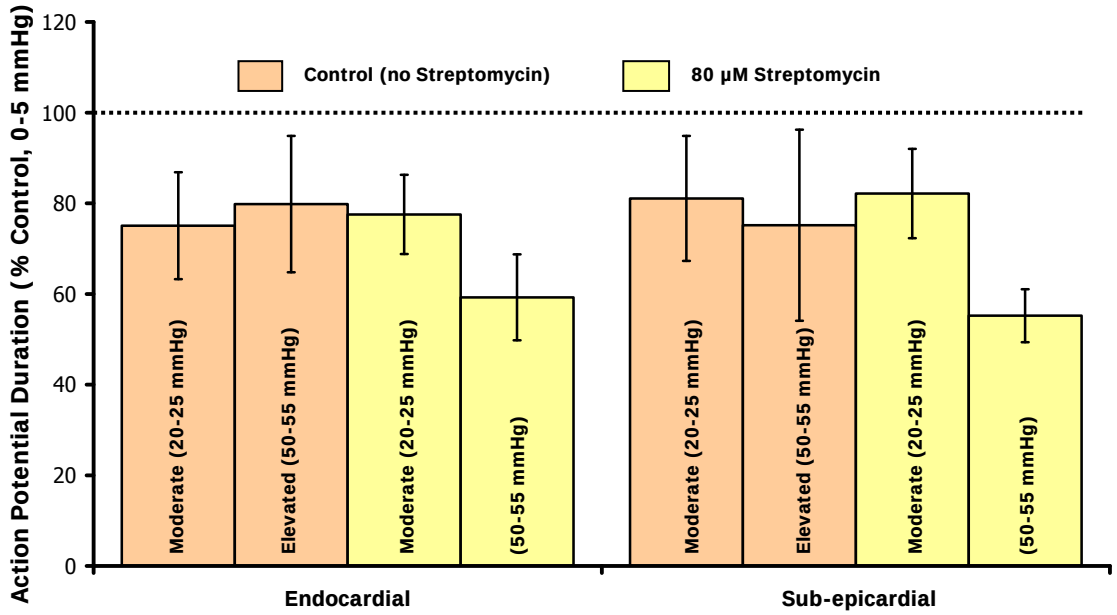

Figure 52: Effect of Stretch on Endocardial and Sub-epicardial APD_{20} in the Guinea pig

Figure showing reduction in APD_{20} following moderate (20-25 mmHg) and elevated (50-55 mmHg) stretch in the presence and absence of 80 μM streptomycin, a known NSAC blocker ($p < 0.05$, ANOVA GLM, $n = 5$). Stretch in general reduced APD_{20} at both EDLVPs tested to the same extent under control conditions ($p > 0.05$) whilst streptomycin evoked a significantly larger decrease in both sub-epicardial and endocardial APD_{20} with elevated stretch.

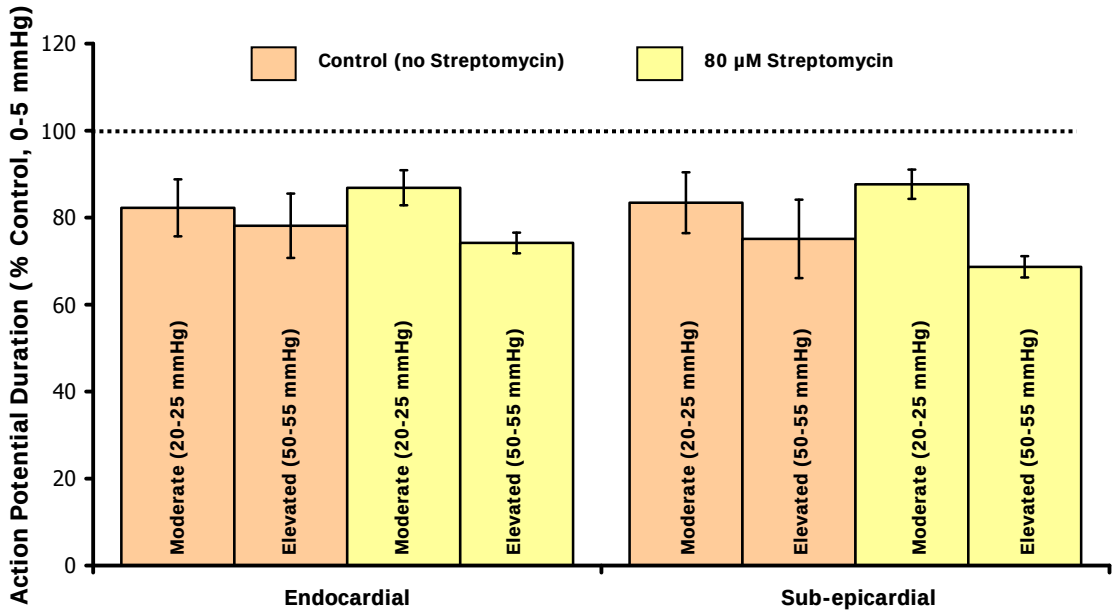

Figure 53: Effect of Stretch on Endocardial and Sub-epicardial APD_{50} in the Guinea pig

Figure showing reduction in APD_{50} following moderate (20-25 mmHg) and elevated (50-55 mmHg) stretch in the presence and absence of 80 μM streptomycin, a known NSAC blocker ($p < 0.05$, ANOVA GLM, $n = 5$). Elevated stretch reduced APD_{50} to a greater extent that moderate stretch in the presence and absence of streptomycin in both sub-epicardial and endocardial MAPs ($p < 0.01$). Surprisingly, there was no statistically significant different between results in the presence and absence of streptomycin ($p > 0.05$).

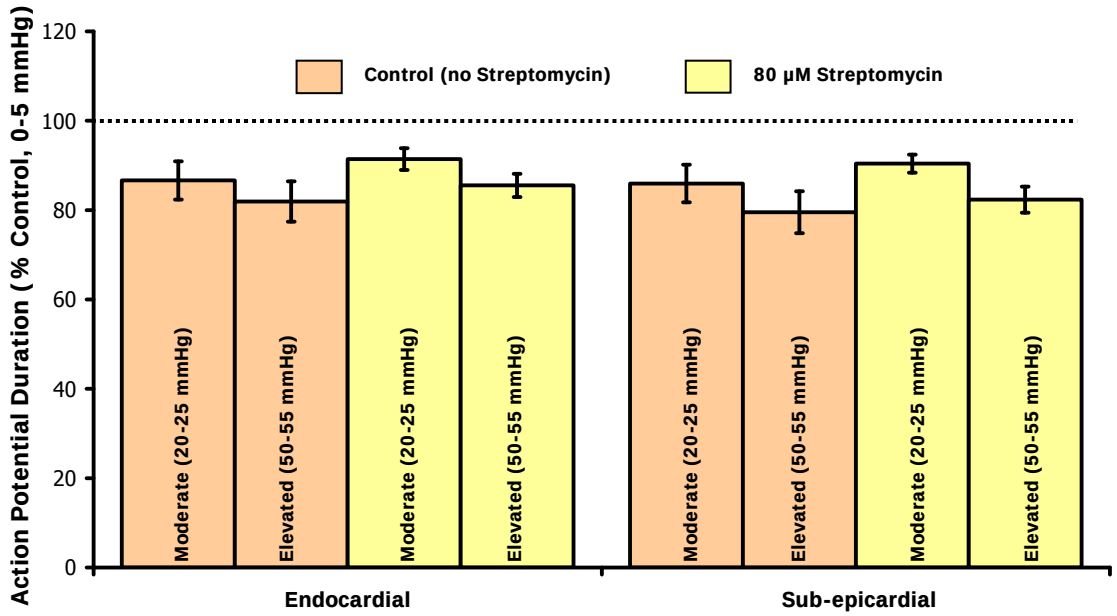

Figure 54: Effect of Stretch on Endocardial and Sub-epicardial APD_{80} in the Guinea pig

Figure showing reduction in APD_{80} following moderate (20-25 mmHg) and elevated (50-55 mmHg) stretch in the presence and absence of 80 µM streptomycin, a known NSAC blocker ($p < 0.05$, ANOVA GLM, $n = 5$). Elevated stretch reduced APD_{80} to a greater extent that moderate stretch in the presence and absence of streptomycin in both sub-epicardial and endocardial MAPs ($p < 0.01$). Surprisingly, there was no statistically significant different between results in the presence and absence of streptomycin ($p > 0.05$).

DISCUSSION

As demonstrated previously in the Langendorff rat heat using transmural electrodes, stretch of the left ventricle through stepwise increases in EDLVP induced MAP shortening in the guinea pig heart. These results provide functional evidence of MEF to the shape of the MAP in the guinea pig heart. However, in contrast to previous results shown in the rat and guinea pig using surface electrodes, moderate levels of stretch (20-25 mmHg EDLVP) were able to induce equivalent reductions in APD 20, 50 and 80 % repolarisation to that observed with elevated stretch (50-55 mmHg EDLVP), a result not seen in the Langendorff perfused rat heart. This would tend to suggest that the guinea pig heart may be more susceptible to stretch induced electrophysiological changes than the rat heart as the decrease in APD was seen at moderate (20-25 mmHg) and elevated (50-55 mmHg) levels of stretch where as in the rat, a reduction was only apparent with elevated stretch. Similarly between both

animal models, MAP amplitudes did not change for endocardial recordings, where as a reduction in amplitude was observed in rat epicardial recordings using the transmural method, but not in the guinea pig. This may suggest that reductions in amplitude may be structurally related since the curvature of the rat heart is greater than that of the guinea pig heart, and stretch may thin the wall, spatially shifting and reducing the number of cells available to establish a recording. It is not clearly understood why a reduction in sub-epicardial MAP duration was observed in the present experiments and not in those involving the suction electrode (refer Figure 29, pg 102). The only justification must involve the physical differences between the electrodes used as outlined earlier.

In the present experiments, there was no difference in MAP duration observed between endocardial and sub-epicardial recordings in the guinea pig heart. Whilst this result is similar to that observed in the rabbit heart (Franz *et al.*, 1992), it is in direct contrast to the results previously obtained using the same techniques in the rat model. Despite the differences, this observation is consistent with epicardial-endocardial MAP recordings made from the dog heart (Zhou *et al.*, 2002). Whilst the reasons remain unclear, as established earlier, MAP recordings only reflect the time course of changes in true intracellular recordings of membrane potential movements. As such, durations may be related to the level of cellular damage at the tip of the respective electrode and not merely a direct reflection of the action potential. If this were the case, then the durations between layers cannot be directly compared for MAP recordings, however, the reduction in MAP duration for each respective electrode with stretch could be compared for the two separate layers. With this in mind it was found that endocardial MAP recordings were always greatest affected by stretch when compared to epicardial recordings. This result appears consistent across both rat and guinea pig (see previous chapters). Of interest is that follow-up experiments in the Langendorff perfused rat heart using temperature and pharmacological agents to modulate this response failed. This suggests the observations are not due to the presence of stretch-sensitive TREK-1 channels known to be present in greater density in the endocardial layer of the heart (Tan *et al.*, 2004). The present results, however, still show that there is a link between stretch induced by increasing EDLVP and associated reductions in MAP duration. Whilst stretch activation of K_{ATP} channels would serve as an excellent possibility, their greater expression in epicardial tissue does not fit with the current stretch model (Morrissey *et al.*, 2005).

It is not understood why there were no significant differences between sub-epicardial and endocardial durations in the guinea pig. Whilst like the rat, it is possible that M-cell signals were obtained instead of epicardial recordings which may have been of similar duration to that of the endocardium - this has not been studied or reported to date. It is also possible that the anaesthetic may have influenced the rate of repolarisation of one or both of the myocardial layers reducing the repolarisation time differences to a non-distinguishable level (Antzelevitch, 2001), but no residual anaesthetic effect was observed in the rat heart. One distinct possibility may be that the silicon coating used to insulate the Endocardial electrode could have rubbed off or scrunched up upon insertion of the electrode into the myocardial wall. This remains a distinct possibility due the added thickness of the left ventricular free wall of the guinea pig heart compared to that of the rat. If this were the case, then both sub-epicardial and endocardial electrodes would pick up essentially similar signals.

Also of interest in the present study was the observation that streptomycin (80 μM) did not inhibit the stretch-induced shortening of MAP APDs in the guinea pig. This is in direct contrast to the result previously demonstrated in the rat whereby streptomycin abolished all stretch-induced shortening of the cardiac action potential. The present observation, however, is consistent with both ideas that the shortening can be contributed to the presence of TREK-1 channels or global/local inhibition of coronary flow due to increasing intra-ventricular pressure exerted against the left ventricular wall by the balloon.

Despite the present observations, others have found that acute atrial stretch in guinea pigs results in inconsistent changes to MAP APD_{50} and 90, with streptomycin inhibiting stretch-induced shortening of APD when it occurred (Babuty & Lab, 2001). Whilst again inconsistent with TREK-1 theory, the results of Babuty *et al* (2001) are consistent with the idea that NSACs can pass both inward Na^+ and Ca^{2+} currents in addition to outward, and hence APD shortening K^+ currents. It was also reported that streptomycin blocked stretch-induced lengthening where it occurred, and that stretch-induced changes in the action potential were independent of pacing frequency.

CONCLUSION

This sub-chapter investigated the effect of changes in EDLVP on the electrophysiology of both sub-epicardial and endocardial layers of the guinea pig myocardium. The present results found (consistent with the rat) an obvious, reproducible reduction on MAP duration with stretch and that this effect was not inhibited by the presence of streptomycin (a know NSAC blocker). More interestingly was that the reduction in MAP duration increased with increasing EDLVP. Due to the presence of possible technical difficulties, the present experiments were unable to distinguish differences in MAP durations between guinea-pig sub-epicardium and endocardium, hence no accurate deduction could be made regarding the contribution of TREK-1 to the present response. However, with the observation that streptomycin had no effect on stretch-induced MAP shortening, it would appear likely that TREK-1 channels or another, undefined mechanism (possibly stretch-induced ischemia or activation of K_{ATP} channels) may explain the present result.

TEMPERATURE MODULATION OF MEF IN THE GUINEA PIG

INTRODUCTION

Following on from the previous experiments in which streptomycin failed to block stretch-induced shortening of the guinea pig action potential, a temperature decrease was applied to enhance the TREK-1 contribution to stretch-induced electrophysiological changes

RESULTS

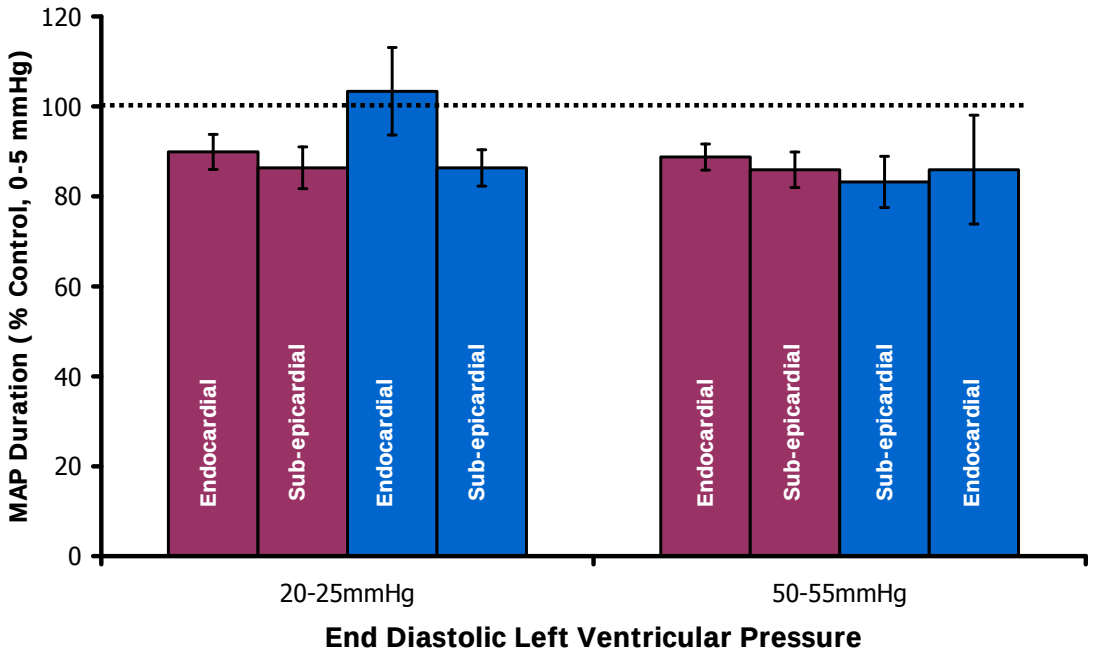

Figure 55: Effect of Temperature on MEF in the Guinea Pig Heart (APD_{80})

Figure showing effect of temperature and stretch on sub-epicardial and endocardial MAP APD_{80} relative to control conditions (no stretch, EDLVP = 0-5 mmHg). Decreasing perfusion temperature to 32 °C (blue columns) had no effect on stretch-induced changes at 20-25 or 50-55 mmHg EDLVP ($p > 0.05$, ANOVA GLM, $n = 4$). Burgandy columns denote normal physiological Langendorff temperature (37 °C).

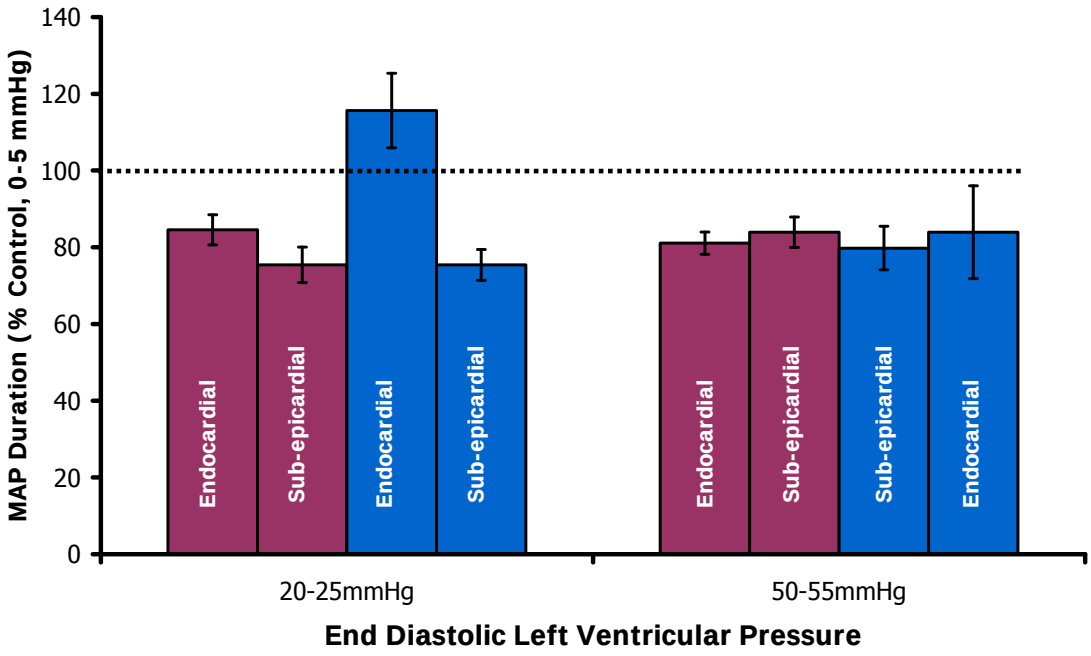

Figure 56: Effect of Temperature on MEF in the Guinea Pig Heart (APD_{50})

Figure showing effect of temperature and stretch on sub-epicardial and endocardial MAP APD_{50} relative to control conditions (no stretch, EDLVP = 0-5 mmHg). Decreasing perfusion temperature to 32 °C (blue columns) had no effect on stretch-induced changes at 20-25 or 50-55 mmHg EDLVP ($p > 0.05$, ANOVA GLM, $n = 4$). Burgandy columns denote normal physiological Langendorff temperature (37 °C).

DISCUSSION

Unfortunately the present results proved to be somewhat inconclusive. Of the 8 Langendorff experiments involving the guinea pig heart and effect of temperature on stretch-induced changes in MAP morphology, only 4 were successful (see Figure 54, Figure 55). As with many of the experiments throughout this thesis, water quality was a considerable problem encountered and often rendered hearts unsuitable for experimentation within minutes of initiating perfusion on the Langendorff setup.

Through manipulation of the perfusate temperature, the present results from both guinea pig and rat Langendorff perfused hearts suggest that TREK-1 channels contribute little to the stretch-induced reductions in action potential duration. Whilst this may seem surprising (and possibly even a bold statement) at first, given the suggested role of TREK-1 channels as a background current involved in controlling the excitability of the membrane (Maingret *et al.*, 2000a; Maylie & Adelman, 2001) and the

substantial number of regulatory molecules of the channel (Maingret *et al.*, 1999b, 2000b; Koh *et al.*, 2001; Patel *et al.*, 2001; Terrenoire *et al.*, 2001; Honore *et al.*, 2002; Danthi *et al.*, 2003; Kang *et al.*, 2005) it would be near impossible to predict the functional status of these channels and their ability to contribute effectively to the stretch response. Likewise, assuming that elevated stretch is suppressing coronary perfusion and the hypoxic nature of the Langendorff heart, it is possible that these channels are already inactivated and unable to make a strong contribution to the overall observed response (Miller *et al.*, 2003) although this has been challenged (Buckler & Honore, 2005). Evidence for this may appear in other models like the implication of TREK-1 is in induction of general anaesthesia. Knockout of TREK-1 protein expression in mice resulted in a small 10s delay to reach anaesthesia compared to control mice (Heurteaux *et al.*, 2004). Whilst this observation implicated TREK-1 channels in determining membrane excitability, a 10s change in the onset of anaesthesia may suggest that the channels contribute only a small current to the overall stability of the membrane potential. Since at physiological temperatures the open probability of these channels is quite high (Maingret *et al.*, 2000a; Kang *et al.*, 2005), then the current they contribute to membrane stability must be quite low to achieve this affect. This possibility is reflected in the present results where a reduction in the heart temperature should have, in theory, increased the dynamic range over which TREK-1 channels could induce a stretch-current. The present results suggest that either (a) the TREK-1 current contributes little to the stretch effect regardless of temperature or (b) that the stretch and temperature sensitivity of the ion channels are un-related and that stretch at lower temperatures does not result in a greater shift in open probability from the lower Po established by the low operating temperature. The observation that TREK-1 knockout had minimal effect an induction of anaesthesia (but did result in greater incidence of epilepsy) suggests that stretch-activation of the hyperpolarising current of TREK-1 is unlikely to account for the significant reductions in APD observed with extreme stretch.

Another possibility may be a combination of ischaemic and TREK-1 regulation by mechanisms other than stretch. TREK-1 is known to be activated by intracellular acidosis (Honore *et al.*, 2002) and metabolised lysophospholipids (Patel *et al.*, 1998; Maingret *et al.*, 2000b; Patel *et al.*, 2001). Localised ischaemia may bring liberation of lysophospholipids from the myocardium and hence TREK-1 activation in a manner similar to that suggested for protection of the brain during metabolic stress (Patel *et*

al., 2001; Honore *et al.*, 2002). The end result would be TREK-1 induced action potential shortening by means other than direct stretch-activation. This however, does not directly address the observations previously made at two different myocardial temperatures.

GLIBENCLAMIDE MODULATION OF STRETCH RESPONSE

INTRODUCTION

In the majority of previous experiments in the rat heart, little or no effect on MAP morphology has been observed at modest end diastolic pressures in the left ventricle except in endocardial recordings. As end diastolic pressure increases toward that of the pressures used to perfuse the coronary vessels of the heart, coronary flow of the left ventricle is likely to be affected (Allaart & Westerhof, 1996; Heslinga *et al.*, 1997; Westerhof *et al.*, 2006). As a result, it remains possible that the present observation of stretch-induced decreases in MAP duration may be the result of local ischaemia induced by the physical pressure that the balloon exerts on the coronary vessels at EDLVPs of 50-55mmHg. In addition, it is recognised that endocardial tissue is more sensitive to ischemic conditions than the epicardial tissue and this may explain the previous results demonstrating larger reductions in endocardial action potential durations as a result of stretch (Westerhof *et al.*, 2006). It would also be reasonable to suggest that as EDLVP increases from moderate to elevated levels of stretch, the degree of flow inhibition through the coronary vessels of the left ventricle would be proportional, and hence so would the response (be it through K_{ATP} channel activation or another undefined mechanism).

The aim of this second set of experiments was to establish if the action potential shortening observed in the rat model with elevated stretch is partly due to localised/global ischaemia of the LV due to activation of K_{ATP} channels that open in response to decreased O_2 supply during low perfusion periods. To test this theory,

Glibenclamide sulphate was used to block the activation of K_{ATP} channels and observe any remaining stretch-induced response through the activation of NSACs and KSACs.

METHODS

Five male Sprague Dawley rats of weights 400-450g were anaesthetised with Ketamine and Domitor as described previously in chapter 3. Hearts were mounted on a Langendorff perfusion setup and retrogradely perfused at a constant flow rate that equated to a coronary perfusion pressure of 60-70 mmHg (~14.7 ± 1.5 ml/min). Hearts were paced through the right atrium at a rate of 5Hz, twice threshold with 10 ms bipolar pulses. Glibenclamide (Sigma-Aldrich) was made up as a stock solution (10 mM) in DMSO and added to the perfusate to make a final concentration of 10 μM when required. MAPs were recorded using the transmural electrode and responses to stepwise increases in EDLVP recorded as previously described for rat and guinea pig. Monophasic action potential durations at 20, 50 and 80% repolarisation were calculated and tested statistically using general linear model analysis of variance.

RESULTS

Under basal conditions, perfusion with 10 μM glibenclamide did not significantly affect any of the action potential parameters (20, 50 and 80% repolarisation) for epicardial and endocardial monophasic action potentials (ANOVA GLM, $p > 0.05$). As with previous experiments, a step in EDLVP from basal (0 mmHg) to moderate stretch (20-25 mmHg) did not significantly alter epicardial action potential duration nor amplitude. By contrast, 10 μM glibenclamide blunted any reduction in epicardial APD_{80} seen at elevated levels of stretch (EDLVP = 50-55 mmHg, see Figure 57 - Figure 59).

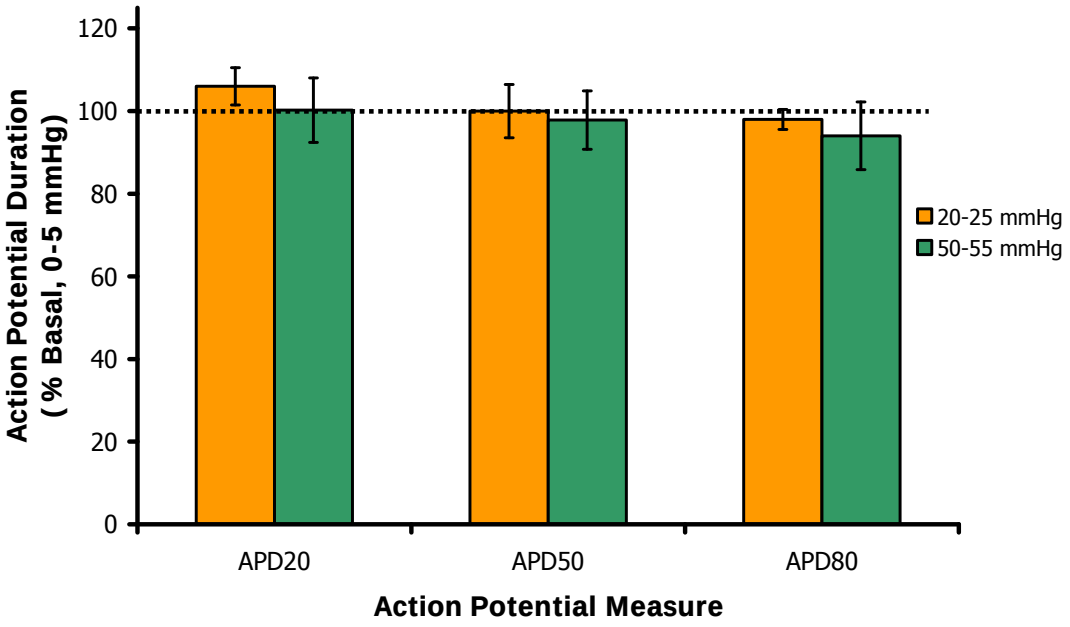

Figure 57: Modulation of Sub-epicardial Stretch Response by Glibenclamide

Figure showing the effect of stretch and 10 μM glibenclamide on stretch-induced changes in sub-epicardial monophasic action potential recordings at 20, 50 and 80 % repolarisation in rat hearts (n=5). Error bars indicate ± SEM, each measurement is the average of 10 action potentials at each level of stretch. There was no significant deviation in MAP durations between basal/control conditions and 20-25 or 50-55 mmHg EDLVP in the presence of glibenclamide ($p > 0.05$, ANOVA GLM).

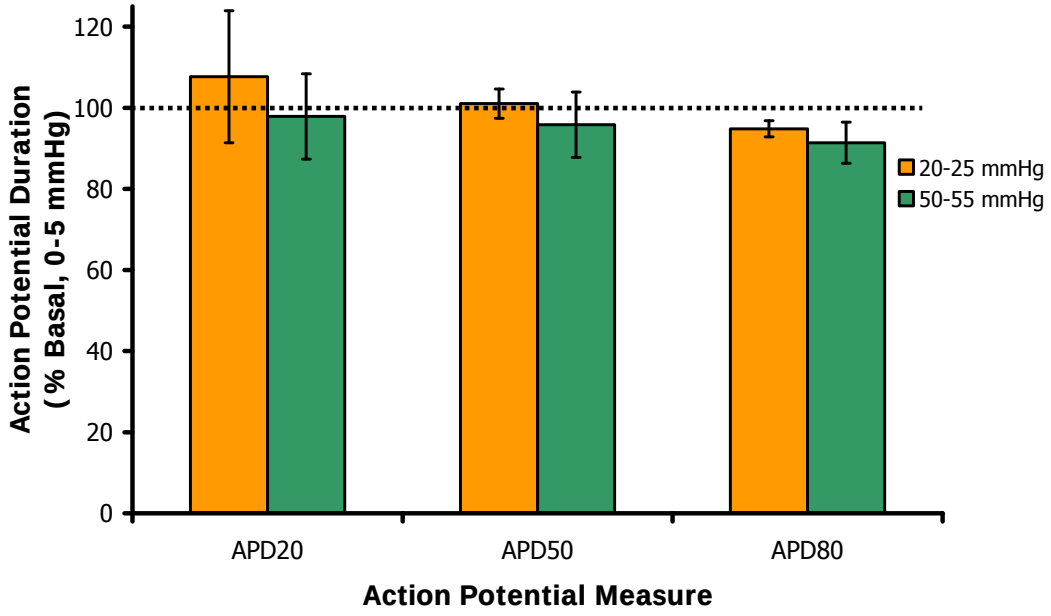

Figure 58: Modulation of Endocardial response to Stretch by Glibenclamide

Figure showing the effect of stretch and 10 μM glibenclamide on stretch-induced changes in endocardial monophasic action potential recordings at 20, 50 and 80 % repolarisation in rat hearts (n=5). Error bars indicate ± SEM, each measurement is the average of 10 action potentials at each level of stretch. There was no significant deviation in MAP durations between basal/control conditions and 20-25 or 50-55 mmHg EDLVP (p > 0.05, ANOVA GLM).

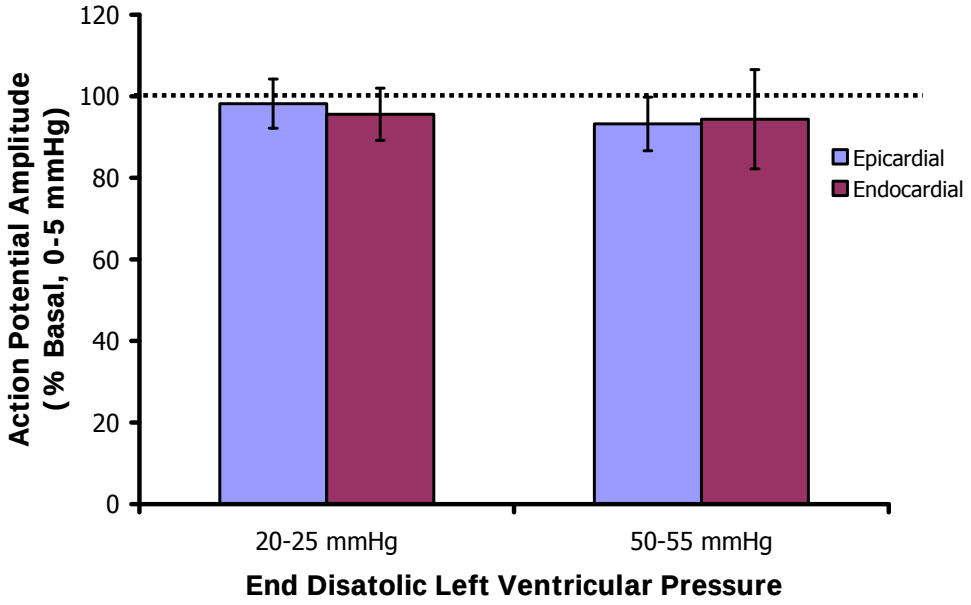

Figure 59: Effect of Stretch and Glibenclamide on MAP Amplitude

Figure showing the effect of 10 μM glibenclamide on stretch-induced changes in endocardial and sub-epicardial monophasic action potential amplitude in rat hearts (n=5). Error bars indicate ± SEM, each measurement is the average of 10 action potentials at each level of stretch. There was no significant deviation in MAP amplitudes between basal/control conditions and 20-25 or 50-55 mmHg EDLVP (p > 0.05, ANOVA GLM).

DISCUSSION

Previously in endocardial recordings (see Figure 39) APD_{80} fell to 82 ±7.1 and 60 ±4.7 % of basal/control conditions for moderate (20-25 mmHg EDLVP) and elevated stretch (50-55 mmHg EDLVP) respectively,whilst 10 μM glibenclamide in the present results greatly blunted this response. Similarly, under control conditions where elevated stretch previously decreased epicardial MAP at 80 % repolarisation to 79 ± 5.6 % of the control (see Figure 38), no change in duration was observed in the presence of glibenclamide. These results suggest that the activation of K_{ATP} channels may play an important role in the observation of MEF in the Langendorff perfused heart. At present there remain two probably and likely explanations of this result.

- Firstly, at least one other author has suggested and demonstrated that K_{ATP} channels are stretch sensitive in the isolated cardiomyocyte model (Van

Wagoner, 1993). Moreover, these channels have been implicated in stretch-induced responses of the heart (Link *et al.*, 1999).

- Secondly, it remains plausable that reductions in action potential duration associated with elevated stretch where EDLVP > 50 mmHg may infact be suppressing coronary flow to endocardial tissues in the rat heart (Allaart & Westerhof, 1996; Heslinga *et al.*, 1997; Westerhof *et al.*, 2006). This is likely to promote local/global ischamemia in the pressure affected regions since coronary flow was set to a constant pressure of between 60-70 mmHg (near that exerted on the endocardial tissue by the left ventricular balloon at elevated pressures).

One possible means of separating ischaemic conditions from contributing to stretch-related changes in the cardiac action potential could be to use isolated papillary muscles in a perfusion bath. In this setup, a constant flow of perfusate nutrients and oxygenation can be provided regardless of the quantity of stretch applied to the muscle. Although it would be somewhat more ischaemic than an entire isolated Langendorff perfused heart, these conditions would at least remain constant throughout tension manipulations. In addition, with the use of intracellular impalements, direct variations in membrane potential can be recorded independently of volume:surface changes that are likely to occur with stretch in the whole heart. These changes which likely thin the wall under the MAP electrode could also contribute to apparent changes in the MAP during stretch manipulations by reducing the number of cells under the recording electrode.

SUMMARY

In this chapter, several novel methods of modulating MEF responses were applied to the langendorff perfused rat heart. The first section described the effects of cardiac cooling and the proposed reduction in KSAC open probabilty and the implications this would have in the possible exaggeration of MEF responses to stretch. The second section looked at the presence of MEF using the transumural electrode to record changes in sub-epicardial and endocardial MAPs in the Langendorff perfused guinea pig heart. This was done since MEF-induced changes were observed in the rat heart at elevated levels of EDLVP in the previous chapter, and thus it was expected that a larger response may be present and detectable in the guinea pig heart using the same electrode technique. Indeed, the results in the second section of this chapter show that

the transmural electrode was able to detect a reduction in action potential duration at moderate (EDLVP = 20-25 mmHg) as well as elevated (50-55 mmHg) levels of stretch in the guinea pig heart, the former result not seen previously in the rat heart. The last section of this chapter investigated the possible involvement of ischaemia and or K_{ATP} channels (which may or may not be stretch sensitive) in the observed MEF responses in the rat heart. The results thus far are consistent with the observations that K^+_{ATP} channels are stretch sensitive (Van Wagoner, 1993) and the observation that glibenclamide had a significant inhibitory effect of *commotion cordis* and related arrhythmia induced by blunt impacts to the chest of swine (Link *et al.*, 1999). However, it remains possible, that TREK-1 channels may be involved through indirect mechanism as discussed above.

It can be seen from the examples and data presented in this chapter that

- MEF is differentially observed across sub-epicardial and endocardial layers in the langendorff perfused rat heart. However, this did not appear to be the case with the guinea pig heart.

- The guinea pig heart is more sensitive to stretch than previously thought with reductions in sub-epicardial and endocardial MAPs observed at modest levels of EDLVP stretch (20-25 mmHg), a result not previously seen in the guinea pig heart using suction electrodes, or the rat heart using suction and transmural electrodes.

- In addition to this observation, 80 μM streptomycin did not inhibit the stretch-induced reduction in action potential duration in the guinea pig heart.

CONCLUSION

The present data show that the transmural method of recording MAPs is especially good at detecting MEF induced changes in cardiac electrophysiology in the isolated Langendorff perfused heart, more so than surface MAP electrode methods. Interestingly, a change in cardiac temperature did not affect the magnitude of reduction in action potential duration (as measured by APD at 20, 50 or 80 % repolarisation) following moderate (20-25 mmHg) or extreme stretch (50-55 mmHg) for either endocardial or sub-epicardial recordings in either the rat or guinea pig heart. Given the added temperature sensitivity of TREK-1 channels, this would suggest

stretch-induced action potential shortening, where present, is the result of another mechanism independent of TREK-1 Channels. The observation that Glibenclimide abolished stretch-induced shortening of the rat MAP suggests that KATP channel activation may play an important roll. Activation of this channel may be directly through stretch or indirectly through elevated levels of EDLVP reducing coronary perfusion and inducing semi-global ischaemic conditions readily modulated by glibenclamide.

CHAPTER 9: MEF IN ISOLATED MUSCLE PREPARATIONS

The ukulele man (playing the banjolele)
WWII English singer & comedian: George Formby
(May 26, 1904 – March 6, 1961)

Chapter 9 - Table of contents

INTRODUCTION

In the previous chapters, the contribution of MEF to mechanical alterations in contractility was observed in response to stepwise increases in EDLVP in the whole heart. In this chapter, intracellular microelectrode recording techniques are used to explore the phenomenon of MEF during manipulation of left ventricular trabeculae and papillary muscles. The first part of the chapter gives a background to intracellular microelectrode recording techniques and justification. The next section deals with a series of experiments to determine the contribution of MEF the stretch-induced increases in cardiac contractility described by the Frank-Starling response. The final part of the chapter provides a detailed discussion of the experimental results as they relate to one another and to the present understanding from past and current literature. It also includes a brief conclusion based on the present results and an explanation. The aim of the present chapter was to demonstrate the contribution of MEF to electrophysiological changes throughout the cardiac cycle in isolated muscle strips.

BACKGROUND

Intracellular Recordings

In previous chapters monophasic action potentials were used to test the involvement of MEF in the cardiac cycle. Monophasic action potentials have the advantage of being non-intrusive. However, their morphology only reflects the time course changes in membrane potential and does not provide a direct means of monitoring electrophysiological changes. Despite their common place in electrophysiological applications, dispute still exists regarding the application of MAP electrodes and their theoretical function or how the monophasic action potential is actually generated from cardiac electrical activity (Franz, 1999; Knollmann *et al.*, 2002; Kadish, 2004; Kondo *et al.*, 2004; Franz, 2005). More so, it has been suggested that monophasic action potentials should be used only to estimate action potential duration (Kanaan *et al.*, 1991) and that there is no means of improving on the accuracy of the monophasic action potential recorded using this technique (Kondo *et al.*, 2004). Even those who endorse their use in the clinical setting openly advise that upon analysis, there is only a high degree of similarity between MAPs and other conventional recording methods, and that the two are not always entirely super-imposable (Franz *et al.*, 1986). More importantly, these variations between MAP and action potentials recorded by conventional means may be of greater significance in animal models with shorter action potential durations compared to the human action potential which can last 500ms or more. This chapter introduces the method of intracellular recordings from tissue samples. Impalement of living cardiomyocytes within cardiac tissue with microelectrodes (intracellular electrodes) is a useful approach and provides a direct measure of changes in membrane potential. This method differs from the MAP technique in many aspects, primarily, that a glass saline-filled microelectrode with a tip only a few microns in diameter is blindly impaled into individual cells of a tissue sample. This has several advantages over the MAP technique as well as some minor disadvantages. The primary advantage of this technique is that it gives the experimentalist direct access to the cell's interior and thus an exact recording of changes in membrane potential (albeit through a high resistance electrode tip). By contrast, the MAP electrode causes damage to the tissue surface, creating an electrically inactive sink from which differences between electrically active cells and the inactive cells. This means that the MAP is only a representation of changes in membrane potential over time and not a direct recording of the actual membrane

potential. In addition, because of the MAP electrode size the recording is a summation of the electrical changes in the cells under electrode contact whilst intracellular recording is representative of only one cell (although its electrical activity is likely influenced by neighbouring cells to which it is electrically coupled). Thus, intracellular recordings provide direct information on action potential amplitude, up-stroke velocity, resting membrane potential as well as the typical durations at 20, 50 and 80 % repolarisation (Edelman *et al.*, 2004). In comparison, only action potential durations of MAP recordings are thought to accurately reflect those obtained using microelectrode techniques. There is no actual value obtained for amplitude as this represents the number of cells being recorded from, no resting membrane potential since MAPs record the difference between a current and sink combination.

The major disadvantage of using intracellular recordings to observe SAC-induced MEF responses is the difficulty in maintaining proper microelectrode impalements. This is especially true when using floating microelectrode techniques on beating isolated Langendorff perfused hears and during mechanical stretch of isolated preparations. As a result, direct evidence from intracellular action potential studies of electrophysiological changes brought about by SAC function and MEF are somewhat limited.

Of interest in one of the first successful microelectrode studies is that mechanical stretch failed to induce any changes in action potentials of cat papillary muscles at any point in the cardiac cycle (Lab, 1980). This result is consistent with that observed using MAP electrode techniques in chapter 4 of the present research. However, Lab also demonstrated that release of stretch when the papillary muscle tension was near its peak prolonged repolarisation or induced a transient depolarisation of resting membrane potential (RMP). This latter effect may be related to Ca^{2+} release from the tropo-myosin complex as a result of altered Ca^{2+} affinity. As a result, the Ca^{2+} may activate the Na^{+}/Ca^{2+} exchanger or other mechanisms to induce a temporary cellular depolarisation in response (Perez *et al.*, 2001).

Previously in isolated cardiomyocytes, others have shown that stretch can depolarise the cellular resting membrane potential up to -30 mV (White *et al.*, 1993) whilst others suggest that this may be the case with modest stretch, extreme stretch has the opposite effect of hyperpolarising the resting membrane potential (Nakagawa *et al.*, 1988). Both observations were made using intracellular techniques, although, the

former was performed on isolated cells using patch clamp methods, whilst the later was performed on isolated ventricular papillary muscles. Both techniques are technically challenging and subject to the development of movement artefacts. In a similar experiment performed using isolated guinea-pig papillary muscles, mild to moderate stretch (100-120 % maximum length) evoked a 6 mV depolarisation in resiting membrane potential. By contrast, extreme stretch (130-140 % maximum length) resulted in a 5 mV hyperpolarisation of resting membrane potential in the same preparation (Nakagawa *et al.*, 1988).

In the present study, isolated papillary muscles are used in conjunction with microelectrode or intracellular recordings to observe mechanosensitive changes in the action potential and resting membrane potential brought about by axial stretch. First introduced in 1959 (Abbott & Mommaerts, 1959), the isolated papillary muscle is a widely accepted preparation and a popular choice for observing both mechanical and electrophysiological (including Mechano-electric) responses (Lab, 1980; Hennekes *et al.*, 1981; Allen & Kurihara, 1982; Nakagawa *et al.*, 1988; Gamble *et al.*, 1992; Schouten *et al.*, 1992; Lab *et al.*, 1994; Bluhm & Lew, 1995; Allaart & Westerhof, 1996; Dijkman *et al.*, 1997a; Heslinga *et al.*, 1997; Bluhm *et al.*, 1998; Dijkman *et al.*, 1998; Alvarez *et al.*, 1999; Nicolosi *et al.*, 2001; Lamberts *et al.*, 2002a; Fulop *et al.*, 2003; Cingolani *et al.*, 2005). The main advantage of the isolated papillary muscle preparation is that it is an extension of the endocardium and unlike other isolated or dissected trabeculae/endocardial tissues, does not suffer extreme damage upon excision.

METHODS

Rat hearts from male Sprague Dawley rats (350-450 g) were prepared as per chapter 3. Hearts were mounted on the cannula of a Langendorff setup and allowed to beat spontaneously whilst perfused with HEPES buffer containing 1.5 mM $CaCl_2$ at 12 ml/min until cleared of blood. Once clear of blood, heating of the perfusate was stopped, the heart allowed to cool for 2-3 minutes and cold HEPES buffer containing 20 mM BDM and 1.5 mM $CaCl_2$ run through the heart. The heart was then removed and placed into ice cold HEPES buffer of the same composition and the left ventricle dissected open and the left ventricular papillary muscles and several trabeculae removed from the left ventricular free wall. All muscle preparation were then kept oxygenated in chilled HEPES buffer on ice until used (within several hours). Trabeculae or papillary

muscles were anchored at one end using one or two fine gauge needles to the base of a purpose made perfusion chamber. Needles remained in place due to a fine coating of silguard (Dow Corning, USA) along the base of the perfusion chamber. The other end of the muscle preparation was hooked onto a fine piece of enamelled wire (diameter 0.125 mm) attached to a micro-force transducer (MLT0202, AD Instruments Australia). Muscle strips remained submerged in HEPES buffer containing 2 mM $CaCl_2$ bubbled with O_2 and perfused at a rate of 10-12 ml/min. Suction was used to remove excess bath solution and to prevent overflow. Samples were field stimulated along the length of the muscle by attaching one of the muscle anchoring needles to one end of a stimulator, whilst chlorided Ag wire ran along the muscle length of the opposite side. Muscle preparations were stimulated at 1 Hz, 2-3 times threshold (generally 3 V) with 1ms bipolar pulses and allowed to stabilise following BDM washout (generally within 15 minutes from start of perfusion).

Glass microelectrodes were pulled from aluminosilicate glass capillary tubes (1 mm OD, 0.5 mm ID, Harvard Instruments - SDR, Australia) using a computer (running PC-DOS 4.0) directed, multi-stage horizontal puller (BB-CH-PC Microelectrode puller, Mecanex SA Switzerland) and cooled at formation with industrial air (pressure = 300 mmHg). Transmembrane action potentials or intracellular recordings were made simultaneously with tension. Glass microelectrodes were filled with filtered (0.2 μm) 3 M KCl and mounted onto a purpose made flexible wire composed of a short length of fine silver wire wound into a spring-like configuration to improve flexibility. Microelectrode tip resistances were above 10-15 Meg ohms and connected to a computer running Chart V software (AD Instruments Australia) by means of a biological amplifier (Model 725 Microprobe Amplifier, W-P Instruments, USA) through a fine length of chlorided silver wire. All recordings were saved and analysis made offline using similar methods to those defined for MAP recordings in chapter 3. Successful intracellular impalements were made by tapping gently on the stage of the micromanipulator (Leitz, Germany) holding the intracellular electrode as previously described elsewhere (Edelman *et al.*, 2004). Only recordings with a rapid change in voltage from 0 V (bath potential) to < -60 mV resting membrane potential were accepted as a successful impalement.

RESULTS

Preliminary Experiments

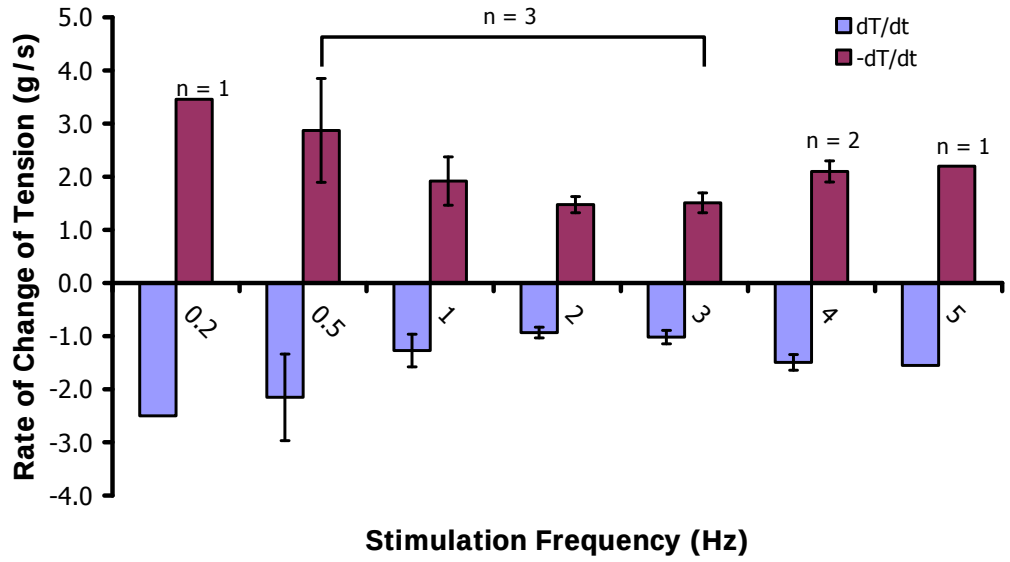

Figure 60: Effect of Stimulation Frequency on Papillary Muscle Contraction Force

Figure showing effect of changes in bath perfusion on isolated papillary muscle contractility at various pacing frequencies. Bath flow rate was tested between 6 and 18 ml/min in random order for a total of 3 papillary muscle preparations. No significant difference in contractility was observed in response to perfusion rate changes when muscles were held at a constant tension of 0.2 g ($p > 0.05$, ANOVA GLM).

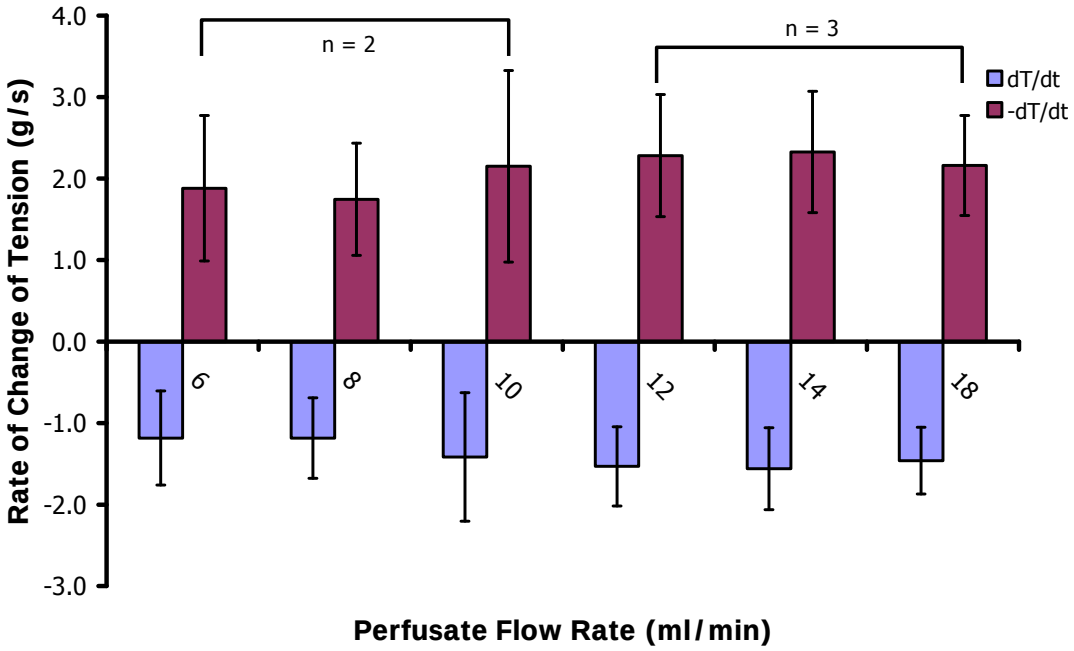

Figure 61: Effect of Perfusion Rate on Papillary Muscle Contractility

Figure showing effect of stimulation frequency on contractility of 3 papillary muscle preparations held at a constant tension of 0.2 g. Frequencies above 1 Hz were difficult to record stable base lines for determining resting membrane potential, whilst frequencies of 0.5 Hz and below were difficult to impale due to considerable contractile movement of the isolated muscle preparation.

For the following experiments, isolated rat papillary muscle preparations were perfused at a rate of 10-12 ml/min. Higher flow rates generated too much turbulence and made obtaining stable intracellular impalements extremely difficult. In addition, higher flow rates had no considerable or obvious effect on the mechanical activity of the isolated papillary muscle preparations (see Figure 61). In addition, papillary muscles in the following sections are stimulated at 1.0 Hz 3x threshold using field stimulation methods. 1 Hz was chosen since it was the easiest to obtain stable recordings from and provided adequate baselines for determining resting membrane potential between contractions. Lower stimulation frequencies allowed for too much contractile movement and generally introduced artefacts or resulted in loss of intracellular recording whilst higher frequencies did not allow enough time for reliable measurement of resting membrane potential before the next action potential was initiated (see Figure 60).

Effect of Stretch

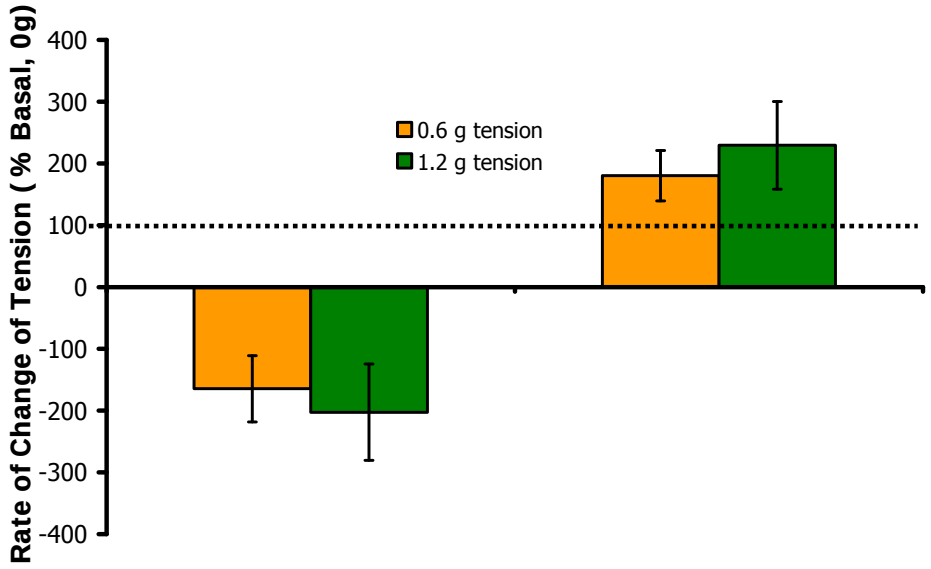

Figure 62: Effect of Stretch on Papillary Muscle Contractility

Figure showing effect of length (tension) changes on contractility of papillary muscles isolated from rat left ventricle (n=7). Data obtained at 0.6 and 1.2 g of tension are expressed as a percentage of contractile strength recorded at 0- 0.1 g tension. As can be seen, contractility significantly increased upon stretch with 1.2 g tension. By contrast, rate of relaxation was not affected (significance tested using ANOVA GLM and Tukey's post hoc pair wise comparisons).

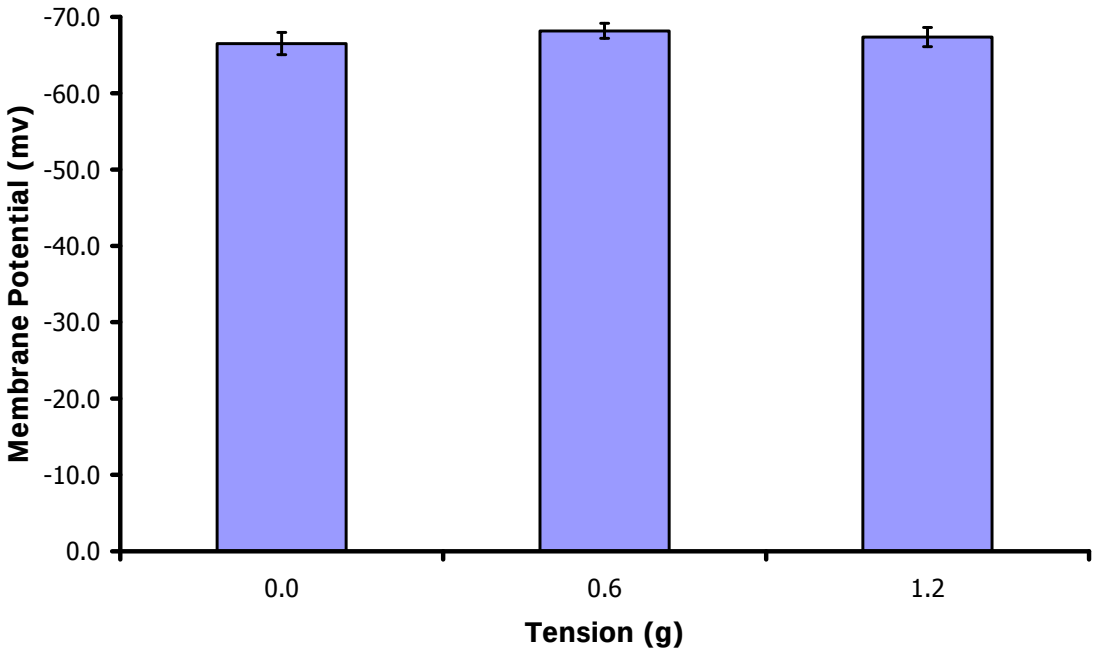

Figure 63: Effect of Stretch on Papillary Muscle Resting Membrane Potential

Figure showing effect of length (tension) changes on resting membrane potential of papillary muscles isolated from rat left ventricle (n=8). There was no significant change in resting membrane potential between either of the tensions tested (0.6 and 1.2 g) compared to basal conditions (0 g) despite a significant increase in contractility ($p > 0.05$, ANOVA GLM).

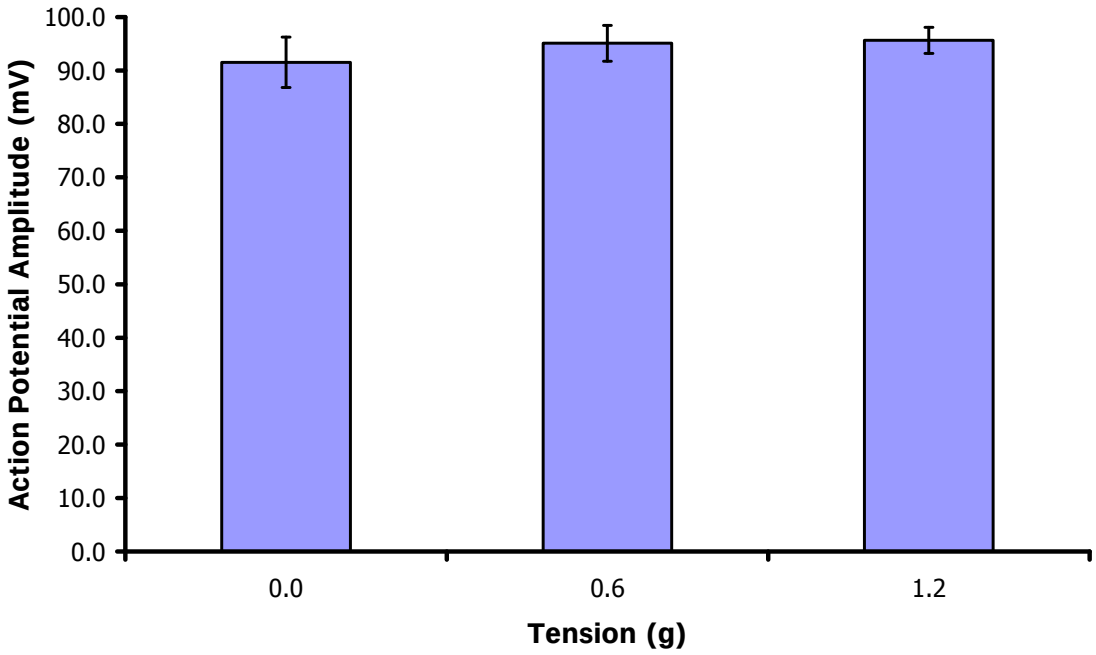

Figure 64: Effect of Stretch on Papillary Muscle Action Potential Amplitude

Figure showing effect of length (tension) changes on action potential amplitude of papillary muscles isolated from rat left ventricle (n=8). There was no significant change in action potential amplitude between either of the tensions tested (0.6 and 1.2 g) compared to basal conditions (0 g) despite a significant increase in contractility. In addition, action potential overshoot did not significantly differ between groups (25.3 ± 3.6, 26.9 ± 2.6 and 28.3 ± 2.8 for 0, 0.6 and 1.2 g respectively, p > 0.01 ANOVA GLM).

There was no significant change in action potential duration between either of the tensions tested (0.6 and 1.2 g) compared to basal conditions (0 g) despite a significant increase in contractility (see Figure 62 - Figure 65). In addition, despite considerable variation in APD_{80} of raw data, when expressed relative to basal conditions (0 g tension) to minimise inter-rat variability, stretch (0.6 or 1.2 g) did not significantly change APD_{80}.

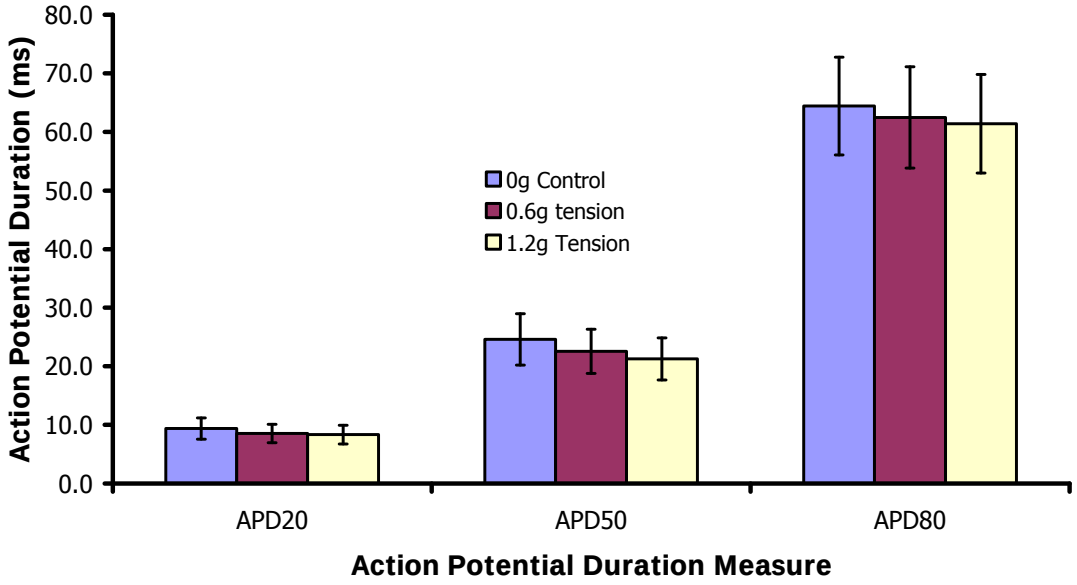

Figure 65: Effect of Papillary Stretch on Intracellular Action Potential Durations

Figure showing variability in transcellular action potential durations in presence and absence of stretch at 20, 50 and 80 % repolarisation. There was no statistically detectable change in action potential duration with stretch ($p > 0.05$, ANOVA GLM, n=8).

Likewise, exposure of papillary muscles to the various SAC modulators in a similar manner used in Langendorff rat hearts revealed no change in Action Potential parameters, consitent with contol conditions (see Figure 66 - Figure 68). In these experiments, 1 mM BDM was used to suppress contractile movements and ensure that contractions were not interfering with the quality of intracellular recordings and hence, the ability to accurately depict changes in amplitude, duration and resting membrane potential.

Effect of SAC Modulators

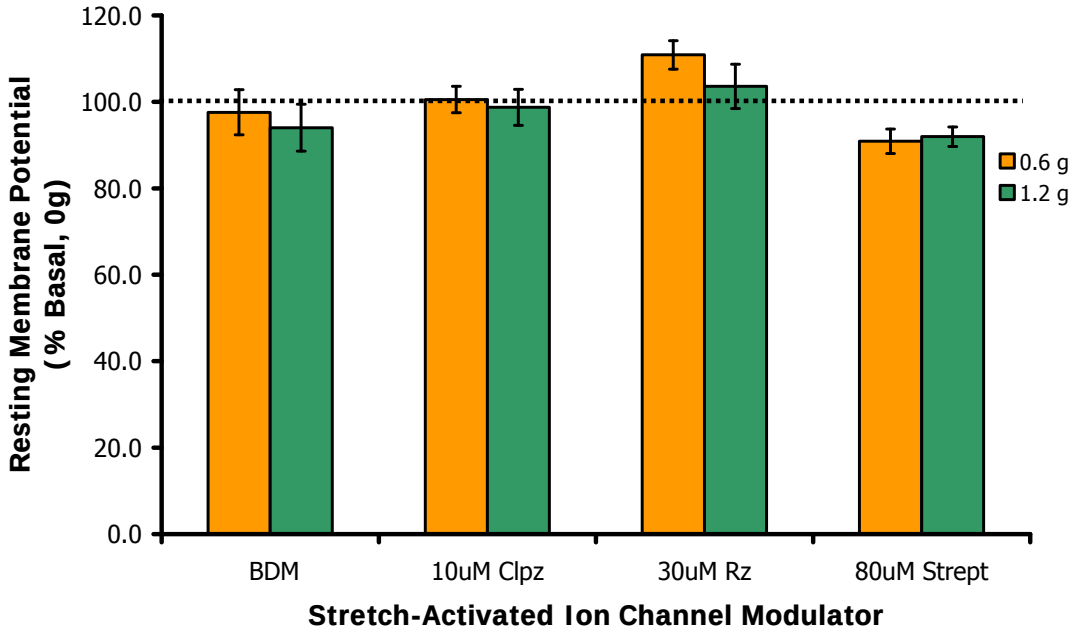

Figure 66: Effect of SAC Modulators on RMP in stretched Papillary muscles

Figure showing effect of SAC modulators (Chlorpromazine TREK-1 inhibitor (n= 6), Riluzole TREK-1 activator (n= 6) and streptomycin NSAC inhibitor (n= 8) and 1 mM 2,3 BDM (2,3-butanedione monoxime to suppress contractility) on papillary muscle resting membrane potential from rat hearts. Error bars indicate ± SEM (each measurement in each heart is an average of 10 action potentials at each level of stretch. Statistical differences were calculated compared to control conditions (no stretch, EDLVP = 0-5 g) using a ANOVA GLM ($p > 0.05$). In general, stretch and modulators of SACs had no effect on resting membrane potential of isolated papillary muscles. By contrast, streptomycin induced a partial hyperpolarisation in RMP from -71 ± 3.2 to – 76. ± 1.5 mV ($p < 0.05$, ANOVA GLM with Tukey's post hoc test).

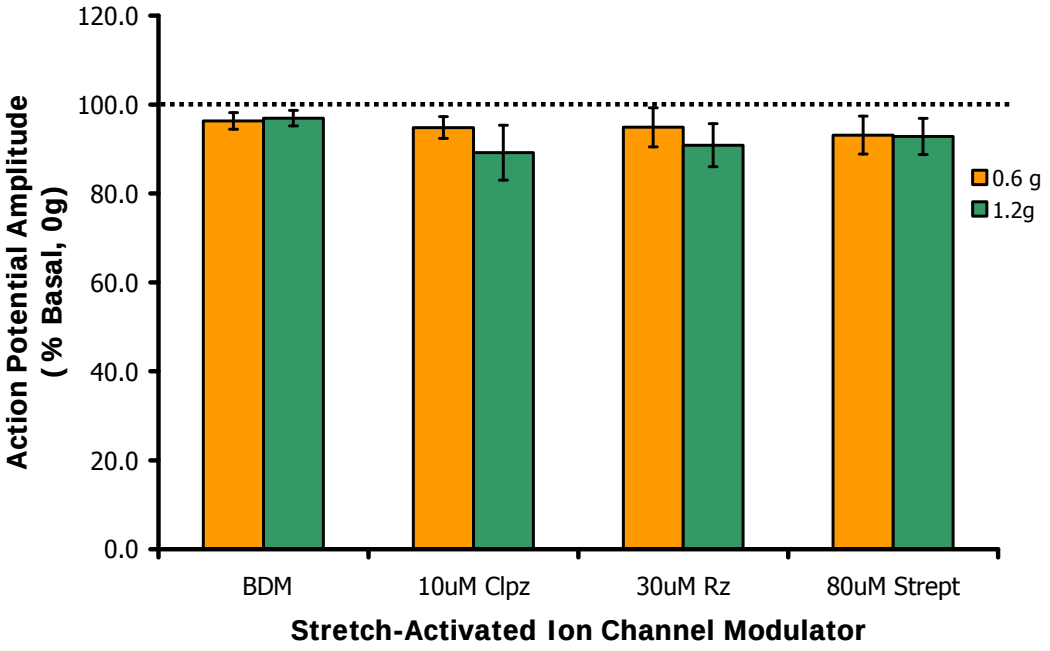

Figure 67: Effect of SAC Modulators on Amplitude in stretched Papillary muscles

Figure showing effect of SAC modulators (Chlorpromazine TREK-1 inhibitor (n= 6), Riluzole TREK-1 activator (n= 6) and streptomycin NSAC inhibitor (n= 8) and 1 mM 2,3 BDM (2,3-butanedione monoxime to suppress contractility) on papillary muscle action potential amplitude from rat hearts. Error bars indicate ± SEM (each measurement in each heart is an average of 10 action potentials at each level of stretch. Statistical differences were calculated compared to control conditions (no stretch, EDLVP = 0-5 g) using a ANOVA GLM ($p > 0.05$). In general, stretch and modulators of SACs had no effect on action potential amplitude. By contrast, streptomycin induced an increase in action potential amplitude from 96 ± 4.8 to 101 ± 5.5 mV ($p < 0.05$, ANOVA GLM with Tukey's post hoc test).

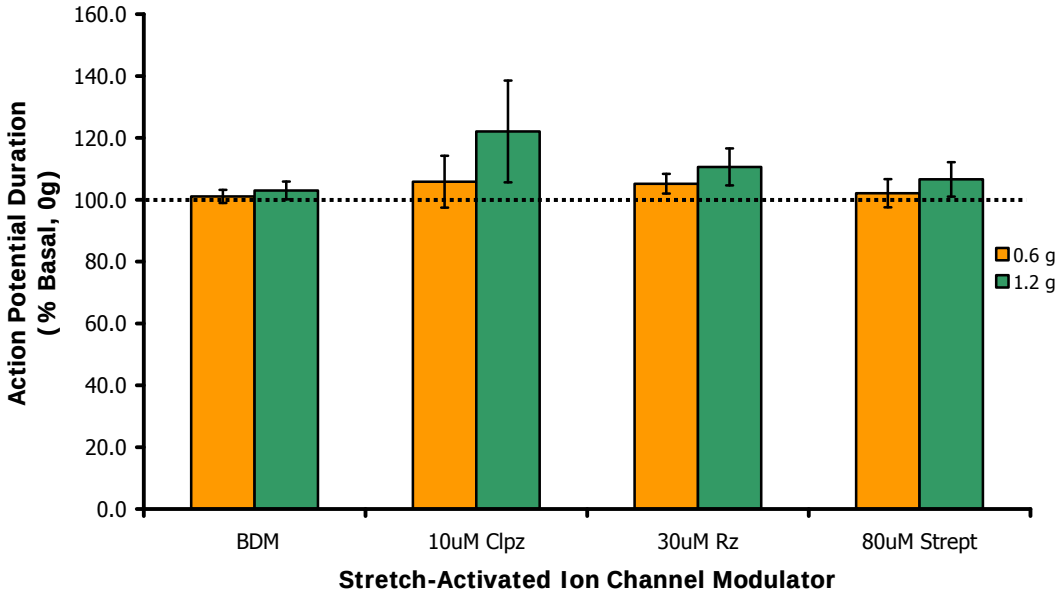

Figure 68: Effect of SAC Modulators on Papillary Muscle APD_{80} During Stretch

Figure showing effect of SAC modulators (Chlorpromazine TREK-1 inhibitor (n= 6), Riluzole TREK-1 activator (n= 6) and streptomycin NSAC inhibitor (n= 8) and 1 mM 2,3 BDM (2,3-butanedione monoxime to suppress contractility) on papillary muscle action potential duration at 80 % repolarisation from rat hearts. Error bars indicate ± SEM (each measurement in each heart is an average of 10 action potentials at each level of stretch. Statistical differences were calculated compared to control conditions (no stretch, EDLVP = 0-5 mmHg) using a ANOVA GLM ($p > 0.05$). In general, stretch and modulators of SACs had no effect on action potential duration at 20, 50 or 80 % repolarisation ($p > 0.05$, ANOVA GLM).

DISCUSSION

Whilst at first these results may seem somewhat contradictory to those observed using Endocardial-Epicardial MAP techniques of Chapter 5, they closely reflect those observations made using surface MAP techniques in Chapter 4. In addition, these results (obtained through reasonably rapid increases in tension) support the observations of (Lerman *et al.*, 1985; Calkins *et al.*, 1989) whereby no observable changes in diastolic potentials of MAP recordings were recorded with rapid ventricular inflations. More importantly, the present results are directly consistent with similar experiments conducted on isolated mammalian tissue samples in the past (Lab, 1980; Hennekes *et al.*, 1981; Kamkin *et al.*, 2000b).

The present results differ from the whole heart model in that inflations of EDLVP using an intra-ventricular balloon have the distinct possibility of inhibiting coronary flow and diverting it from the left ventricle as the EDLVP approaches the coronary perfusion pressure (Westerhof *et al.*, 2006). In the isolated papillary muscle preparation, whilst it could be considered more hypoxic, there are no flow-related changes in tissue perfusion since the preparation itself is not perfused (see Figure 61). This reduces the likelihood that MAP duration changes may reflect alterations in local or left ventricular wall perfusion and thus action potential shortening through K_{ATP} channel activation (Van Wagoner, 1993; Miyoshi *et al.*, 1996; Hamada *et al.*, 1998; Wirth *et al.*, 2000; Morrissey *et al.*, 2005).

Thus, the present results demonstrate that there are no electrophysiological consequences of physiological and pathophysiological levels of stretch in the isolated rat papillary muscle (see Figure 62 - Figure 68). This is consistent with the findings by (Lab, 1980) in cat papillary muscles and in rat atria (Kamkin *et al.*, 2000b), but in direct contrast to the observed results in similar guinea-pig preparations (White *et al.*, 1993).

During the present work, stretch of isolated papillary muscle preparations whilst recording action potentials often resulted in movement artefacts that resembled diastolic depolarisations. These were most obvious or occurred immediately prior to losing an intracellular impalement recording and were not reversed or abolished by releasing the stretch. The present results regarding the lack of consistent obvious diastolic depolarisations, except when corresponding with failing impalements is entirely consistent with that observed in intracellular impalements of atrial tissue by (White *et al.*, 1993; Tavi *et al.*, 1998). This study suggested that depolarization of the resting membrane potential (during diastole) was always associated with the changes in the cell-electrode impalement (resistance and capacitance) caused by the electrode leaving the cell. By excluding recordings demonstrating dramatic falls in seal resistance, Tavi *et al* were able to deduce that stretch was not associated with non-selective ion currents that altered resting membrane potential. Whilst this was not possible with the present setup used for intracellular recordings from the isolated papillary muscles, by excluding those recordings that displayed un-reversible depolarisation-like artefacts, a similar result regarding a lack in diastolic depolarisation with stretch was obtained. However, the current results differ from that of Tavi *et al*

and (White *et al.*, 1993) in that no action potential changes accompanied myocardial stretch.

With this in mind, a series of experiments was performed in the presence of 2,3-BDM since its inhibitory effects on mechanical movement help reduce the possibility of developing motion artefact during intracellular recordings without obvious electrophysiological changes (Kettlewell *et al.*, 2004), (see Figure 63 - Figure 68)

Like isolated cardiomyocytes, it remains possible that the isolated papillary muscle is an inadequate means of addressing the physiological importance of stretch-induced changes in cardiac electrophysiology. Whilst this method provides a higher complexity than isolated myocytes alone, the preparation may not be adequate as a result of differing action potential profiles and distribution of stretch-activated ion channels throughout the myocardium (Li *et al.*, 2002; Tan *et al.*, 2004). However, even with this in mind, since the largest changes in action potential duration were previously observed in the endocardium using transmural MAP electrodes, it was expected and not unreasonable to assume that a similar result would have been observed when using intracellular recording techniques of endocardial tissue. This clearly was not the case in the present result, suggesting stretch-induced changes in cardiac perfusion may play an integral role in observed MEF responses.

In spite of this, isolated cardiomyocytes have presented their own problems in reporting consistent stretch-related electrophysiological responses. Stretch of cardiomyocytes along the longitudinal axis has been shown to depolarise the cells during diastole (Hansen *et al.*, 1991; Kohl *et al.*, 1999; Zhang *et al.*, 2000) whilst others have suggested this is associated with poor impalement quality (White *et al.*, 1993; Tavi *et al.*, 1998). More interesting is the variations in APD with both decreases (White *et al.*, 1993) and increases (Zeng *et al.*, 2000) previously reported. In the present experiments, however, no such changes were observed.

CONCLUSION

Consistent with experiments performed in Langendorff perfused rat hearts, the present results demonstrate that there are no electrophysiological consequences of physiological and pathophysiological levels of stretch in the isolated rat papillary muscle. Despite this observation, 80 μM streptomycin was found to affect basic

electrophysiological properties by hyperpolarising resting membrane potential and marginally increasing action potential amplitude.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation for Dr Daniel Ninio of the Queen Elizabeth Hospital (and fellow PhD student) for his instruction on setting up the above experiments and the method of extracting data and analysis. His appreciation of the difficulty of the above experiments and general helpfulness throughout difficulties with electrode manufacture were most appreciated.

EFFECT OF TEMPERATURE ON MEF IN ISOLATED PAPILLARY MUSCLES

INTRODUCTION

In keeping with observations made in the Langendorff heart, a final set of experiments looking at the potential effects of temperature modulation of stretch-induced changes in isolated papillary muscle electrical activity were performed. Since the setup and experimental conditions were identical to that previously described, no methodological section is provided.

RESULTS

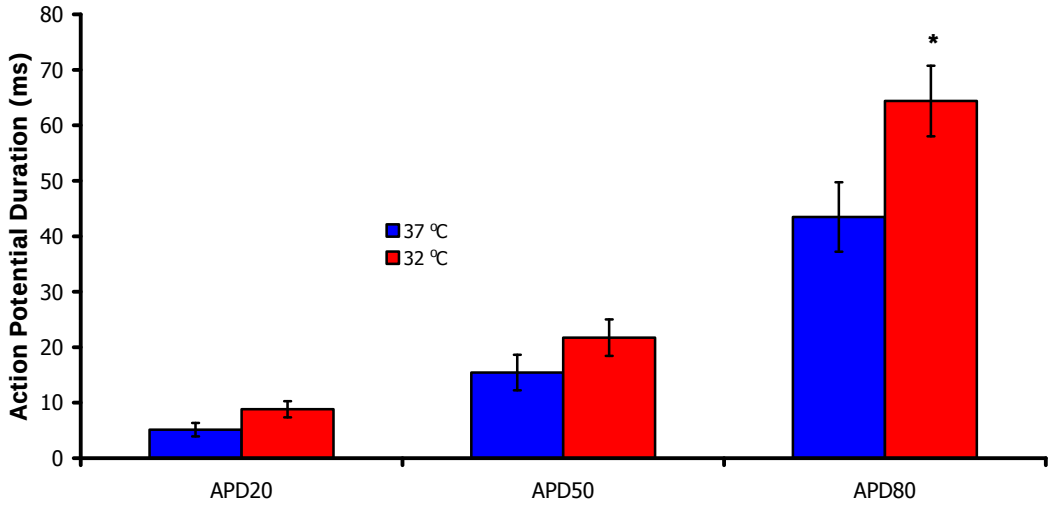

Figure 69: Effect of Reducing Bath Temperature on Action Potential Parameters

Figure showing effect of decreasing perfusion temperature on papillary muscle action potential duration at 20, 50 and 80 % repolarisation from rat hearts. Error bars indicate ± SEM (each measurement in each heart is an average of 10 action potentials at each temperature for n= 6 hearts). Statistical differences were calculated compared to basal/control conditions (no stretch, EDLVP = 0-5 mmHg, p > 0.05). Decreasing perfusion temperature from 37 to 32 °C (in random order) significantly lengthened basal action potential duration at 80 % repolarisation (denotes p < 0.05, ANOVA GLM with Tukey's post hoc pair wise comparisons).*

Decreasing perfusion temperature from 37 to 32 °C (in random order) significantly lengthened basal action potential duration at 80 % repolarisation (Figure 69). However, in the absence of stretch, resting membrane potentials did not significantly change upon a decrease in temperature (-75 ±3.4 under control conditions and -70 ±2.7 mV at 32 °C). In addition, basal action potential amplitude did not change with the decrease in temperature remaining at 96 ±3.9 at 32 °C and 101 ±4.4 mV under control conditions (n=6).

Likewise for stretch induced changes at normal perfusion temperatures (37 °C) and at cooler temperature of 32 °C, there was no significant change in action potential duration at 20, 50 or 80 % repolarisation when subject to 0.6 or 1.2 g tension (Figure 70).

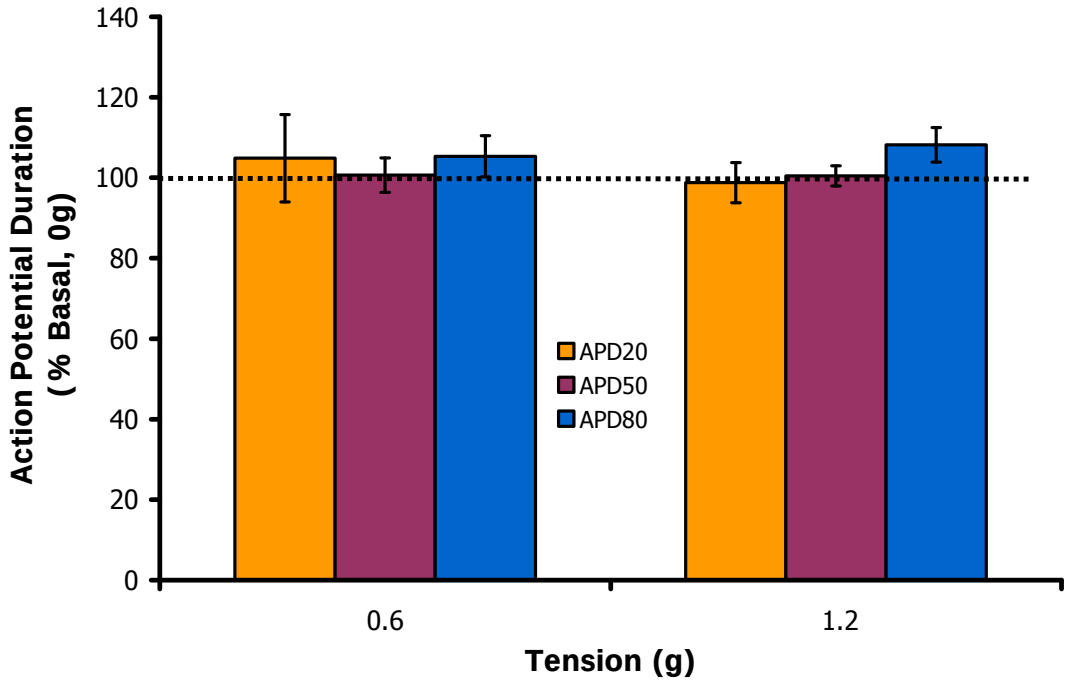

Figure 70: Effect of Temperature on Stretch-induced Changes in Electrophysiology

Figure showing effect of decreasing perfusion temperature on papillary muscle action potential duration at 20, 50 and 80 % repolarisation from rat hearts (n = 6). Error bars indicate ± SEM (each measurement in each heart is an average of 10 action potentials at each level of stretch). Statistical differences were calculated compared to basal/control conditions (no stretch, EDLVP = 0-g) using a ANOVA GLM (p > 0.05). Decreasing perfusion temperature from 37 to 32 °C (in random order) did not alter stretch responses at any level of repolarisation tested (p > 0.05).

DISCUSSION

In this section, the electrophysiological response of the rat heart to changes in end diastolic MEF events if it is taking a recording from a considerably large population of cells.

Unlike the rat heart in the Langendorff model, the isolated papillary muscle described here is not perfused by the coronary vasculature and therefore (unlike whole heart preparation) not subject to perfusion induced changes in APD as a result of stretch induced ischaemia (caused by increasing EDLVP). Furthermore, the papillary muscle is essentially a two dimensional structure and thus not exposed to the complex 3-dimensional distributions of stretch that likely occur in the ventricular wall of the whole heart during stretch. The other differences are as pointed out earlier in that the recordings made using transcellular methods are from a considerably smaller population of cells compared to the MAP electrode. As such, these differences must be kept in mind when comparing isolated papillary muscle preparations to those results observed in the whole heart during Langendorff perfusion and manipulations of EDLVP.

Whilst the present results convincingly demonstrate a lack of MEF in the isolated papillary muscle, this has been previously confirmed in cat papillary muscle by at least one other study (Lab, 1980). However, it has also demonstrated in other models that stretch can indeed shorten the action potential (Lab, 1980, 1982). The main difference between the present results and those observed by (Lab, 1982) is likely a result of differences in internal calcium handling between mammalian and amphibian species. However, the major question regarding the present findings and those using isolated papillary muscles is their relevance to the intact mammalian heart. In no way does an anchored isolated papillary muscle preparation exhibit the same stressors as those found in the intact left ventricle. Nor is it likely that stretching papillary muscles in a single plain reflex the complex array of strains on cardiomyocytes that make up the left ventricular wall. With this in mind, however, and the observed difficulty in visualising changes in APD and resting membrane potential it is fair and reasonable to assume that MEF plays an insignificant roll in cells that make up functional contractile tissue when stretched within normal-physiological and optimal contractile ranges.

As with the previous experiments in the Langendorff perfused rat heart, temperature was modulated as a means for altering the electrophysiological response of the preparation to stretch. Consistent with previous findings, this had little effect.

Moreover, with the absence of coronary flow-impairment due to either contractions or endothelial flow restriction as a result of EDLVP, no reduction in action potential durations was observed. This provides further evidence for the possibility that MEF-induced shortening of APD may reflect flow-impairment and its associated electrophysiological consequences (Van Wagoner, 1993; Miyoshi *et al.*, 1996; Wirth *et al.*, 2000; Morrissey *et al.*, 2005)

CONCLUSION

In this chapter the effect of step-wise increases in axial stretch of papillary muscles isolated from the left ventricle of rats was used to investigate MEF. This model was chosen to complement MAP recordings since it provides a direct electrophysiological measure of activity at the cellular level. In accordance with previous findings, stretch had no effect on electrophysiological parameters of the multicellular preparation. Furthermore, SAC inhibitors, activators and temperature did not modulate any response present.

CHAPTER 10: INTRACELLULAR RECORDINGS IN LEFT ATRIAL TISSUE

Popular American singer: Ruth Etting
(Nov 23, 1896 – Sept 24, 1978)

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BACKGROUND

The previous section dealt with intracellular recordings taken from isolated papillary muscles under tension and exposed to various concentrations of known SAC modulators. This section looks at stretch-induced changes in isolated rat left atrial tissue using similar methods to that previously described (Kamkin *et al.*, 2000b).

METHODS

Rats weighing 350-400 g were prepared as previously described for intracellular recordings (ie, heart was rinsed of blood on Langendorff setup, and the right ventricle dissected out under ice-cool conditions (in the presence of O_2). Whole left atrium or left ventricular strips were cut from the hearts of male Sprague Dawley rats (290-300 g). Atrial strips were perfused in a standard bicarbonate buffered physiological solution containing 1.5 mM $CaCl_2$ and paced at 0.5 Hz. Tissue was then stretched between a fixed point and micro-tension transducer attached to a micro-manipulator. Action potentials were recorded during intracellular impalements performed on areas under tension using a modified floating glass micro electrode as outlined previously (see Figure 71 - Figure 73).

Criteria for an acceptable experiment were: a stable recording with a resting membrane potential (RMP) of at least -60 mV and an Action Potential Overshoot of at least 5 mV. Values for Action Potential Duration (APD) at 20, 50 and 80 % repolarisation as well as RMP and amplitude were recorded for each action potential.

RESULTS

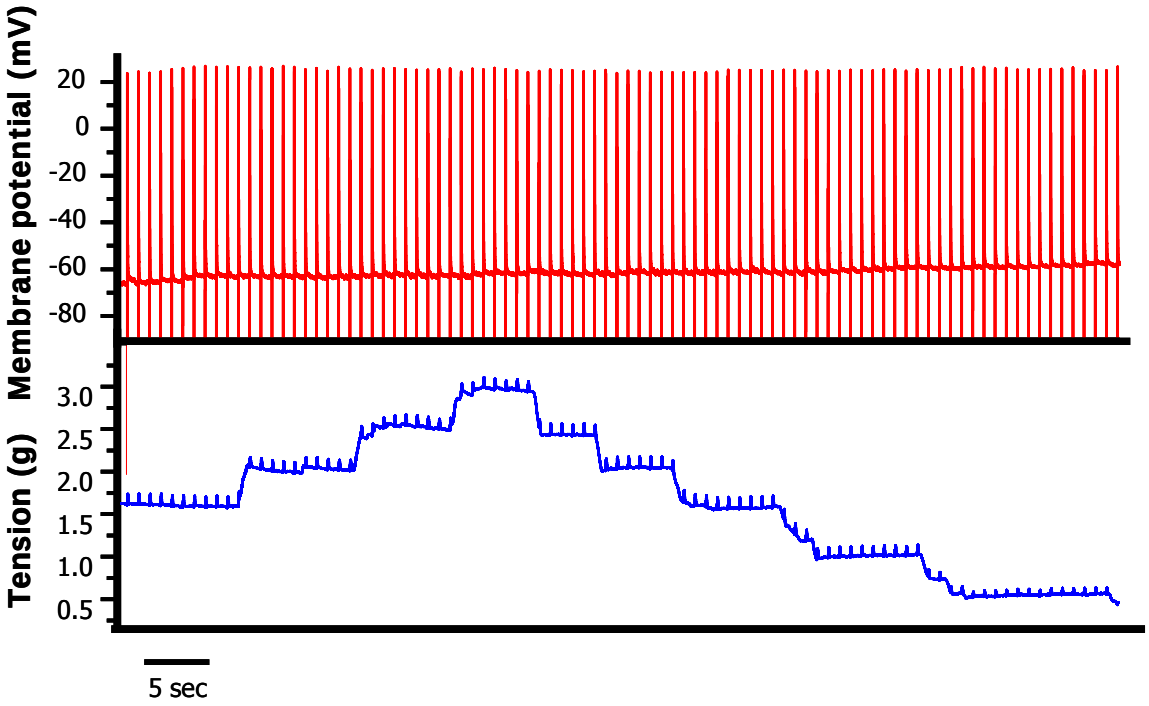

Figure 71: Stepwise changes in resting tension on atrial contractility and electrophysiology.

Figure showing a continuous impalement transcellular recording made from one left atrial appendage. Upper trace (red) shows membrane potential and is composed of individual action potentials. The solid red, horizontal line depicts the actual resting membrane potential (in mV). Lower trace (in blue) shows the papillary muscle tension (both passively applied via stretch and actively developed by contraction). Despite changes in resting tension, there was no obvious change in resting membrane potential or action potential duration until late in the recording where the impalement began to fail.

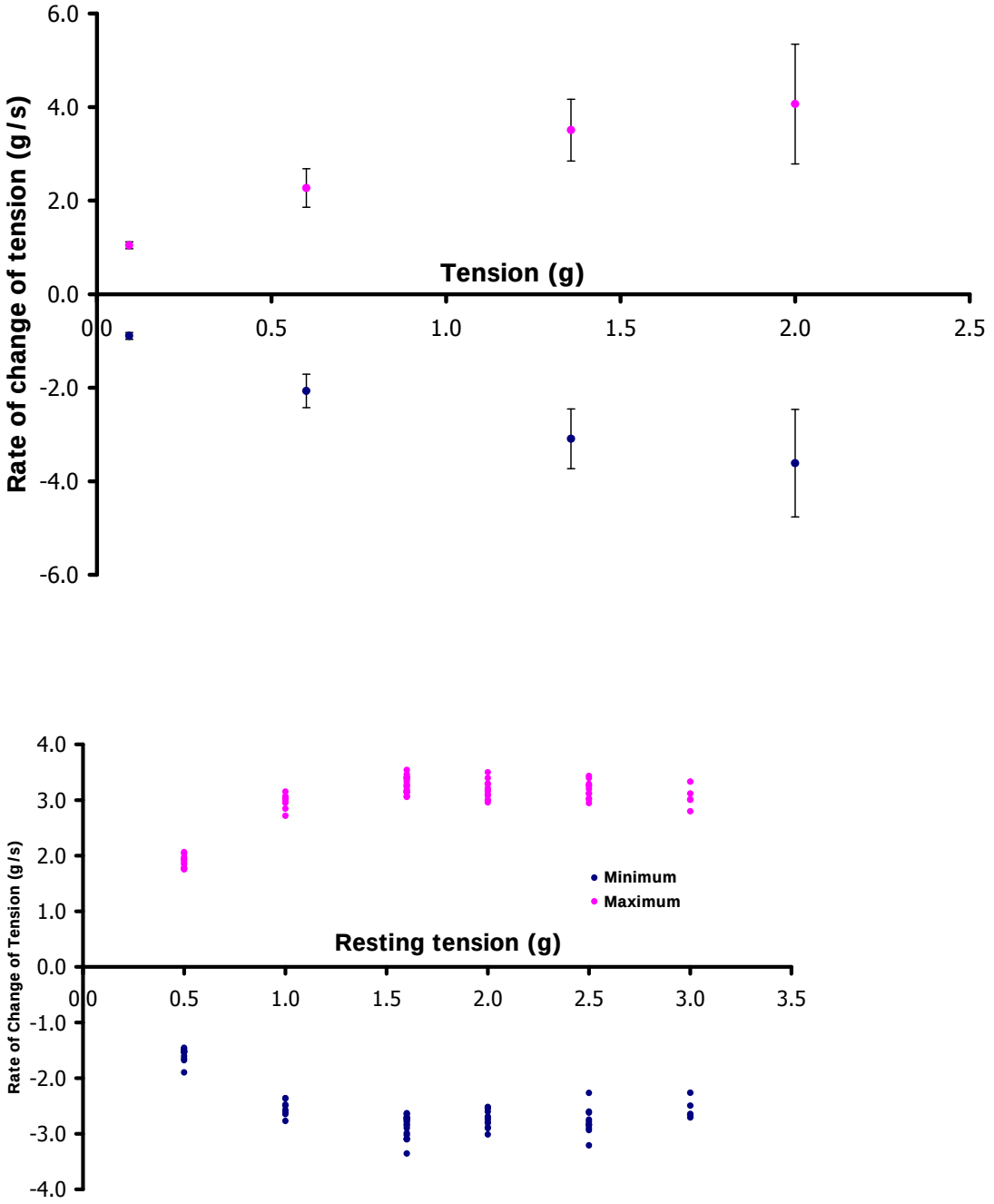

Figure 72: Effect of physiological tension on contractility in isolated left atrial tissue.

Axial stretch of rat atrial tissue significantly increased contractility from ~ 1 ±0.1 g/s to 4.1 ±1.3 g/s, the optimal tension-contractile relationship at a tension of ~1.4 g. Even at pathological tensions (<2.5 g) where contractility failed to increase further or decreased (lower panel) stretch-induced ectopic beats were not observed in any of the tissues. Main panel shows average results obtained from n=4 rats whilst lower insert shows results from a typical experiment.

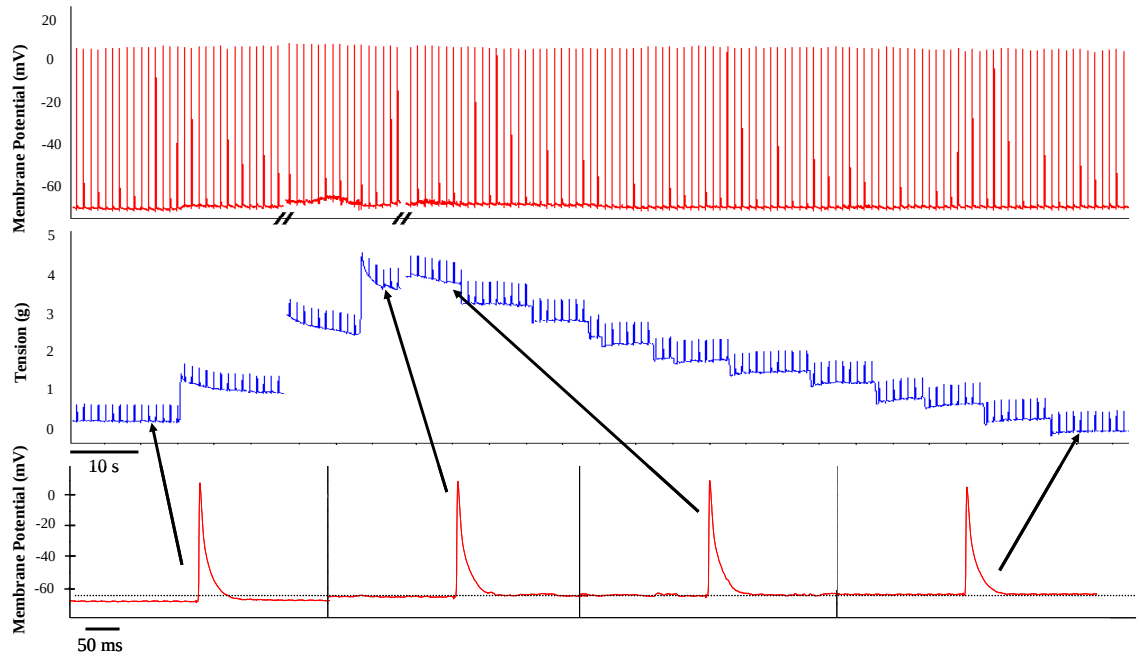

Figure 73: Continuous Isolated Atrial Tissue Impalement During Stretch Manipulations.

Continuous impalement showing no obvious change in action potential amplitude and small depolarisation with extreme stretch. Upper panel (red) shows membrane potential recordings made with intracellular electrode (containing 3 M KCL), blue trace shows resting membrane tension and contractions whilst lower traces show examples of individual action potentials obtained throughout manipulations in resting tension.

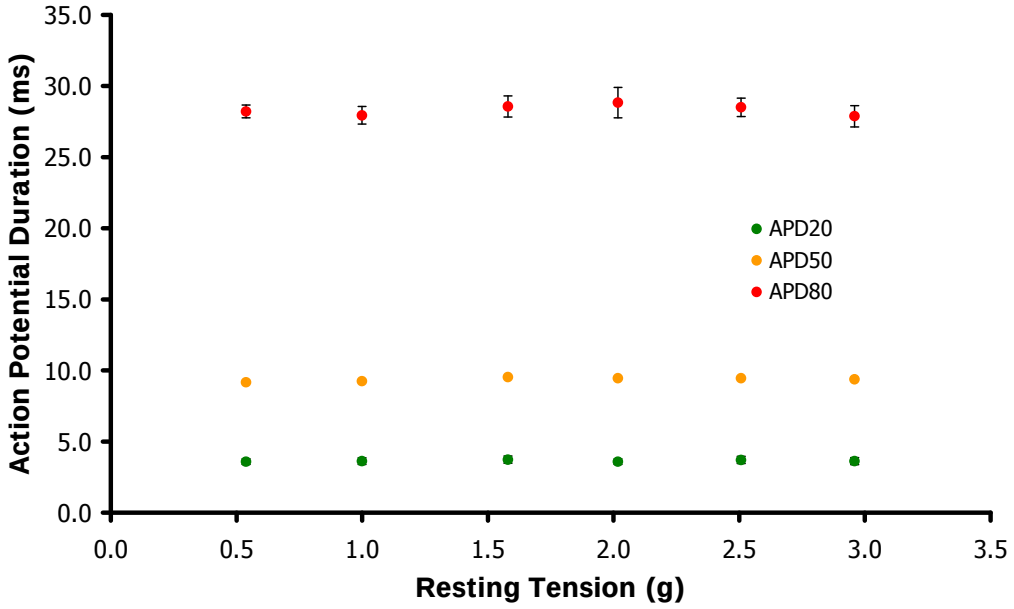

Figure 74: Effect of Stretch on Isolated Left Atrial Tissue Action Potential Duration

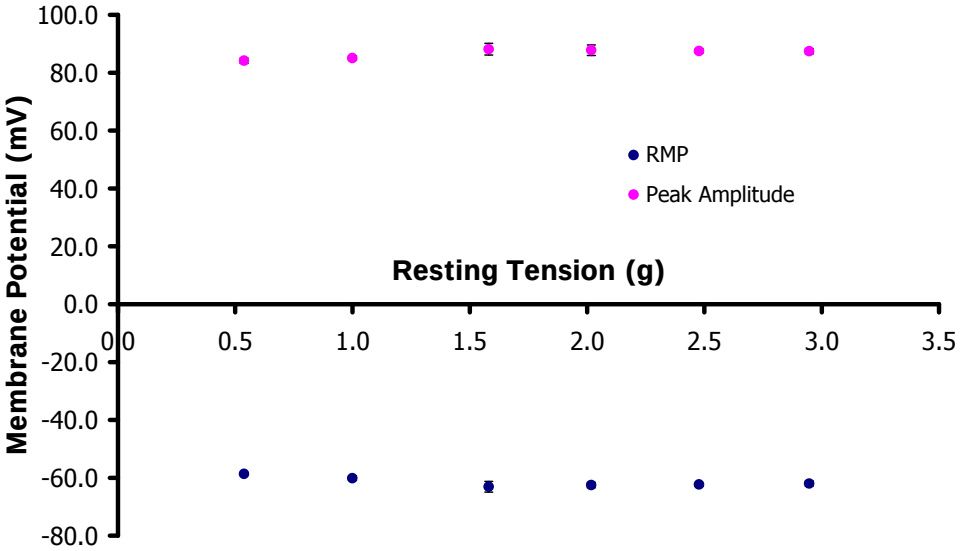

Figure 75: Effect of Stretch on Isolated Left Atrial Tissue Action Potential Amplitude and Resting Membrane Potential

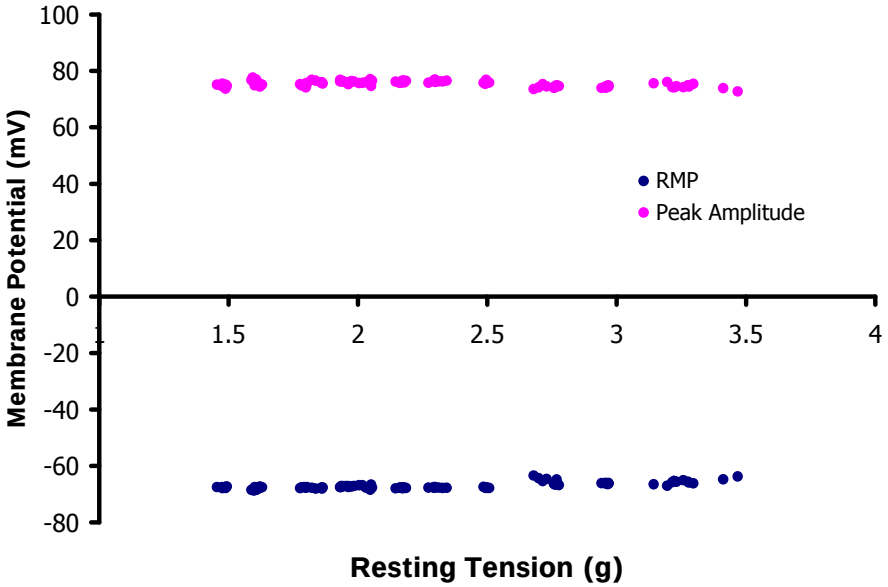

Figure 76: Effect of Stretch on Isolated Left Atrial Tissue Action Potential Amplitude and Resting Membrane Potential

Figure 74 - Figure 76 are typical results obtained from a single atrial appendage showing the average action potential durations at 20, 50 and 80 % repolarisation as well as action potential amplitude and resting membrane potential. There were no significant changes in action potential measures with stretch (ANOVA GLM, p > 0.05, see table 2 for n).

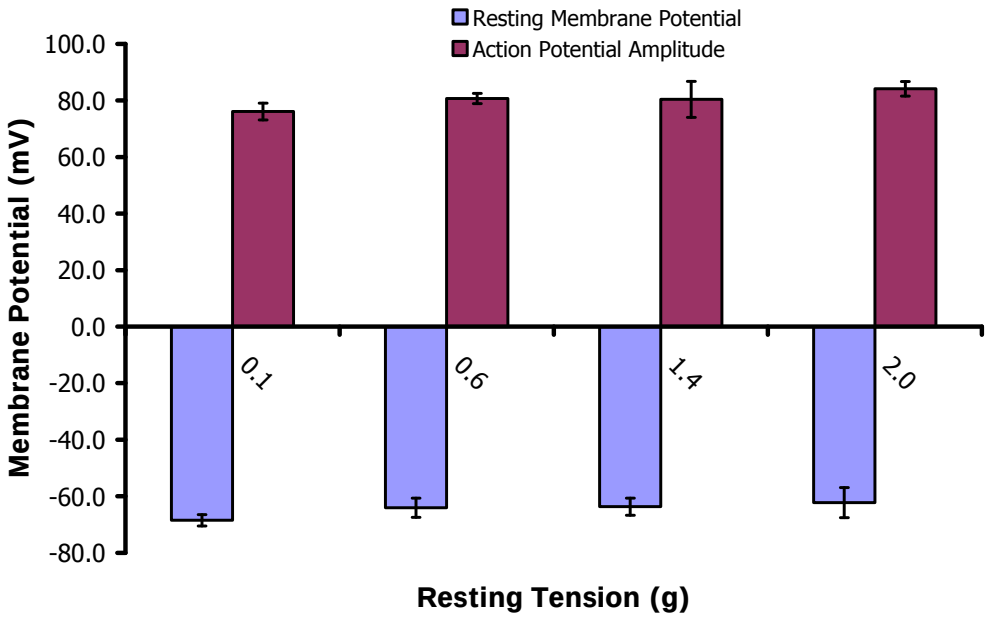

Figure 77: Effect of Stretch on Isolated Left Atrial Tissue Action Potential Amplitude and Resting Membrane Potential

Figure showing the effect of stretching atrial appendage on action potential amplitude and resting membrane potential for n=4 rats. Despite significant stretch (up to 2.0 g resting tension) there was no obvious nor consistent change in action potential amplitude ($p > 0.05$, ANOVA GLM).

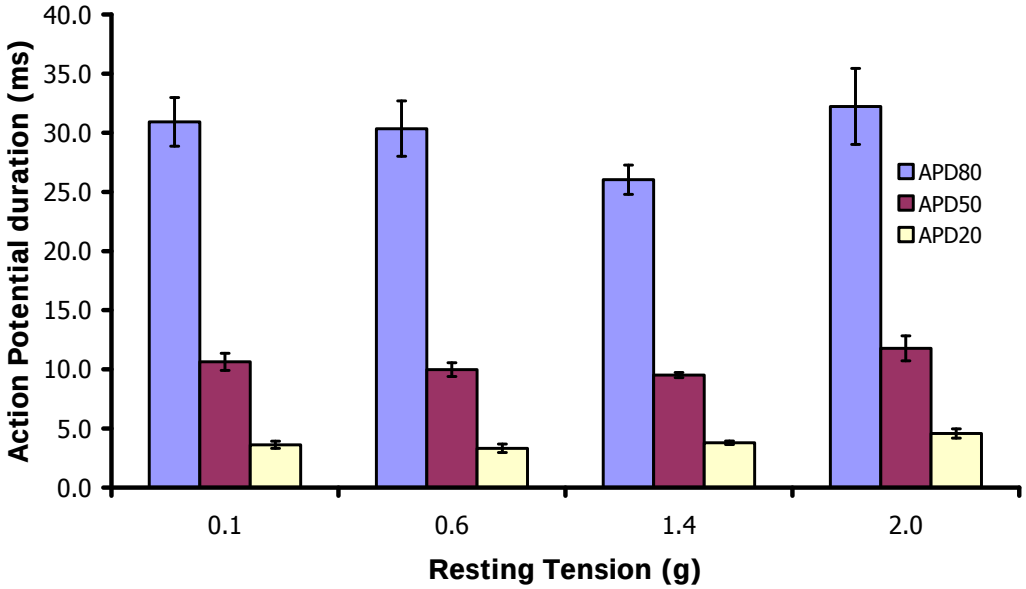

Figure 78: Effect of Stretch on Action Potential Duration in Left Atrium of Rat

Figure showing the effect of stretching atrial appendage on action potential duration for n=4 rats. Despite significant stretch (up to 2.0 g resting tension) there was no obvious consistent change in action potential amplitude or duration at 20, 50 or 80 % repolarisation (p > 0.05, ANOVA GLM).

Atrial	APD20	APD50	APD80	RMP	Amplitude	Ventricular	APD20	APD50	APD80	RMP	Amplitude
No Change	n=20 (10)	n=15 (8)	n=13 (8)	n=10 (9)	n=12 (8)	No Change	n=2 (2)	n=4 (2)	n=2 (2)	n=2 (2)	n=5 (3)
Increase	n=2 (2)	n=3 (3)	n=5 (4)	n=7 (6)	n=3 (3)	Increase	-	-	-	-	n=1 (1)
Decrease	n=4(3)	n=6 (6)	n=4 (4)	n=9 (5)	n=12 (6)	Decrease	n=6 (3)	n=4 (3)	n=5 (3)	n=5 (2)	n=2 (2)
Inconsistent	n=2 (2)	n=4 (4)	n=6 (6)	n=2 (2)	n=1 (1)	Inconsistent	-	-	n=1 (1)	n=1 (1)	-

Table 2: Summary of Stretch-induced Electrophysiological Effects in Isolated Rat Atrial Preparations.

Table showing variability in action potential parameters during stretch of both atrial and ventricular tissue. ("n" denotes the number of experimental stretches and numbers in brackets denote animal preparations used. E.g., of 20 stretches involving 10 atrial preparations from 10 rats, no change in APD_{20} was observed.). All data were compared to basal resting conditions (no stretch) using paired t-test (significance accepted as $p < 0.05$).

DISCUSSION

In general, for all experiments, changes in APD fell well below expectations with most changes observed being smaller than 1 ms (see Figure 74, Figure 78). In addition, any changes in amplitude and RMP were also considerably smaller than previously reported, most being < 1-3mV and statistically insignificant (see Figure 75 - Figure 77) as previously described in stretched rat atria (Kamkin *et al.*, 2000b). These experiments were prompted by previous demonstrations of MEF in atrial tissue (Allen & Kurihara, 1982; Bode *et al.*, 2001) *in vivo* and *in vitro*, usually seen as a reduction in refractory period (Ravelli & Allessie, 1997). In an attempt to better understand the ionic basis of MEF, action potentials were recorded from isolated atrial tissue. All efforts failed to convincingly demonstrate stretch-induced changes in the action potential, even at extreme tensions, due to the wide variety of effects observed. Furthermore, the action potentials recorded from the atrial myocytes did not reflect the changes in resting tension. This may suggest that SACs do not directly modulate the action potential of atrial myocytes but may be involved in other indirect mechanisms. It may also be that the insult to the tissue during dissection and other neural/hormonal factors may influence the function of SACs (Franz, 1996). However, the present results directly reflect that observed previously in atria from rats having undergone left ventricular infarction (Kamkin *et al.*, 2000b) with the exception that APD_{80} lengthening and premature action potentials were not observed with significant stretch.

In general, the quantity of stretch required to generate the Frank-Starling response was near double that observed in isolated papillary muscles from rats of similar age and weight. Whilst the reasons for this discrepancy are not apparent, it may reflect myocyte orientation and the orientation of the atrium under stretch rather than technical difficulties.

CONCLUSION

Although intended as preliminary experiments to the impalement of isolated papillary muscles, the present study utilised small (0.1 -0.3 g) steps in tension to observe the electrophysiological consequences of stretch. It was found that fast, swift increases in tension retained cellular impalement better than slow, gentle increases. Interestingly, as a result, no electrophysiological changes were readily observed as a consequence of these abrupt changes in tension. Instead, any changes in cellular electrophysiology were associated with failing impalements and not MEF.

CHAPTER 11: THE CONTRIBUTION OF MEF TO THE GREGG EFFECT

Popular French singer: Lucienne Delyle:
(Paris, France, April 16, 1917 —1962, dying from Leukaemia)

Chapter 11

Chapter 11 - Table of contents

INTRODUCTION

In this chapter, the Langendorff perfused rat heart model with the implementation of the sub-epicardial and endocardial transmural MAP electrode and surface MAP electrode will be used to study other physiological responses that may involve MEF, namely the Gregg effect to see if coronary flow changes may be contributing to the responses observed. The first section provides a general overview to the Gregg effect and the experiments that follow. The second section comprises the experiments used to investigate the contribution of MEF to the Gregg effect using surface MAP electrodes. In this section the electrical changes (from MAPs) are recorded during stretch resulting from alterations in the rate at which the heart is perfused with physiological solution (termed the Gregg Effect). The increase in perfusion of the heart (or rate of flow through the heart) is known to increase contractility of the heart. In addition, this effect on contractility is more pronounced than when altering ventricular stretch directly through manipulations in EDLVP observed in previous chapters (the Frank-Starling effect). The third section applies the use of the transmural electrodes to observe changes in cardiac electrophysiology during the Gregg effect. A fourth section featured in the following chapter implements a new technique not yet addressed in this thesis, namely the use of isolated, perfused tissue combined with intracellular impalements to obtain a direct recording of the changes in membrane potential that may be associated with the Gregg effect. The final section of this chapter examines each approach and examines the outcomes. Thus, for each experiment, each approach is explained and discussed as to how they differ. This chapter will form the basis of examining the contribution of MEF to the Gregg effect in the rat heart and the likely importance. Finally, some conclusions are made regarding the function of the Gregg effect and any role that MEF through SACs may play.

BACKGROUND

The Gregg Effect is defined by an increase in myocardial contractile force in response to an increase in coronary perfusion pressure or flow. It was first observed in 1958 (Mc *et al.*, 1958) and is dependent to some degree on the autoregulation of the coronary vessels that supply the heart (ie, the ability of the coronary vessels to self regulate local flow changes).

Whilst some past studies of the Gregg effect suggest that increased perfusion within the normal autoregulation operative range of the heart does not result in an increase in myocardial contractility (Schulz *et al.*, 1991), others have suggested a strong link between the Gregg Effect and perfusion induced increases in contractility and associated activation of SACs (Suarez *et al.*, 1999; Lamberts *et al.*, 2002a). More interestingly, these responses have been blocked with SAC modulators such as gadolinium. Regardless of the mechanism, the Gregg effect is complicated by the ability of the coronary vasculature to maintain a constant blood flow despite changes in perfusion pressure but remains readily visible in the isolated Langendorff perfused heart. Moreover, small coronary vessels that run along in close contact with cardiomyocytes help provide an environment suitable for cellular processes to continue. It is thought that these "micro-vessels" are able to modulate and influence the contractile properties of the cells in response to various factors (McClellan *et al.*, 1992; Dijkman *et al.*, 1996).

Effect of Contraction on Coronary Flow

In addition to these effects there are also regional differences in the anatomical structure of the vessels that supply the epicardial and endocardial layers of the ventricular wall (Westerhof *et al.*, 2006). These differences account for a higher coronary flow in the sub-endocardial layers compared to sub-epicardial layers. As a result, it is reasonable to expect that changes in coronary flow and intraventricular pressures as highlighted earlier will have a greater effect on endocardial layers compared to the epicardial layers. This should be reflected as a greater change in action potential morphology in the endocardial MAPs when compared to epicardial MAPs when taking into account the possible role of SACs (Suarez *et al.*, 1999). With this in mind, it is generally accepted that the effect of cardiac contraction on endocardial flow results in negligible endocardial coronary perfusion during systole leaving endocardial perfusion to take place only during diastole (Westerhof *et al.*, 2006). As a result, the present experiments are conducted with an EDLVP of 0-5 mmHg so as not to inhibit endocardial flow during diastolic periods. This should avoid the previous concerns regarding the effects of elevated stretch on diastolic coronary flow suggested earlier.

Involvement of SACs

As mentioned, research to date has shown that the inotropic nature of the Gregg effect is blunted in the presence of the SAC blockers gadolinium (10 μM) and streptomycin (40 or 100 μM) but not verapamil (0.1 μM) (Suarez *et al.*, 1999; Lamberts *et al.*, 2002b). This is an interesting observation given Gd^{3+} inhibition of the Gregg response was in the presence of bicarbonate buffer (Caldwell *et al.*, 1998). However, despite blunting of the effect and its attribution to SACs, it remains unclear as to the electrophysiological changes that may accompany the Gregg effect.

The mechanism behind the Gregg effect itself still remains somewhat controversial. It has been postulated that an increase in microvascular volume opens NSACs in the vascular cells or surrounding myocardiocytes resulting in Ca^{2+} influx followed by an increase in Ca^{2+} sensitivity of the contractile apparatus (Lamberts *et al.*, 2002b). These effects combine together to increase contractility significantly greater than that observed during independent manipulations of EDLVP alone. This theory is supported by the observation in isolated perfused rat papillary muscle that neither limiting the oxygen supply and inducing hypoxic conditions, nor altering muscle length could accurately explain the relation between perfusion and force of contraction (Schouten *et al.*, 1992). Gregg effect perfusion-induced increases in force are generally larger than the maximum increase in force induced by stretch alone and the time course of the responses are also different. Moreover, these perfusion related force changes are accompanied by increased $[Ca^{2+}]_i$ transient amplitudes that return to basal levels after 2 minutes but without obvious changes in diastolic $[Ca^{2+}]_i$ (Lamberts *et al.*, 2002a).

In the following experiment I investigate these observations further by observing these perfusion related, stretch-induced changes in heart electrophysiology using MAP electrodes and intracellular recording techniques. The MAP technique, as before, will enable us to demonstrate changes in the electrophysiology of the whole heart during the Gregg effect. In addition, I aim to demonstrate that these changes are altered in the presence of stretch-activated ion channel blockers. The second technique is somewhat novel and more complicated but will enable direct, quantitative measurements of any changes in the electrophysiology of individual heart cells during the Gregg Effect.

The hoop/circumferential stress model of the Gregg effect involves increased coronary perfusion induced deformation of cardiomyocyte membranes. Increased perfusion

pressure is known to increase intramyocardial pressure as demonstrated in isolated, perfused rat papillary muscles (Heslinga *et al.*, 1997). The resultant membrane stretch, in theory, activates SACs and results in an increase in contractility through an undefined mechanism. It has been established, however, that the changes in contractility are not brought about by soluble factors released from the endothelium in response to coronary-pressure induced stretch (Dijkman *et al.*, 1997a).

METHODS:

As with all previous experiments, changes in the heart action potential were recorded indirectly by use of special MAP electrodes

Male Sprague Dawley rat hearts were prepared as outlined in chapter 3 from rats weighing 350-480 g. Hearts were paced at 4 Hz and perfused with bicarbonate buffer containing 1.5 mM $CaCl_2$ at coronary perfusion flow rates of between 4 and 18 ml/min in randomised order. In additions to this, a second series of experiments utilised constant coronary perfusion pressure instead of flow rate. MAP recordings were taken at each coronary flow rate once contractility had stabilised (in general, between 3 and 5 minutes was adequate before changing the coronary flow rate). To simplify analysis, 10 monophasic action potentials were taken at each coronary perfusion step to identify changes in MAP morphology. All MAPs were taken only when the EDLVP was in the range of 0-5 mmHg. Data in each coronary flow range were then averaged and any changes in MAP duration (as measured by action potential duration, APD, at 20, 50 and 80 % repolarisation calculated and presented.

RESULTS

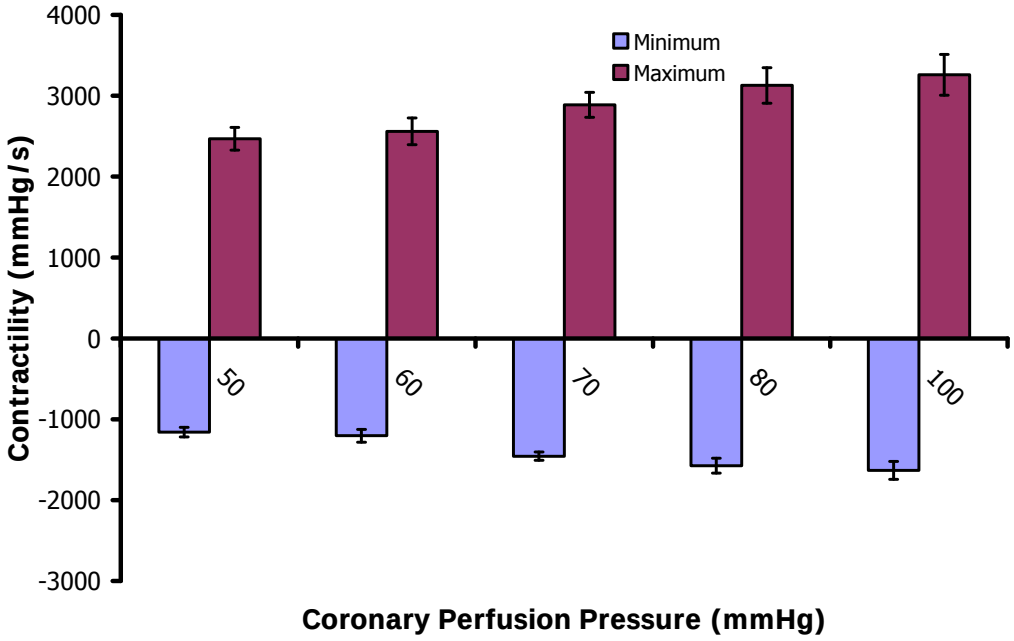

Figure 79: Effect of Coronary Perfusion Pressure on Myocardial Contractility

Figure showing increasing contractility with increasing coronary perfusion pressure (Gregg effect). It should be noted that increased coronary perfusion pressure was associated with increased coronary flow rate with perfusion pressures of 50, 60. 70, 80 and 100 mmHg associated with coronary flow rates of 10 ±1.0, 12.1 ±0.9, 13.1 ±1.1, 15.6 ±1.1 and 19.5 ±1.7 ml/min respectively. Bars indicated average of n=5 rats and error bars denote ± SEM.

Gregg Effect Observations with Suction MAP Electrode

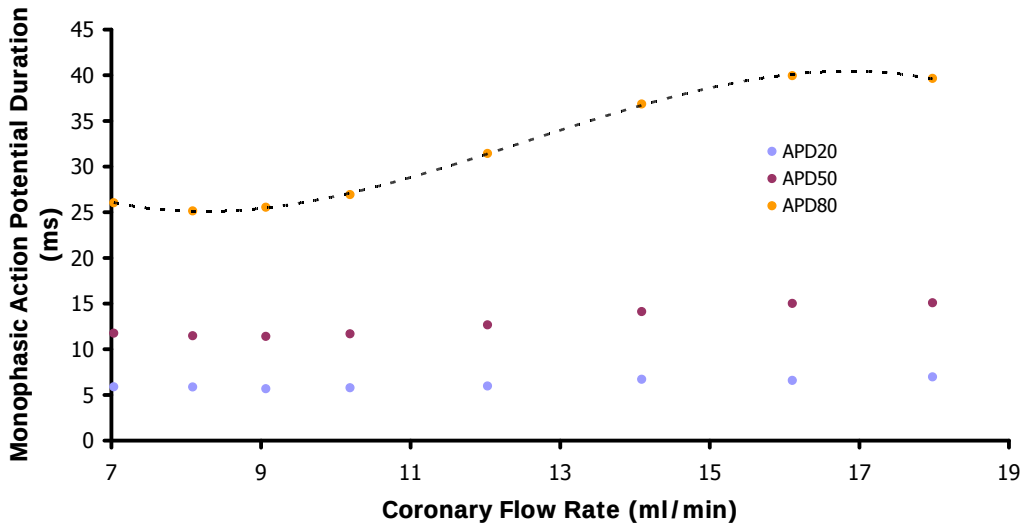

Figure 80: Preliminary Experiment Showing Effect of Flow on MAP durations

Figure showing increasing MAP duration with increasing flow rate for one Langendorff perfused rat heart. MAP duration ranged from 26 ms to nearly 40 ms at coronary flow rates of 9 to 16 ml/min respectively.

Several preliminary experiments (Figure 79, Figure 80) suggested that flow rates chosen from the range of 8 – 16 ml/min would provide a reasonable distribution of MAP durations for adequate representation of the Gregg effect in the rat heart.

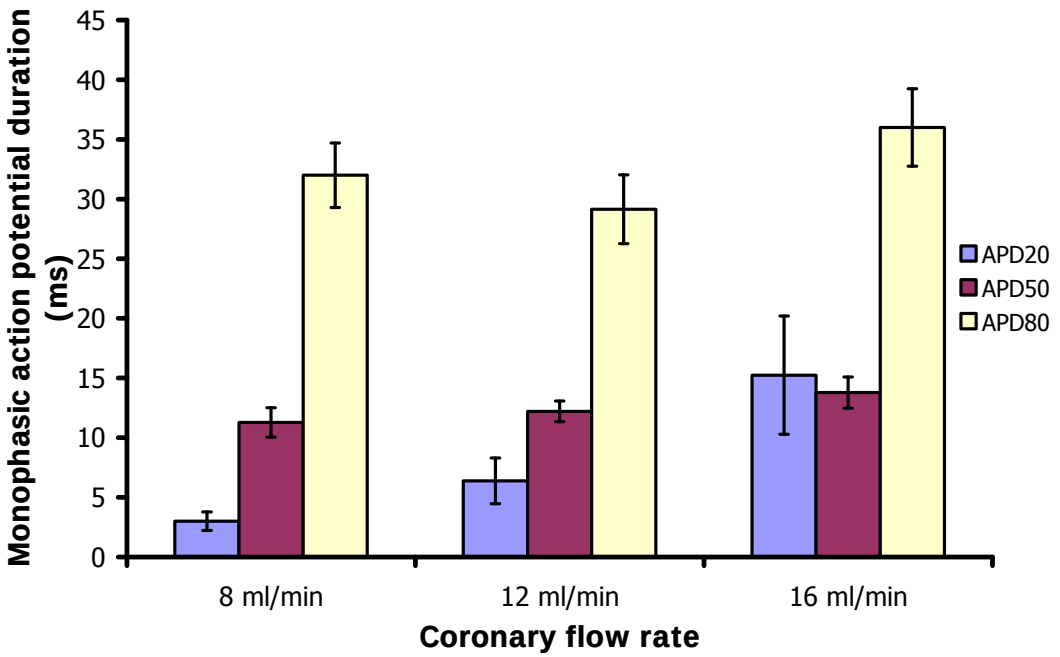

Figure 81: Effect of Coronary Flow Rate on MAP Durations

Figure showing effect of Coronary flow rates of 8, 12 and 16 ml/min on MAP durations recorded using the suction electrode n the rat heart (n=9). Despite the result of preliminary experiments, coronary flow rate did not appreciably change MAP durations at any of the levels of repolarisation test ($p > 0.05$, ANOVA GLM).

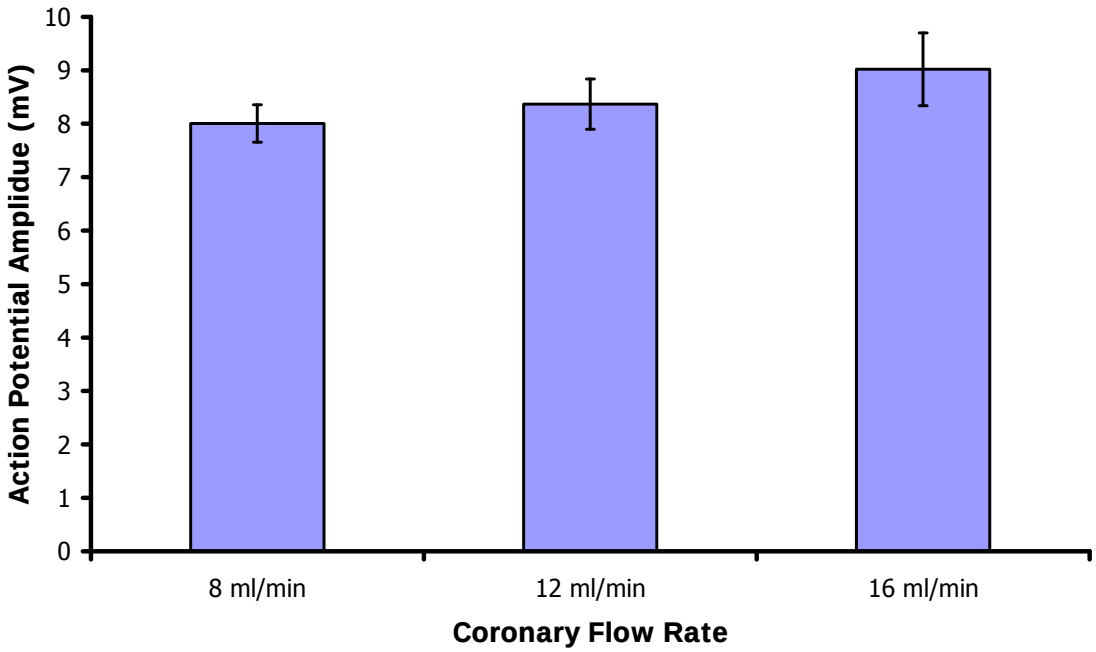

Figure 82: Effect of Coronary Flow Rate on MAP Amplitude in the Rat

Figure showing effect if Coronary flow rates of 8, 12 and 16 ml/min on MAP Amplitude recorded using the suction electrode n the rat heart (n=9). Despite the result of preliminary experiments, coronary flow rate did not appreciably change MAP durations at any of the levels of repolarisation test ($p > 0.05$, ANOVA GLM).

Gregg Effect Observations with Surface Contact Electrode

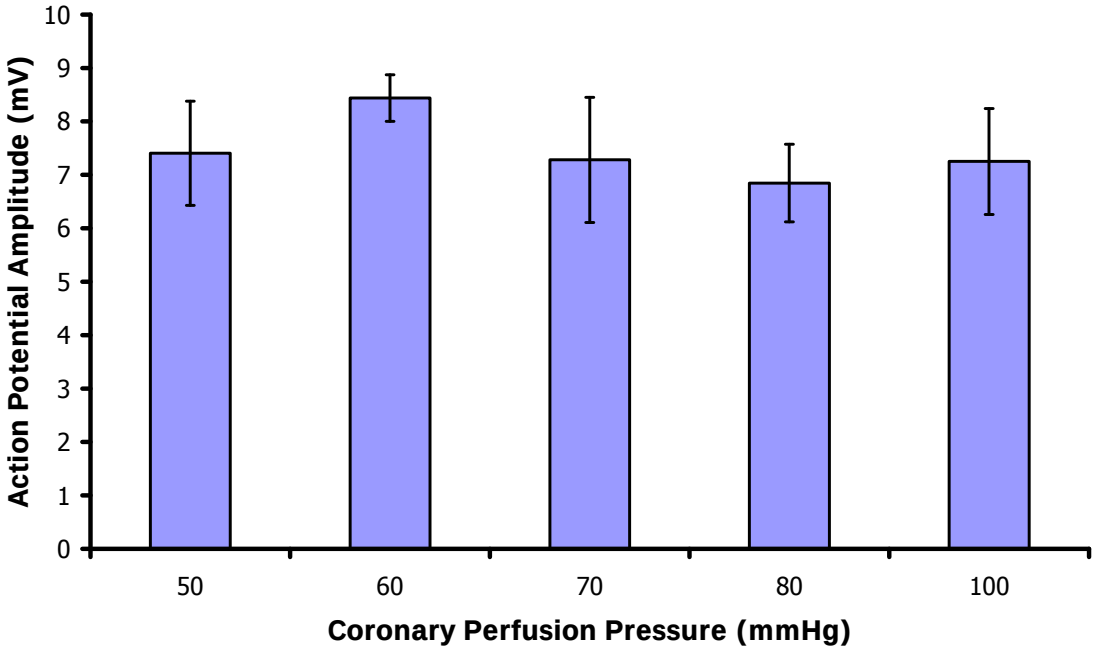

Figure 83: Effect of Changes in Coronary Perfusion Pressure on MAP Amplitude

Figure showing effect of changing coronary perfusion pressure on MAP Amplitude as measured by Franz contact MAP electrode. EDLVP was maintained < 7 mmHg, whist MAP recordings were made at various coronary perfusion pressures in randomized order. MAP Amplitude did not significantly change at any of the pressures tested (p >0.05, ANOVA GLM, n=9).

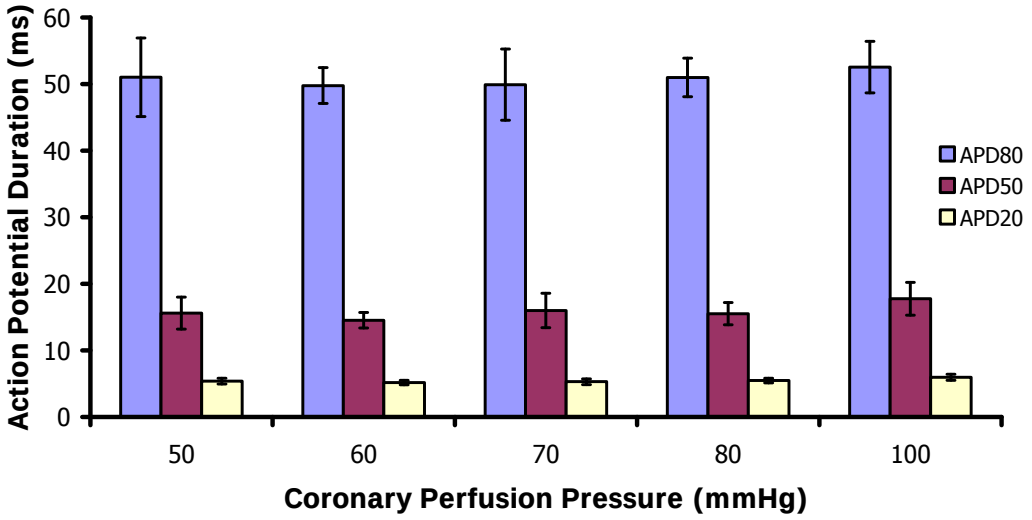

Figure 84: Effect of Coronary Perfusion Pressure on MAP Durations in Rat

Figure showing that despite earlier observations regarding changes in comparable changes in coronary flow, that Coronary perfusion pressure does not readily modulate MAP durations in the rat heart (p >0.05, ANOVA GLM, n=9).

Despite preliminary experiments, increasing the sample number to n=9 showed that changes in coronary flow rate (Figure 81Figure 82) or coronary perfusion pressure (Figure 83Figure 84) had any obvious effect on MAP durations or amplitude as recorded by surface MAP electrodes in the rat heart.

Gregg Effect Observations with Transmural MAP Electrode

In a similar manner to previous experiments, where surface electrodes failed to record changes in MAP durations and cardiac electrophysiology, the sub-epicardial – endocardial transmural electrode was used to determine if there was electrophysiological changes deeper in the ventricular wall.

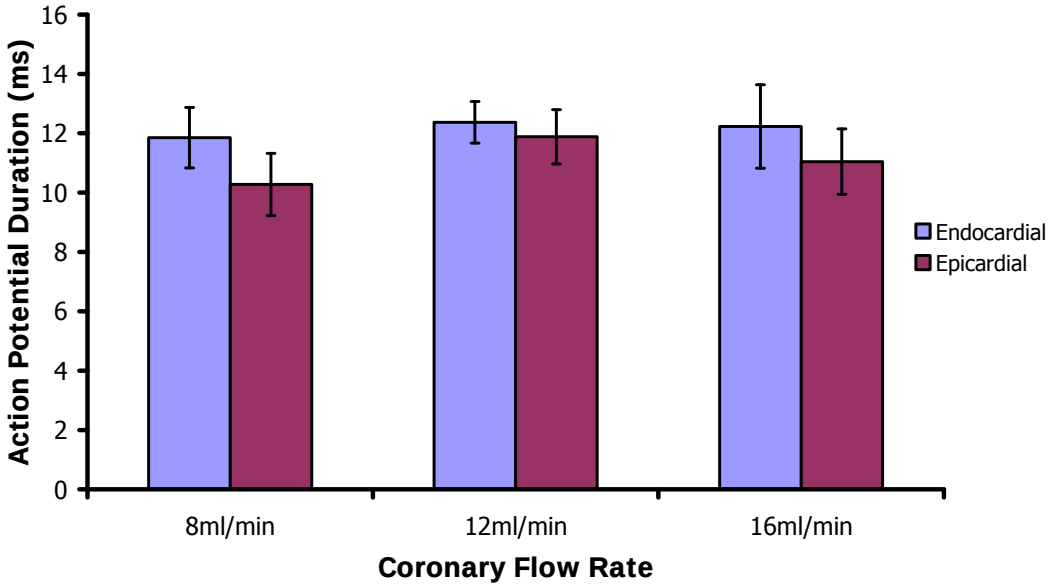

Figure 85: Effect of Coronary Flow Rate on MAP APD_{50} in the Rat

Figure showing that contractile changes associated with the Gregg effect were not accompanied by changes in APD_{50}. Data expressed as averages ± SEM from 10 action potentials taken at each coronary flow rate for n = 6 rats (p >0.05 ANOVA GLM).

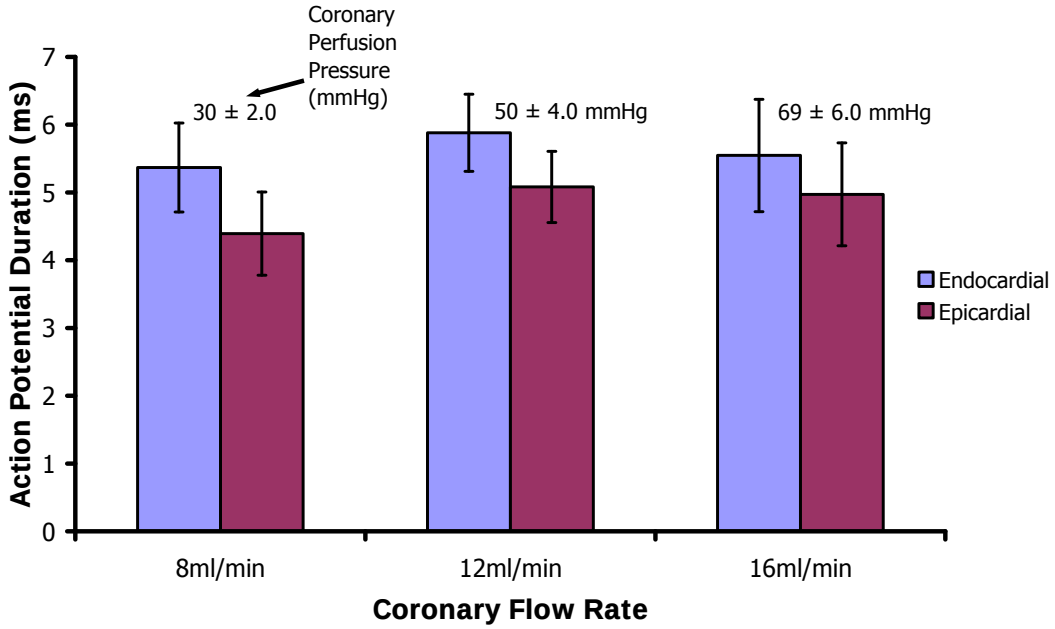

Figure 86: Effect of Coronary Flow Rate on MAP APD_{20} in the Rat

Figure showing that contractile changes associated with the Gregg effect were not accompanied by changes in APD_{20}. Data expressed as averages ± SEM from 10 action potentials taken at each coronary flow rate for n = 6 rats (p >0.05 ANOVA GLM).

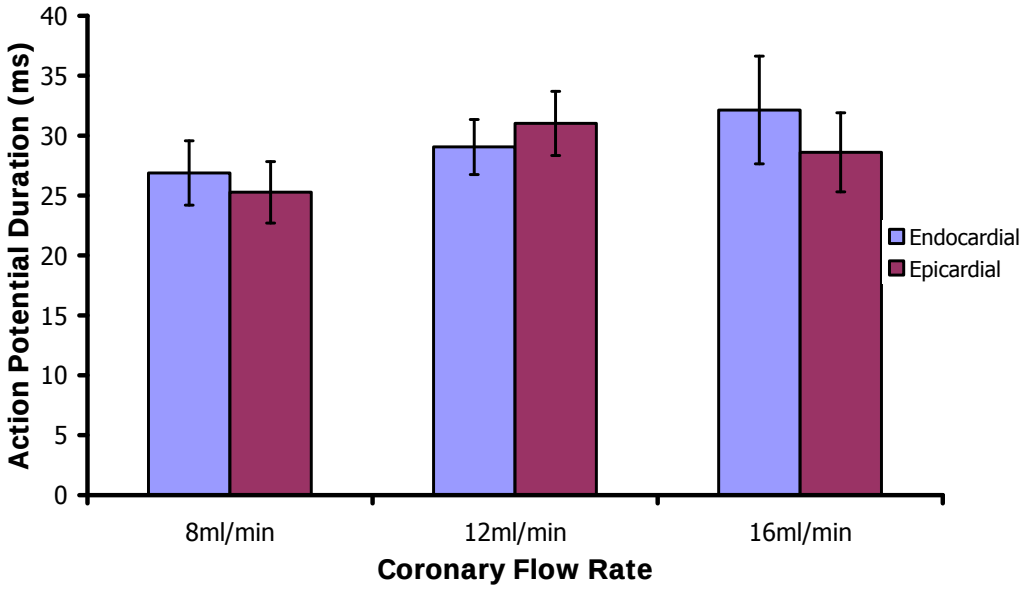

Figure 87: Effect of Coronary Flow Rate on MAP APD_{80} in the Rat

Figure showing that contractile changes associated with the Gregg effect were not accompanied by changes in APD_{80}. Data expressed as averages ± SEM from 10 action potentials taken at each coronary flow rate for n = 6 rats (p >0.05 ANOVA GLM).

Consistent with surface MAP recordings, transmural recording of sub-epicardial and endocardiam MAPs showed no change in morphology in response to changes in coronary flow rate (see Figure 85 - Figure 87).

DISCUSSION

The Gregg effect is defined by the ability of the coronary vasculature to maintain a constant blood flow despite changes in perfusion pressure. However, contracting muscle not only generates a force and resultant ventricular pressure, it also shortens, thickens and increases the stiffness of the ventricular wall (Westerhof *et al.*, 2006). As one would expect, such changes will impose restrictions on the coronary vasculature and hence coronary flow (Heslinga *et al.*, 1997).

The present results demonstrate that stretch of cardiomyocytes associated with increasing coronary perfusion pressures does not alter action potential morphology in the rat heart. This observation is consistent with the conclusion that Gregg's phenomenon is not related to changes in fibre length, i.e. the hypothesis of pressure-induced stretch ('garden hose' effect) (Schouten *et al.*, 1992)

Whilst it was observed that Gregg's phenomenon induces an observable change in contractility (Figure 79), it was not associated with any observable changes in cardiac electrophysiology (Figure 81- Figure 84, Figure 85 - Figure 87). Thus, it is more probable that the Gregg effect is induced by inotropic factors released by the endothelium rather than ionic factors relating to the presence of SACs. However, this is not supported by the observation that when effluent from tissue undergoing coronary perfusion changes is used to perfuse a 2nd papillary muscle sample, the changes in contractility associated with the perfusion changes is not transmitted to the 2nd preparation (Dijkman *et al.*, 1997a). This suggests that the Gregg effect must involve cardiomyocytes directly or a paracrine response is involved and not soluble inotropic factors released from the endothelium into the effluent/perfusate (Schouten *et al.*, 1992; Wang & Feng, 2004).

The Gregg effect was initially described by the "garden hose" effect whereby perfusion related changes resulted in better overlap of actin and myosin filaments in cardiomyocytes. Thus, the increase in contractility seen with the Gregg effect by this theory is brought about by the Frank-Starling mechanism of increased force development. However, the increase in contractility associated with the Gregg effect is larger and the increase in [Ca^{2+}] is sensitive to Gd^{3+} (Lamberts *et al.*, 2002a; Lamberts *et al.*, 2002b). In addition, despite increasing muscle diameter with increasing perfusion pressure, muscle segment length has been reported not to increase (Dijkman *et al.*, 1998). A number of confounding factors complicate this finding, primarily the influence that Gd^{3+} has on L-type Ca^{2+} channels that would also readily inhibit increases in the Ca^{2+} transient and the availability of Gd^{3+} in the presence of carbonate buffers (Lacampagne *et al.*, 1994; Caldwell *et al.*, 1998; Zhang & Hancox, 2000)

The present results are also inconsistent with others (Lamberts *et al.*, 2002b) who showed that the Gregg effect was indeed blunted with the use of streptomycin (40 μM and 100 μM) although electrophysiological changes were not reported nor recorded. Moreover, they demonstrated that verapamil (0.1 μM) had no effect on the Gregg response indicating that Gd^{3+}-induced blunting of the Gregg effect was not related to L-type channel inhibition. This alone is an intriguing result given that verapamil resulted in a 56% reduction in basal contractility.

Whilst the physiological role of the Gregg effect has yet to be established, it could be involved in local regulation of contractility. As noted by others, increases in perfusion result in increased contractility and decreases in reduced contractility (Dankelman *et al.*, 1996; Dijkman *et al.*, 1996; Dijkman *et al.*, 1997a; Dijkman *et al.*, 1997b; Heslinga *et al.*, 1997). By contrast, it is known that stronger contractile responses of cardiac tissues reduce coronary flow even in the perfused isolated muscle preparation (Allaart & Westerhof, 1996). Thus, the Gregg effect could provide a localised negative feedback mechanism where by cell contractile activity regulates its own blood supply and hence contractile response. Such a mechanism would act to equalise strains in cardiac tissue during the normal contraction, thus regulating contractile responses on a beat-to-beat basis.

The major limitation with the present study is that both coronary flow and coronary perfusion pressure increase simultaneously. As such, the two effects on contractility and action potential changes cannot be separated (ie, they are one of the same thing). With this in mind, the present study still confirms that the Gregg effect was not associated with any electrophysiological changes induced by SACs. This effect is supported by recent literature that demonstrates it is changes in coronary flow and not pressure that contribute to the increased contractility associated with the Gregg response (Karunanithi *et al.*, 1999). Other research suggests that stretch and the Gregg effect mediate increases by different, independent mechanisms (Lamberts *et al.*, 2002a). Since increased perfusion pressure is not a prerequisite for the increase in contractility, the role of SACs in the Gregg response can be theoretically challenged. This is despite earlier studies suggesting that Gregg-related changes in contractility are related to perfusion pressure related changes in shear stress whilst changes in coronary flow related to increased oxygen consumption (Dijkman *et al.*, 1996).

Also of interest was that the lowest of the flow rates used here (8 ml/min) did not significantly shorten MAP durations as a result of ischaemia. This is unfortunately an oversight as the present experiments also intended to show ischaemic/hypoxic induced shortening of the MAP duration in the Langendorff perfused heart. It would appear that a further reduction in the coronary flow rate was required that was not evident from preliminary experiments.

CONCLUSION

The present results failed to demonstrate coronary flow related changes in MAP duration and thus contribution of SACs to the Gregg effect. Whilst previous published data have suggested an involvement for SACs in the response, this is the first demonstration of to include electrophysiological recordings through the use of both contact (suction and Franz) as well as transmural MAP electrodes. It would appear from the present experiments that the ionotropic effect associated with the Gregg effect is independent of electrophysiological changes, and thus mechanical in nature.

CHAPTER 12:

INTRACELLULAR RECORDINGS OF ISOLATED PERFUSED RIGHT VENTRICLE

American jazz singer: Annette Hanshaw
(Octo 18, 1901 - March 13, 1985)

Chapter 12 - Table of contents

BACKGROUND

The previous section dealt with the Gregg effect in the whole isolated Langendorff perfused heart and measuring changes in left ventricle electrophysiology indirectly through changes in the morphology of monophasic action potentials. This section looks at the Gregg effect in a novel isolated, coronary perfused right ventricular preparation. With this method, a direct recording of changes in membrane potential can be obtained from individual cells with the use of micro-electrode techniques.

The right ventricle is better model for observing perfusion-induced changes in contractility and action potential durations since autoregulation is generally poor under physiological conditions compared to the left ventricle (Westerhof *et al.*, 2006). This is important as perfusion-induced changes in contractility are more readily observed in preparations that lack adequate coronary autoregulation through protection of changes in capillary pressure (Dijkman *et al.*, 1998). Whilst Ca^{2+} is thought to play a major role in the process, the supporting data is not particularly solid. Previously it was demonstrated that the Gregg effect is larger when performed in low [Ca^{2+}] conditions compared to high [Ca^{2+}] conditions. Whilst this does suggest the involvement and dependence of the mechanism on Ca^{2+} as suggested (Westerhof *et al.*, 2006), it is also equally likely that high [Ca^{2+}] saturates the contractile mechanisms through increase L-type Ca^{2+} currents and maximal activation of the contractile apparatus leaving little or no room for contractile improvements through other mechanisms. Of particular interest to the present study however, is that both the force and Ca^{2+} alterations associated with the Gregg effect are inhibited by blockade of NSACs with either gadolinium, or streptomycin. (Lamberts *et al.*, 2002a; Lamberts *et al.*, 2002b). Alterations in Ca^{2+} induced by the Gregg effect can be expected to be accompanied by changes in the morphology of the action potential. As such, it is hoped that by using the isolated perfused right ventricular preparation the following experiments will maximise any observed effect of the Gregg effect on action potential morphology due to the inherent poor autoregulation of the right ventricle.

The main and most obvious advantage of the isolated, perfused right ventricular wall preparation is that unlike other isolated preparations (namely the papillary/trabeculae) it is perfused through the coronary arteries. This has two main advantages in the present study: Firstly, it allows an isolated detailed electrophysiological investigation of the Gregg effect using microelectrode techniques not readily possible or technically

challenging in the Langendorff perfused heart or isolated papillary muscle preparation. Secondly, unlike isolated papillary muscle preparations, perfusion through the coronary artery attached to the isolated right ventricle ensures that the tissue does not become hypoxic or ischemic during the course of the experiment. The inclusion of 2,3 BDM to the perfusate to inhibit contractile movements and aid quality intracellular impalements should also abolish any autoregulation present by inhibiting the contractile apparatus associated with the vasculature as well. Although 2,3-BDM has been shown to inhibit L-type Ca^{2+} channels (Eisfeld *et al.*, 1997) and I_{to} (Xiao & McArdle, 1995), others have demonstrated that concentrations of up to 20 mM have no electrophysiological effects on the cardiac action potential (Kettlewell *et al.*, 2004).

METHODS

Rats weighing 350-400g were prepared as previously described for intracellular recordings (ie, heart was rinsed of blood on Langendorff setup, and the right ventricle dissected out under ice-cool conditions in the presence of O_2). Once the heart had been cleared of blood and contractions stopped with ice-cold HEPES buffer containing 20 mM BDM and Ca^{2+} free, the heart was placed on a dissection tray in oxygenated solution of the same type. Both atria were initially removed. One blade of the dissecting scissors was then inserted into the right ventricle and used to lift it and find the junction of the right ventricle and septum. The Right ventricle and major coronary artery was then carefully dissected from the septum and left ventricle (the remainder of the heart), making sure to keep the RV lifted and isolated from the remainder of the heart while cutting. The large coronary vessel was then canulated onto plastic 18-guage or equivalent cannula of ~ 2 cm length and tied in place with 6-0 silk suture to prevent leakage. Successful cannulation was apparent once perfusion started as the right ventricular wall would partially inflate and become less flaccid. (For a more detailed explanation of the process refer to (Maddaford T.G *et al.*, 1996)). The cannulated preparation was transferred under ice-cold conditions to a purpose built bath and placed epicardial free-wall facing up before being attached to the perfusion portal. One attached, perfusion with HEPES buffer aerated with 100% O_2 containing 1.5 mM $CaCl_2$ at 37 ^{o}C was started immediately at a flow of ~ 10-12 ml/min. A regulated suction line removed excess solution as the bath volume increased. The right ventricle was paced at a rate of 4Hz with 1ms, bi-polar pulses at 2-3 times threshold. With this method, the isolated right ventricle remained perfused, beating and submerged in the external bath solution. Contractions were subsequently

suppressed with 20 mM BDM added to the perfusate to aid in maintenance of successful microelectrode impalements. Flow rate was monitored via the control voltage signal sent to regulate peristaltic pump speed, perfusion pressure of the right ventricular preparation was monitored using a pressure transducer (MLT844, AD Instruments, Australia) attached to the perfusion line immediately before the cannula. Once setup, the preparation was perfused at different flow rates in random order. Impalements were made using aluminosilicate glass microelectrodes, filled with 3 M KCl having a resistance of > 20 MΩ to bath.

RESULTS

Inhibition of contractility to aid in impalement and microelectrode recording of membrane potentials was based on observation (no data recorded). The isolated right ventricle of one rat was perfused at a constant pressure of 60 mmHg, paced at 4 Hz and exposed to stepwise increases in BDM of 1, 2, 5, 7, 10, 15 and 20 mM until no visible contractions were present. Based on observation, 15 mM BDM significantly inhibited right ventricular movements whilst 20 mM abolished the majority of movement. As such, 20 mM BDM was used to aid in stable impalements of the isolated, perfused right ventricle preparation.

With this novel method of perfusing the right ventricle, considerable variation was seen in the perfusion pressures at the 4 different levels of coronary flow rate (4, 8, 12 and 16 ml/min). In general, perfusion pressure was 21 ± 8 mmHg at a coronary flow of 4 ml/min and up to 41 ± 9 mmHg at coronary flows of 16 ml/min. The variable nature of these pressures is likely to have been a direct result of dissecting the right ventricle from the reminder heart tissue. As such, all data are presented in relation to the maximum perfusion pressure obtained for each preparation (ie, 16 ml/min gave 100 % of the maximum pressure, and all calculations are a proportion of this, see Figure 88).

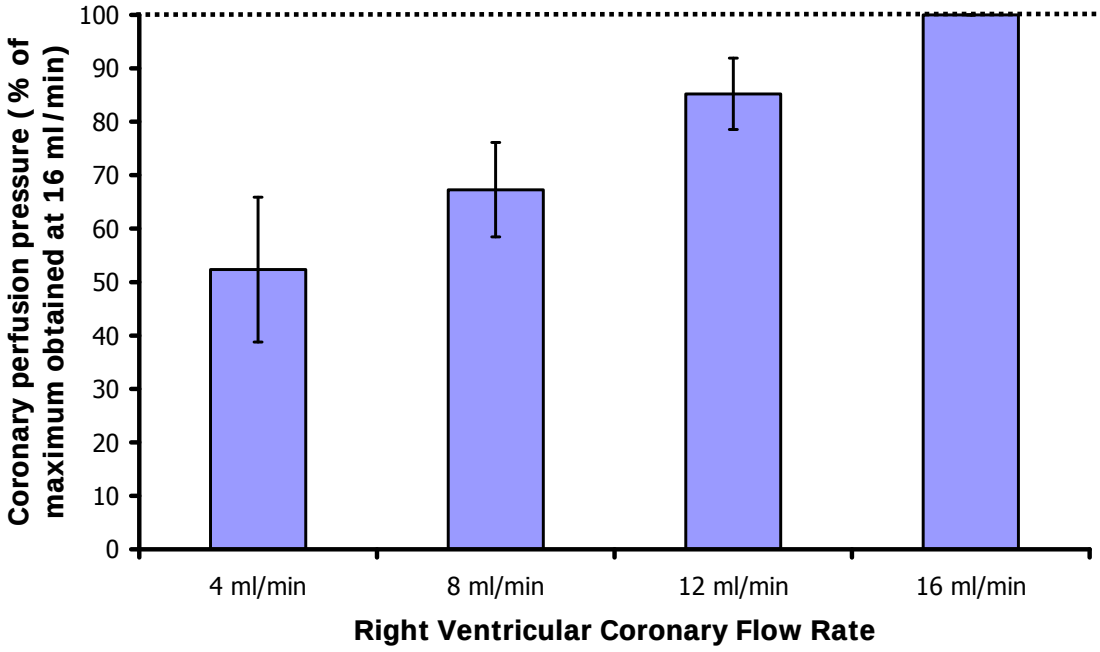

Figure 88: Effect of Perfusion Rate on Perfusion Pressure in Isolated, Perfused Right Ventricle

Figure showing effect of right ventricular coronary flow rate on perfusion pressure in isolated right ventricular preparations at flow rates of 4, 8 12 and 16 ml/min in randomised order for 5 rats. Right ventricles were perfused with HEPES buffer oxygenated with O2 containing 1.5 mM $CaCl_2$, contractions suppressed with 20 mM BDM at 37 ^{o}C and paced at 4 Hz 2-3 times threshold. Data are presented as a percentage fraction of the maximum perfusion pressure obtained at the maximum coronary flow rate of 16 ml/min, and error bars indicate ± SEM, stars denote significance $p < 0.05$. Slower coronary flow rates of 4 and 8 ml/min significantly reduced coronary perfusion pressure when compared to that obtained at 16 ml/min (ANOVA GLM followed by Tukey's pairwise comparisons).

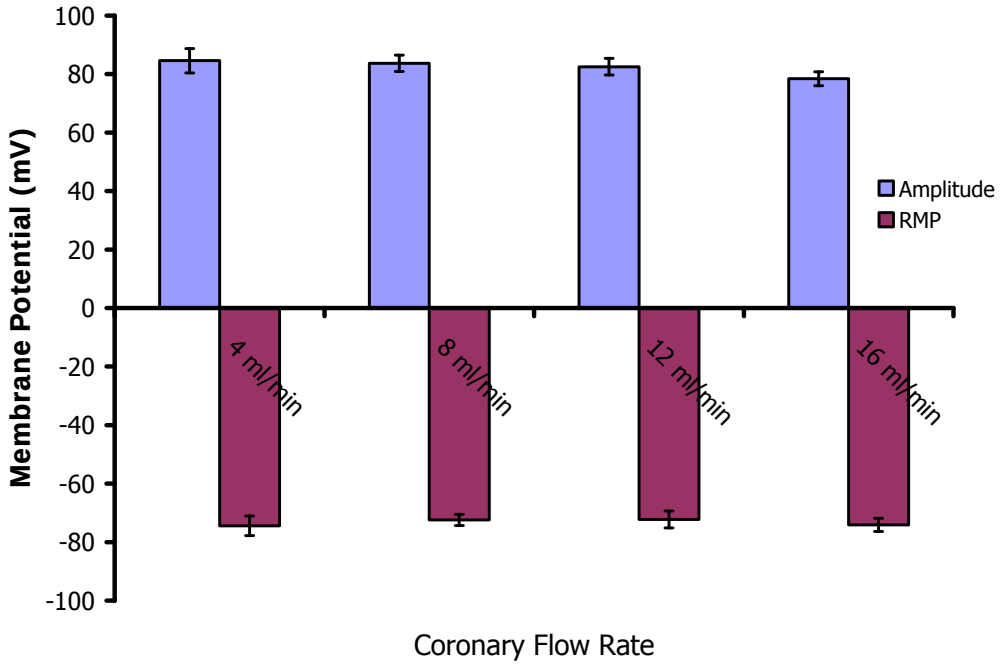

Figure 89: Effect of Perfusion Rate on Action Potential Amplitude and RMP in Isolated, Perfused Right Ventricle

Figure showing action potential amplitudes and resting membrane potential at different coronary flow rates in randomised orderData expressed as average ± SEM for n=5 rats (Error bars indicate ± SEM, each measurement in each heart is an average of at least 10 action potentials at each step in perfusion). Action potential amplitude and resting membrane potential did not change significantly with alterations in coronary flow remaining at 82 ± 3.0 and -73 ± 2.5 mV respectively across all flow rates (p >0.05, ANOVA GLM).

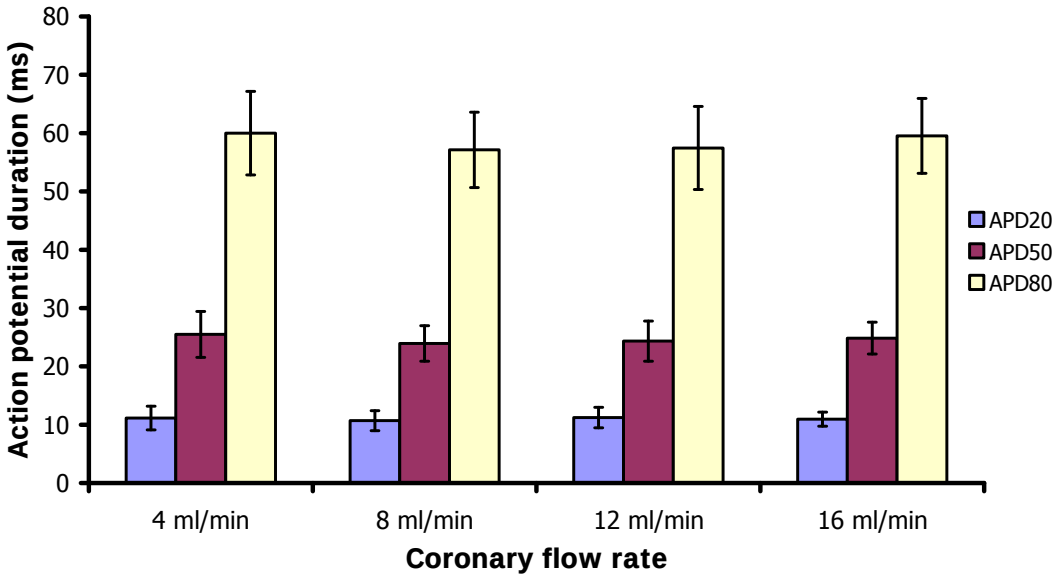

Figure 90: Effect of Perfusion Rate on Action Potential duration in Isolated, Perfused Right Ventricle

Figure showing the recorded changes in action potential duration as tested at 20, 50 and 80 % repolarisation for n=5 rats. Action potential durations did not significantly change between different coronary flow rates despite considerable increases in contractility & perfusion pressure. Action potential durations remaining at 11 ± 1.6, 25 ± 3.1 and 59 ± 6.3 ms for APD_{20}, 50 and 80 respectively (n=5) across all flow rates. Data expressed as average ± SEM for n=5 rats (Error bars indicate ± SEM, each measurement in each heart is an average of at least 10 action potentials at each step in perfusion, p > 0.05, ANOVA GLM).

Overall, little changed electro-physiologically in the isolated, perfused right ventricle as a result of changes in coronary flow (Figure 89 - Figure 90). Despite a four fold increase in coronary flow rate, there was no observable change in right ventricular action potential duration, amplitude or resting membrane potential.

DISCUSSION

Of particular interest in previous studies was that both the force and Ca^{2+} alterations associated with the Gregg effect are inhibited by blockade of NSACs with either gadolinium, or streptomycin (Lamberts *et al.*, 2002a; Lamberts *et al.*, 2002b). As such it was hypothesised that these alterations in Ca^{2+} induced by the Gregg effect should be accompanied by changes in the morphology of the action potential.

As mentioned previously, the Gregg effect is most commonly observed under poor autoregulation conditions and in the right ventricle (Westerhof *et al.*, 2006). The first step in the mechanism behind the Gregg effect has been proposed to involve the opening of NSACs, thereby increasing $[Ca^{2+}]_i$ and Ca^{2+} sensitivity of the contractile apparatus. However, there are also numerous studies that suggest stretch of cardiomyocytes is not accompanied by increases in $[Ca^{2+}]_i$ but rather $[Na^+]$ that result in cellular depolarisation and changes in action potential morphology.

- The results of the present study may suggest it is pressure that is important and not flow rate. With the isolated right ventricular preparation, normal coronary pressures observed in the Langendorff rat heart were not reached even when using comparable flow rates (Figure 88). Whilst this likely reflects losses through dissected coronary vessels, it also shows that the Gregg effect is somewhat dependent on flow changes and not just coronary pressure changes. With this in mind, however, it demonstrates that the Gregg effect and associated increases in contractility can operate independently of electrophysiological changes as well as increases in coronary perfusion pressure.

Of the limitations in the present study are the results regarding 4 ml/min flow rate through the right ventricle, which should have been considerably ischaemic. Moreover is the fact that with the described method the data obtained from the right ventricle may not necessarily correspond with the left ventricle or the whole heart. Despite these possibilities, there was no statistically observable change in action potential morphology when compared to the other flow rates where a reduction in duration may have been expected. The results of this are unclear since it is quite possible that 4 ml/min may have been adequate for perfusion of the right ventricle alone, which is not case for the whole Langendorff perfused heart. Despite this possibility, in the present experiments, changes in coronary flow rate significantly altered perfusion pressure without the occurrence of electrophysiological changes.

One of the major limitations with this preparation is the need to suppress contractile activity of the right ventricle in order to obtain stable intracellular microelectrode recordings. In the present study, this was achieved through the use of 20 mM 2,3-Butanedione Monoxime (a pharmacological phosphatase). The main disadvantage of this method is that 2,3-BDM is known to reversibly inhibit L-type Ca^{2+} channels within

the concentration range that suppresses cardiac contractility (Eisfeld *et al.*, 1997). The result is a reduction in the amplitude of I_{Ca}^{2+} in addition to the direct inhibitory effects on the contractile machinery.

CONCLUSION

Intracellular assessment of perfusion related changes in the isolated, perfused right ventricle from rats found that modulation or perfusion pressure and flow rate were not associated with any electrophysiological changes. With this in mind, the present results suggest that the Gregg effect is not the result of SAC involvement in coronary flow changes but other indirect or specifically mechanical mechanisms

CHAPTER 13:

CONTRIBUTION OF MEF TO RAPID-STRETCH INDUCED ECTOPIC BEATS

American actress & singer : Dorothy Lamour
(Dec 10, 1914 – Sept 22, 1996)

IN VIVO DEMONSTRATION OF MEF

INTRODUCTION

In the previous chapters stretch was applied via reasonably slow increases in EDLVP or tension to investigate possible MEF effects. This stretch was applied throughout the duration of the cardiac cycle and in the presence of various pharmacological SAC modulators. In the present chapter, MAP electrodes are used to explore the phenomenon of MEF in response to rapid, controlled stretches of the left ventricle at specific times in the normal cardiac cycle. The first part of the chapter gives a background to rapid stretch-induced ectopic beats whilst the next section deals with a series of experiments to determine the contribution of MEF through SACs to the stretch-induced ectopic beats. The final part of the chapter provides a detailed discussion of the experimental results as they relate to one another and to the present understanding from past and current literature. It also includes a brief conclusion based on the present results and an explanation. The aim of the present chapter was to demonstrate that rapid-stretch induced ectopic beats could be inhibited or enhanced using known SAC channel modulators.

BACKGROUND

In Langendorff experiments, inflation of a small balloon inserted into the left ventricle induces depolarisations in the ventricular tissue during diastole (Franz *et al.*, 1992; Stacy *et al.*, 1992) (see Figure 91). In single ventricular myocytes, these stretch-induced depolarisations of the membrane potential are associated with a large rise in diastolic $[Ca^{2+}]_i$, and an increase in intracellular sodium concentrations ($[Na^{+}]_i$).(Gannier *et al.*, 1994; Gannier *et al.*, 1996; Hu & Sachs, 1996; Calaghan & White, 1999; Zeng *et al.*, 2000; Calaghan *et al.*, 2003). Furthermore, the size of the ventricular depolarisations increased with the amount of ventricular stretch until a threshold is reached, above which a ventricular action potential is generated with each stretch pulse (see Figure 91). This threshold is associated with the initiation of extrasystoles (Lab, 1978a, 1980; Franz *et al.*, 1989; Franz *et al.*, 1992; Lab, 1996; Dick & Lab, 1998). Ventricular arrhythmias are often observed in patients with ventricular dysfunction be it dilated cardiomyopathy, overload or other contractile problems and may be explained by these depolarisation induced changes through stretch. Moreover, strong impacts to the chest during vulnerable periods of the cardiac cycle can induced premature ventricular beats that may initiate runs of ventricular tachycardia or even ventricular fibrillation (Kohl *et al.*, 2001) (Janse *et al.*, 2003).

To date, few studies have investigated the effect of transient stretches on the electrophysiology of the left ventricle. Of particular interest and an initiating factor of the present series of experiments was the previous work carried out by (Franz *et al.*, 1992). Using a computer-controlled servo device, it was demonstrated that rapid inflations of the LV induced premature ventricular excitations which were triggered more easily by either faster volume pulse rise velocities or by larger pulses in the LV pressure (see Figure 91). The observation of increasing stretch-induced depolarisations become progressively larger and reach the threshold required to elicit ectopic activity with increasing levels of stretch was also observed by (Stacy *et al.*, 1992). By contrast, gradual volume increases (as performed in previous chapters of this thesis) rarely induced premature ventricular excitations (Franz *et al.*, 1992). As a result, by using MAP recordings the authors concluded that membrane depolarisation is caused by both gradual and rapid ventricular stretch, but that premature ventricular excitations were more easily induced by rapid stretch alone (Franz *et al.*, 1992).

Other Effects of Stretch

Because of these pioneering experiments, many mechanical heart disorders are now thought to alter the heart's electro-physiological properties through these stretch-induced changes (MEF). Experiments based upon sudden chest impact (Kohl *et al.*, 2001) and volume or pressure changes in whole hearts (Franz *et al.*, 1992) have demonstrated changes in heart electrophysiology. These effects may lead to induction of arrhythmias including episodes of ventricular tachycardia or even ventricular fibrillation (Janse *et al.*, 2003)

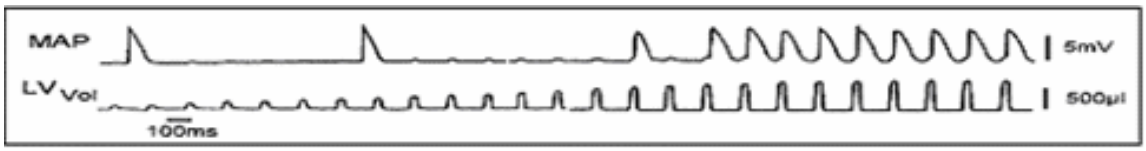

Figure 91: Stretch-induced effects on the whole heart

A monophasic action potential recording from the epicardial surface of a rabbit ventricle in response to volume pulses (change in volume is shown in lower trace) applied to a balloon inserted into the left ventricle. Volume pulses induced a transient membrane depolarization, which increased amplitude parallel to increases in volume pulse amplitude. Above a certain volume, the transient depolarisations produced premature ventricular excitations (extrasystoles). Diagram from Franz e.t al., (1992).

The present study investigates MEF effects in the whole heart using a servo controlled inflation model to predictably elicit controlled pressure changes at timed intervals in the cardiac cycle. In addition to streptomycin, $NiCl_2$ was used to inhibit the Na^+-Ca^{2+} exchange mechanism based on the possibility that stretch may modulate the cardiac action potential indirectly through the Na^+-Ca^{2+} exchange mechanism and changes in the Ca^{2+} transient associated with stretch (duBell *et al.*, 1991; Bouchard *et al.*, 1995; Tavi *et al.*, 1998). In rat cardiomyocytes, Na^+-Ca^{2+} exchange activity is proportional to $[Ca^{2+}]_i$ (Sham *et al.*, 1995), which is more than likely to be modulated as a result of Ca^{2+} affinity changes of contractile apparatus during sudden stretch and release (Tavi *et al.*, 1998)

Importance of Na^+-Ca^{2+} exchanger

The majority of experiments thus far have focused on the direct contribution of NSACs and KASCs to possible MEF-induced changes to cardiac action potential morphology. However, as mentioned in the review of past and current literature, the Na^+-Ca^{2+} exchanger also plays an important role in cardiac electrophysiology. This exchanger enables the electrogenic exchange of Na^+ and Ca^{2+} ions across the cardiomyocyte membrane in one of two directions. In the forward direction, the exchanger moves 3 Na^+ ions into the cell whilst removing 1 Ca^{2+} ion from the intracellular space. Thus, the Na^+-Ca^{2+} exchanger forms another mechanism by which intracellular Ca^{2+} may be extruded from the intracellular space during relaxation and diastole. The forward mode operates over the majority of the action potential duration at potentials more negative than ~-33 mV. This however, comes at the cost of depolarising the cell membrane with the addition of one extra positive charge from the movements of Na^+ into the cell. At membrane potentials more positive than ~-33 mV during the period immediately after excitation, the reverse mode of the exchanger helps cardiomyocyte contraction and calcium-induced calcium release by facilitating Ca^{2+} movement into the cell and Na^+ removal. As such, changes in Na^+-Ca^{2+} activity brought about indirectly by NSACs could also potentially contribute to MEF and changes in the morphology of action potentials. Moreover, changes in $[Ca^{2+}]_i$ resulting from alterations in myofilament Ca^{2+} sensitivity could also contribute to cellular depolarisation through reverse mode operation of this exchanger.

METHODS

Rat hearts were prepared as per chapter 3. Hearts were paced at 4 Hz and perfused with bicarbonate buffer containing 1.5 mM $CaCl_2$ at a constant flow rate corresponding to a perfusion pressure of 60-70 mmHg once stable. The servo-controlled injector was an addition to the previous setup by means of a standard 3-way tap joined to the balloon catheter. Intra-ventricular volume changes were performed both manually (to set resting diastolic pressure) and mechanically using the servo regulated syringe driver operating a water filled, gas tight 1ml syringe (Hamilton instruments, USA) . The syringe plunger was driven by a high torque DC servo with feedback loop (Jayco, model, USA) mounted on the same horizontal plane of the plunger using a purpose made movement transfer device designed by staff of the engineering dept, Adelaide University. The variable plunger movement and hence balloon volume commands were generated by a purpose designed control box that also received input from the pacing source used to drive the heart rate of the Langendorff perfused heart. Servo-controlled inflations of the intraventricular balloon were timed so that EDLVP would increase to the desired level at the end of the previous contraction when activating the circuit. With the left ventricular balloon in place and the heart perfused in the constant perfusion mode, left ventricular diastolic pressure was increased in small 5 mmHg steps using the servo control to obtain the threshold for ectopic activation (see Figure 92, pg 236). Since MAP recordings are uniquely suited to detect after depolarisations in the heart (Franz, 1994), a standard contact MAP electrode was used on the free wall of the left ventricle to observe the induction of stretch-induced ectopic beats. Ectopic threshold was taken as the rapid EDLVP required evoking an ectopic 4 times consecutively.

In preliminary experiments, a difference in ectopic threshold was found when approaching the threshold from higher or lower EDLVPs. As such, in the present set of experiments, ectopic threshold was obtained by gradually increasing the volume injected into the intra-ventricular balloon. In addition, based on other research, stretches were timed to occur at the end of the previous action potential during late diastole (see Figure 92, pg 236), the most vulnerable period to stretch-induced ectopic beats (Zabel *et al.*, 1996)

In the present study, because of the use of a servo controlled setup, I was unable to alter the rate at which various EDLVPs were obtained other than by increasing the final

EDLVP. As such, I could not investigate rate-related phenomena regarding the induction of ectopic beats but inducibility as determined by the final EDLVP required to obtain an ectopic beat with the present setup. Where ectopic beats were induced, regardless of where in the transient stretch period, adequate time (30s-1 min or as required) was allowed for heart contractility to return to normal before the next test of trial transient stretch.

RESULTS

Rat hearts from 400-500 g rats were perfused with bicarbonate perfusate at a constant coronary flow rate to obtain a final coronary perfusion pressure of 60-70 mmHg. Coronary flow rate was on average 10 ± 1 ml/min (n=7). Under control conditions, the threshold for ectopic induction was tested for each heart. Once a satisfactory and reproducible threshold was established, the hearts were exposed to either streptomycin (80 μM, n=7), low external calcium conditions (0.5 mM $CaCl_2$, n=7), 20 μM Verapamil (n=6), a combination of 80 μM streptomycin and 20 μM Verapamil (n=6) or 5-10 μM $NiCl_2$ (n=4), see Figure 93.

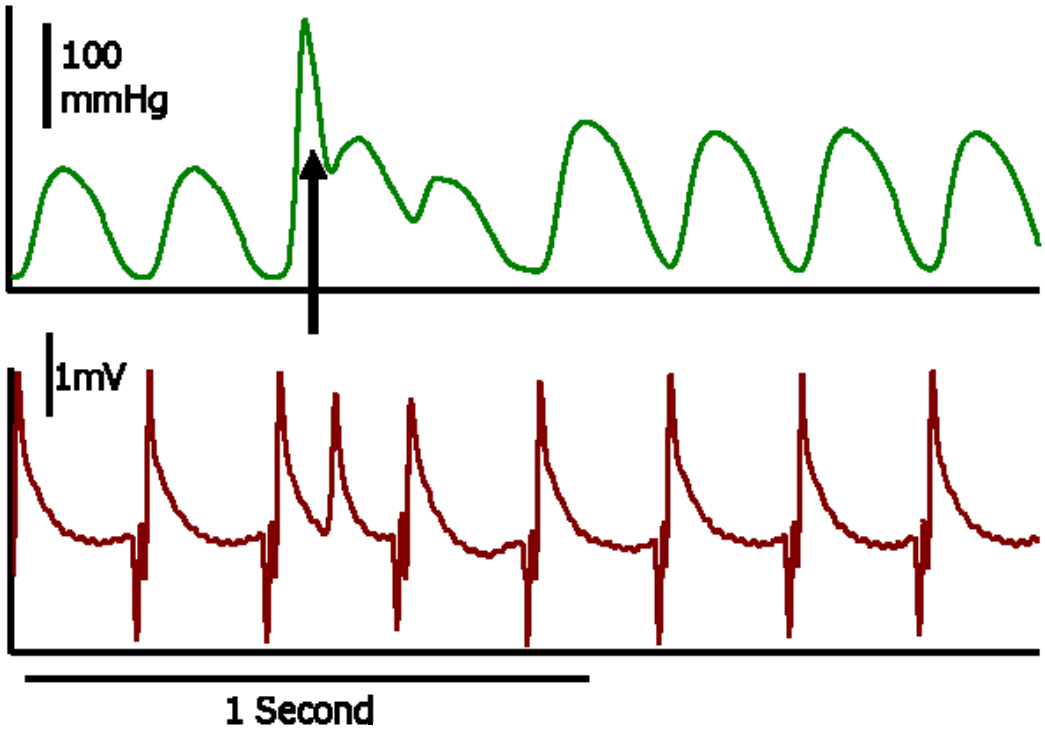

Figure 92: Timing of Stretch-induced Ectopic Beat

Figure showing a rapid increase in end diastolic left ventricular pressure resulting in an ectopic action potential and beat. Upper trace shows left ventricular pressure (LVP) (diastolic of 0 mmHg, systolic 110 mmHg, with EDLVP of the stretch ~20 mmHg). Arrow denotes initiation of stretch device, and the pressure artifact (a result of insufficiencies in the pressure transducer and placement of the rapid syringe driver in the LVP system).

On average, ectopic beats were inducible when EDLVP was rapidly increased to 12 ± 3 mmHg but this threshold ranged from 7 to 23 mmHg in all experimental control groups. As such, to decrease inter-rat variability as previously reported (Hansen *et al.*, 1990), all results are expressed relative to the threshold obtained for each heart under control conditions (see Figure 93). These variations are likely due to some differences in the timing of the applied pressure pulse/step, heart geometry and irritability. In the present results, ectopic beats or runs of ectopic beats that occurred after the initial rapid inflation were discounted, and only those stretches that induced an ectopic beat immediately upon inflation were included in the final result. However, as with the observations made by (Hansen *et al.*, 1990), many of the stretches once past a given threshold were able to initiate excitations during or shortly after the volume increase.

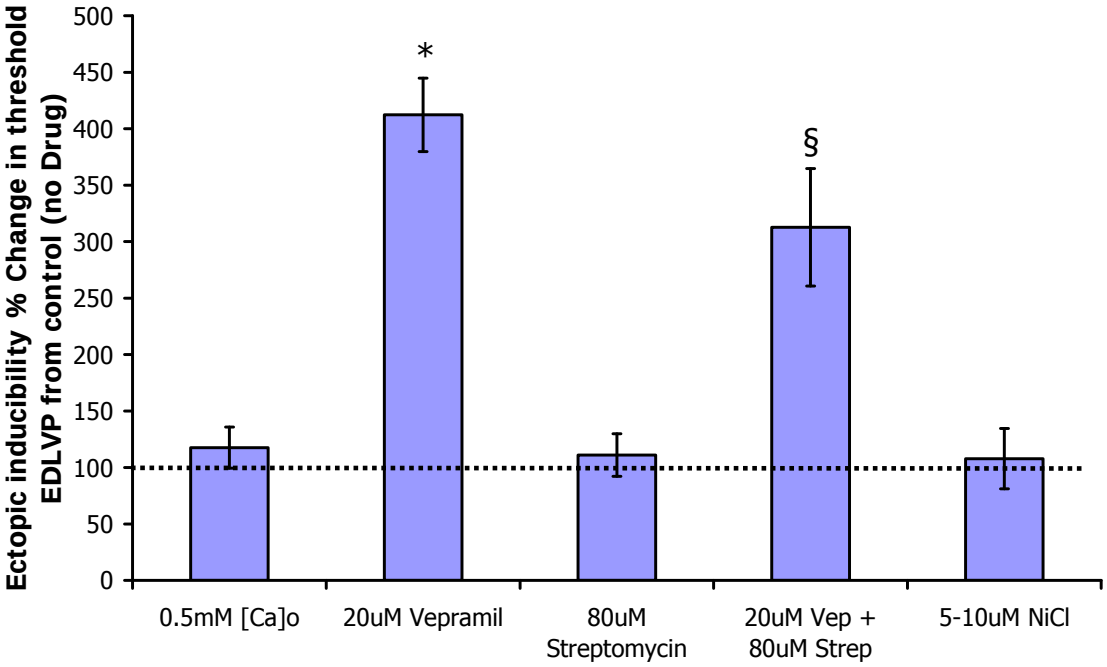

Figure 93: Pharmacological Modulation of Stretch-induced Ectopic Beats

Figure showing that only solutions containing verapamil inhibited stretch-induced ectopic activity in the isolated, Langendorff perfused rat heart. Data were compared to respective controls, significance accepted < 0.05. 20 μM verapamil significantly increased ectopic threshold (denoted p = 0.01) as did a combination of verapamil and streptomycin (§ denotes p = 0.08, ANOVA GLM followed by Tukeys pairwise comparisons).*

Upon exposure to various conditions it was noted that the NSAC modulator, streptomycin (80 μM) had no effect on the inducibility of ectopic activity with rapid stretch. In addition to this observation, decreasing external $[Ca^{2+}]_o$ to 0.5 mM or inhibiting I_{NCX} activity with $NiCl_2$ also had no effect. By contrast, a reasonably high concentration of verapamil had a significant effect on ectopic inducibility resulting in a four fold increase in ectopic threshold (Figure 93).

10 μM SKF, a TRP channel blocker was also tested however inconsistent results were obtained (n=3). Due to cost and available quantity considerations, only a small number of experiments were able to be performed rendering insignificant data.

DISCUSSION

Through the use of a servo-controlled mechanism in conjunction with isolated rat heart preparations, the present study sought to modulate stretch-induced ectopic activity using known modulators of NSACs. The present servo model is much simpler than those previously used or described elsewhere (Hansen, 1993; Zabel *et al.*, 1996) because it utilised pre-constructed components currently available through many hobby shops. These servo components already include the adequate feedback mechanisms regarding position, but unlike previous literature, lacked the ability to easily alter the rate of movement. However, this method was chosen for its ease of application and cost effectiveness.

As previously observed, the occurrence of ectopic ventricular activity occurred rarely when EDLVP was gradually adjusted when setting the diastolic baseline. By contrast, rapid inflation of the balloon using the servo-controlled syringe consistently produced ectopic beats above a particular threshold (see Figure 92). This observation is consistent with previous studies (Franz *et al.*, 1992) demonstrating that transient stretch of the left ventricle by means of controlled volume increases induced left ventricular ectopic beats. Although several studies have described this phenomenon in response to sudden stretch (Franz *et al.*, 1992) evidence for NSAC modulation of the response is somewhat limited. By examining the ectopic response to transient stretches in the presence of streptomycin (a known NSAC modulator) amongst other pharmacological agents we can obtain a better understanding of the possible electrophysiological mechanisms underlying stretch-induced ectopic beats.

As previously observed in a similar study by (Hansen *et al.*, 1990) considerable variability was seen in the pressure required to induce ventricular depolarisation for different ventricles/Langendorff preparations. As such, the results presented above were re-calculated and expressed relative to the diastolic pressure required to induce ectopic beats under control conditions (no pharmacological modulators of NSACs present) to minimise inter-heart variability. The data presented clearly establish that only when external sources of Ca^{2+} influx were inhibited (verapamil) were rapid stretch-stretch induced ectopic beats significantly inhibited. By contrast, streptomycin, a drug shown to block NSACs at a concentration of 80 μM failed to alter rapid stretch-induced ectopic thresholds. This is inconsistent with (Stacy *et al.*, 1992) who showed in blood-perfused isolated canine left ventricles that the NSAC blocker Gd^{3+} at 10 μM

produced a potent inhibition of stretch induced arrhythmias in their model. Whilst the use of Gd^{3+} in such an environment is questionable (Caldwell *et al.*, 1998), like streptomycin, Gd^{3+} is known to inhibit NSAC currents in the mammalian heart. It should be noted, however, that the ability of these pharmacological modulators of NSACs to inhibit stretch-induced ectopic activity could be related to the blockage of other major contributing currents to the cardiac action potential. Gd^{3+} is known to block I_{Na} and I_{NCX} at concentrations near 10 μM (see chapter II). One possibility may be that these rapid stretch-induced ectopic beats are the result of changes in Ca^{2+} flux across the membrane be it through changes in the reverse mode of the Na^{+}-Ca^{2+} exchanger, I_{Ca} or NSACs. Streptomycin (50 μM) has been previously found not to block the Ca^{2+} influx through NSACs in isolated mouse cardiomyocytes (Kondratev & Gallitelli, 2003). However, since 50 μM streptomycin inhibits the major contributor to I_{NSAC}, namely Na^{+} influx, this does not explain the present results since 80 μM streptomycin should have been adequate to suppress Na^{+} influx via I_{NSAC}. Consistent with the present findings, the role of stretch activated channels in chest wall impact induced arrhythmia has recently been dismissed *in vivo* (Garan *et al.*, 2005). Here, streptomycin was administered intramuscularly such that serum levels were in the order of 115 μM (similar to that used in vitro) and animal received precordial impacts. As with the present study, there was no difference in the ability to induce ectopic behaviour (or arrhythmia in swine) before or after streptomycin was administered. Whilst the authors suggest that the results offer no evidence that streptomycin-sensitive NSACs play a role in the induction of ventricular fibrillation by low impact to the chest, there are no studies to suggest that streptomycin-insensitive NSACs exist. It has been shown that the Ca^{2+} component of NSACs is partially insensitive to streptomycin in isolated mouse cardiac myocytes (Kondratev & Gallitelli, 2003), this is unlikely to have any bearing of the above results since others have demonstrated that Na^{+} influx through NSACs is more crucial to the generation of stretch-induced electrophysiological changes (Youm *et al.*, 2006).

As such, it can be reasonably concluded that NSACs have little role in this pathological mechanism. This may suggest that rapid stretch induces damage that may be microscopic as opposed to macroscopic, and thus not readily detected using anatomical or histological techniques.

Another probable possibility is that the present results can be considered consistent with the theory that stretch may alter $[Ca^{2+}]_i$ through intracellular mechanisms such as

altered Ca^{2+} sensitivity of the contractile apparatus (Lab, 1980; Allen & Kurihara, 1982; McDonald *et al.*, 1995; Wakayama *et al.*, 2001). It has previously been demonstrated that rapid changes in stretch and release of muscle can alter the dissociation rate of Ca^{2+} from troponin (Allen & Kentish, 1985; Wakayama *et al.*, 2001). Such an effect could promote cellular depolarisation large enough to invoke an action potential through the reverse mode operation of the Na^{+}-Ca^{2+} exchanger. Indeed, it has been shown in isolated guinea pig cardiomyocytes that release of caged Ca^{2+} or Ca^{2+} from the SR into the intracellular space leads to Na^{+}-Ca^{2+} exchange activity and can account for the development of after depolarisations such as those observed in the present experiment with rapid stretch (Spencer & Sham, 2003). Whilst it was proposed that this current could account for up to 50 % of the normal action potential duration in guinea pig cardiomyocytes, this is unlikely to be the case in the rat due to the significantly shorter action potential duration and different morphology (Janvier & Boyett, 1996). However, as with the study by (Spencer & Sham, 2003), our results regarding the effects of verapamil on ectopic inducibility suggest the involvement of L-type Ca^{2+} channels in the generation of after depolarisations (Adachi-Akahane *et al.*, 1996). Moreover, it has been demonstrated in mouse ventricular cardiomyocytes that streptomycin (50 μM) blocked all NSAC Na^{+} movements into the cell, but only partially blocked Ca^{2+} (Kondratev & Gallitelli, 2003).

Consistent with this possibility, and earlier study based on action potentials recorded from rat atrial myocytes showed that stretch-induced changes in the action potential were modulated by the sarcoplasmic reticulum (Han *et al.*, 1999). Here, Ryanodine, thapsigargin and caffeine were all successfully used to inhibit stretch-induced increases in action potential duration suggesting an important role of the SR and $[Ca^{2+}]_i$ in the modulation of the action potential during stretch. Given the contribution of the SR to the Ca^{2+} transient, this observation supports the possibility that changes in Ca^{2+} sensitivity and Ca^{2+} release from the contractile apparatus as a means for altering cellular excitability with rapid stretch.

A similar experiment was performed by (Dick & Lab, 1998) who found that stretch-induced ectopic activity was followed by a "mechanoelectric adaptation period" during which another ectopic could not be induced for at least 1 minute. This is an interesting observation, and again not consistent with NSAC behaviour at the isolated cellular level (see chapter II). In the present experiments, ectopic beats could be generated at intervals shorter than 1 min apart, although this was not tested for specifically, rather,

contractile responses were allowed to stabilise before the next inflation was given. Moreover, others have shown disruption of the cytoskeleton with colchicine and cytochalasin-B had no effect on the ectopic threshold, again, contrary to experiments performed in isolated myocytes (Isenberg *et al.*, 2003). As such, the question as to the involvement of the cytoskeletal matrix and hence NSACs in MEF responses is inconsistent across preparations.

In the present results, ectopic beats or runs of ectopic beats that occurred after the initial rapid inflation were discounted, and only those stretches that induced an ectopic beat immediately upon inflation were included in the final result. However, as with the observations made by (Hansen *et al.*, 1990), many of the stretches once past a given threshold were able to initiate excitations during or shortly after the volume increase. In addition, the quality of the MAP signal in the present study was not considered paramount and as such no detail is provided on morphological changes. With this in mind, however, an "all or nothing" response was noted in that diastolic depolarisations in the MAP signal were not observed as previously described in the rabbit model by (Franz *et al.*, 1992). There was no observable diastolic MAP depolarisation that correlated with increasing intra-ventricular volumes. Instead, the present results found an ectopic was either generated or it wasn't. This may relate to slightly different timing of the stretch compared to (Franz *et al.*, 1992) but otherwise remains unclear. Stretch was applied during the later phase of the rat ventricular action potential, which has previously been shown to induce after depolarisations observable in the MAP signal, an observation not seen in the present experiments.

The present results are somewhat consistent with the observation that 50 μM streptomycin was unable to inhibit wall-stress-induced arrhythmias upon aortic occlusion in the rat heart (Salmon *et al.*, 1997). More interestingly, 200 μM streptomycin was effective, but at this concentration, it is more than likely other channels important to normal cardiac myocyte function were influenced.

CONCLUSION

From the present results it is concluded that whilst MEF can be observed in the form of stretch-induced ectopic activity, the mechanism remains undefined but is unlikely to involve NSACs since the stretch-induced disturbances in electrophysiology were not hindered in the presence of NSAC inhibitors (80 μM streptomycin).

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to Mr Alban O'Brien and Mr Ian Linke of the Adelaide University Electronics Engineering Department for their development of the servo controlled device used to drive EDLVP changes in the present chapter. I would like to especially thank Mr O'Brien for his persistence and flexibility with the design and application. Without his help and knowledge, the present experiments would not have been possible.

IN VIVO DEMONSTRATION OF MEF

BACKGROUND

In vivo demonstration of MEF has been conducted in both man and mammalian species with varying effects (Taggart *et al.*, 1992; Horner *et al.*, 1994; Horner *et al.*, 1996). The overall conclusion is that stretch of the left ventricle by means of aortic occlusion results in a decrease in MAP duration that is time dependent (Horner *et al.*, 1994) or restored upon release (Taggart *et al.*, 1992) . Unlike the methods presented earlier within this thesis, the method of demonstrating MEF in the *in vivo* model is compounded by both autonomic and hormonal influences. The main advantage with this method is that MEF can be observed in the live animal model with the nervous system intact and heart perfused with blood rather than by artificial means. With these observations in mind, this series of experiments was aimed at observing similar MEF responses in the anaesthetised rat *in vivo*.

METHODS

Rats were anaesthetised as set out in chapter 3. A tracheotomy was then performed and the rat put on artificial ventilation. The femoral artery was then canulated to obtain blood pressure readings using a standard physiological pressure transducer (AD Instruments Aust, MLT844). Once canulated, a small volume of heparinised saline was injected to flush the line. Following successful cannulation, 0.4 ml of blood was

taken for O_2, CO_2 and pH analysis. Ventilator settings were then adjusted accordingly to maintain these values within the appropriate ranges. A heating pad provided heat to maintain thoracic temperature at 37 ^{o}C and once the animals' temperature and blood-gas concentrations were stable, the thoracic cavity was opened to enable access to the heart. Body temperature (rectal temperature) was monitored throughout experiments and maintained at 37 ± 0.3 ^{o}C. After the chest cavity was opened, another blood sample was taken for analysis, with a 3rd blood sample taken upon completion of the experiment. Once the heart was exposed, a transmural MAP electrode was inserted into the left ventricular free wall and baseline MAP signals recorded. A set of haemostats was then used to clamp the whole of the aorta for up to 20s during which the MAP electrode remained in the LV free wall. MAP durations and BP signals were analysed offline for basline values as well as changes during aortic occlusion and following resoration of aortic flow (see Figure 94).

PRELIMINARY RESULTS

Preliminary experiments were conducted in un-paced hearts. It was found that touching the aorta prior to occlusion with haemostats was sufficient enough to induce a series of ectopic beats.

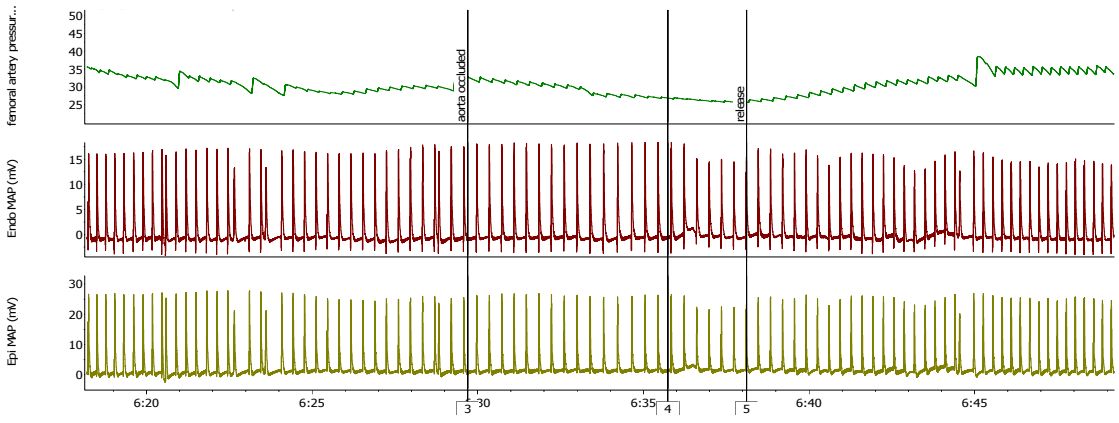

Figure 94: Effect of Aortic Occlusion on Heart Rate and Mean Arterial Pressure

Figure showing (a) change in heart rate during aortic occlusion (seen from spacing of MAP signals in both lower traces) and (b) mean arterial pressure (upper green trace) during aortic

occlusion of rat heart in vivo. Ectopic beats can be clearly seen when movement was inflicted upon the heart during periods immediately before aortic occlusion and following release.

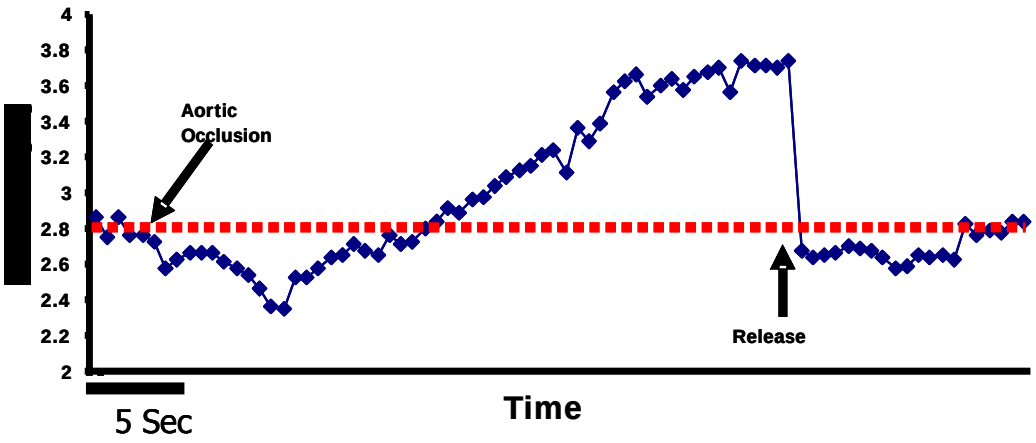

Figure 95: Effect of Aortic Occlusion on HR *in vivo*

Figure showing changes in beat-to-beat interval (Hz) before, during and following aortic occlusion in vivo. In this case, following aortic occlusion, basal heart rate decreased to 2.4 Hz before steadily rising to a maximum of 3.8 Hz prior to release. Following release, basal heart rate was re-established

DISCUSSION

As can be seen from Figure 94 and Figure 95, aortic occlusion was demonstrated to induce an immediate reduction in basal heart rate, followed by a gradual increase over 20 seconds (not seen in the first figure due to the short duration of the clamp). Since changes in heart rate influence MAP durations, it was evident that the heart must be paced at 4-5 Hz whilst in-vivo to prevent flow mediated changes in APD from influencing the results. This result itself is inconsistent with observed reductions in MAP duration (Taggart *et al.*, 1992; Horner *et al.*, 1994; Horner *et al.*, 1996) since reductions in heart rate or pacing frequency are generally associated with longer action potential durations. As could be expected with the absence of pacing in such conditions, the present experiments presented inconsistent results regarding MAP duration before, during and immediately after aortic occlusion. The other problem with such experiments is the inability to regulate ischemia and diastolic pressure with aortic occlusion. As such, the next series of experiments present are from animals paced at 4 Hz throughout the duration of the experiment to avoid the influence of heart rate on action potential durations.

RESULTS

Rats were prepared as above with 3-4 blood samples taken for immediate analysis. These hearts were paced at 4 Hz in order to ensure that MAP duration changes were a result of aortic clamping and not HR variations.

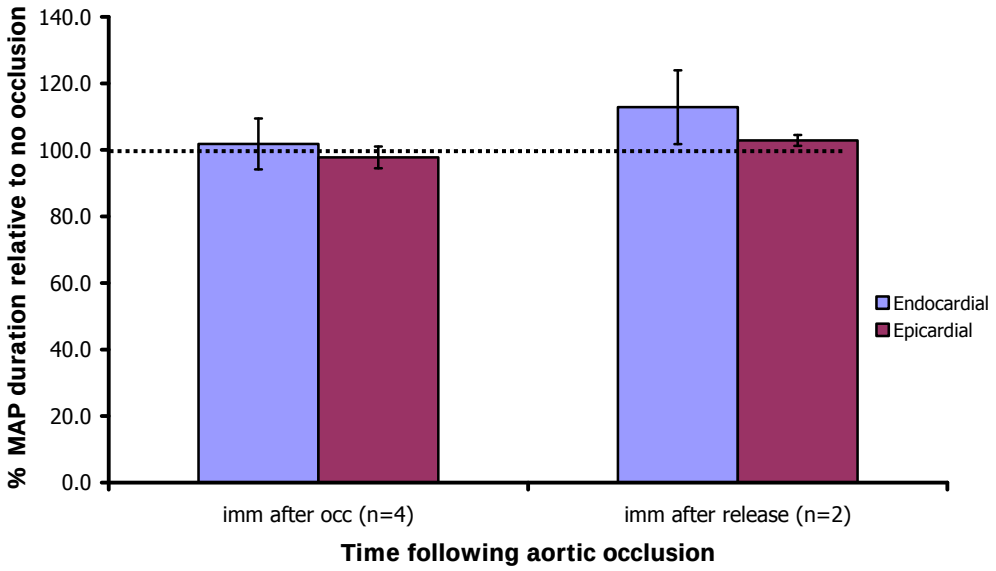

Figure 96: Effect of Aortic Occlusion on Endocardial and Sub-epicardial MAP APD_{80} immediately after occlusion and immediately after release

Figure showing no change in monophasic action potential duration at 80 % repolarisation of endocardial and sub-epicardial MAP recordings. Data are compared relative to control conditions (no occlusion) with MAP durations immediately after occlusion (imm after occ) and immediately after release of aortic occlusion (imm after release). Data expressed as mean ± SEM, $p > 0.05$ using paired t-test relative to control conditions.

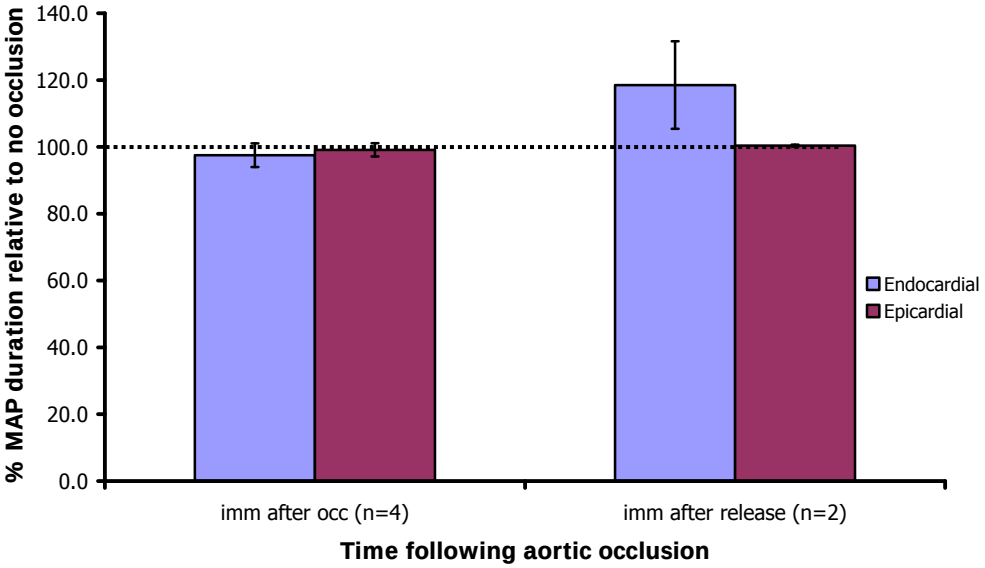

Figure 97: Effect of Aortic Occlusion on Endocardial and Sub-epicardial MAP APD_{50} immediately after occlusion and immediately after release

Figure showing no change in monophasic action potential duration at 50 % repolarisation of endocardial and sub-epicardial MAP recordings. Data are compared relative to control conditions (no occlusion) with MAP durations immediately after occlusion (imm after occ) and immediately after release of aortic occlusion (imm after release). Data expressed as mean ± SEM, $p > 0.05$ using paired t-test relative to control conditions.

DISCUSSION

Upon removing heart-rate associated changes in MAP durations, aortic occlusion using haemostats, whilst able to induce ectopic beats had little effect on MAP durations immediately following aortic occlusion of after release (Figure 96, Figure 97). It is agreed that this is a rather crude method for looking at the effects of LV stretch and MEF on action potential duration. Whilst manipulating the aorta, every effort was taken to avoid stimulating or interfering with the vagal nerve. However, due to its close proximity to the heart and aorta, it remains possible that direct stimulation may have occurred and resulted in the initial decrease in HR seen in preliminary experiments.

From the rate controlled experiments, however, there was still no observable change in MAP APD in either epicardial or endocardial recordings immediately following occlusion or release. This may have been due to the small number of animals tested and the difficulty in setting up the procedure. The observation that ectopic beats were readily seen when clamping and releasing the aorta still demonstrate the presence of MEF to some extent. These results suggest that MEF may be originating from another source, more likely the junction between the aorta and cardiomyocytes or even pulmonary veins (the latter known to be involved in many cardiac arrhythmia (Gerstenfeld *et al.*, 2003; Kalifa *et al.*, 2003; Perez-Lugones *et al.*, 2003). This result is partly consistent with similar aortic clamping methods applied to the dog heart in vivo (Takagi *et al.*, 1999). However, aortic clamping in the dog resulted in no change in endocadial MAPs at either APD_{50} or 90, but a significant reduction in epicardial APD_{50} and 90 not seen in the present study.

The present results regarding induction of ectopic beats upon occlusion of the aorta are consistent with that observed previously in situ dog and pig experiments (Franz *et al.*, 1989; Dean & Lab, 1990)

CONCLUSION

A number of studies have shown stretch-induced ectopic activity in both the whole heart and at a cellular level (Franz *et al.*, 1992; Stacy *et al.*, 1992; Hansen, 1993; Zabel *et al.*, 1996; Kong *et al.*, 2005). However, the interpretation of these results is difficult as the necessary follow up experimentation with currently accepted NSAC modulators and the confirmation of the involvement of NSACs has not been undertaken. Of the studies reviewed, only one example of chest impacts delivered in vivo was found to use streptomycin (at a plasma concentration of 115 μM) as an NSAC blocker, to which there was no effect on the experimental outcomes. This is contrary to one other study in blood perfused canine ventricle which found 10 μM Gd^{3+} to be potent inhibitor of stretch induced arrhythmias despite the unsuitability of Gd^{3+} in such applications (Caldwell *et al.*, 1998). Furthermore, no replication of similar findings *in vitro* is evident since the discovery of the "selective" NSAC blocker GsMTx-4 (Suchyna *et al.*, 2000; Oswald *et al.*, 2002) leading to the conclusion that existing experiments are probably non-repeatable as conferred by the variability in eliciting ectopic responses in both the present study and by others (Hansen *et al.*, 1990). This leads to the

conclusion that the involvement of NSACs in these ectopic responses is still controversial.

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I would again like to acknowledge Dr Lorraine Mackenzie for sharing her ideas, skills and expertise which brought about the above *in vivo* experiments. Without her experience in small animal surgery and preparation, the above results would not have been possible.

CHAPTER 14: CONCLUSION

1st woman to sing into a microphone over the radio
American jazz singer : Vaughn De Leath
(Sept 26, 1894 – May 27, 1943)

Chapter 14 - Table of contents

INTRODUCTION

As with any Langendorff heart preparation, MEF is regularly observed as alterations in rhythmic contractions when handling the heart. In vivo, these ventricular arrhythmias can be potentially life threatening and contribute to significant problems in the clinical setting. Various electrophysiological changes can facilitate the development of arrhythmia, namely reductions in action potential duration that can bring about the development of re-entry circuits. This chapter presents a summary of the main findings of this thesis and an overall discussion of their application and contribution to MEF theory as it presents today. The results of the experiments are summarised below:

SUMMARY OF RESULTS

Chapter 5 - Contribution of MEF to the Frank-Starling Relation:

Physiological levels of stretch associated with the Frank-Starling (length-tension) phenomenon of the heart were not associated with any change in heart electrophysiology as viewed using monophasic action potentials (both suction and contact MAP electrode techniques). These effects were absent regardless of the possible influences of perfusion pressure (or coronary flow rate). Further more, it did not appear to matter if small 2.5 mmHg stepwise increases in EDLVP or large steps were used, no electrophysiological changes were observed.

Chapter 6 - Further Investigations of MEF in the Rat Heart

After the negative result established in chapter 5, it was important to confirm that the MAP electrode utilised could visualise the small possible electrophysiological changes associated with physiological levels of stretch. 4-Aminopyridine (10μM) was used to partially inhibit K^+ channels and lengthen the action potential a small amount. This effect was observable using contact electrode techniques.

The possibility that the rat action potential was to short too adequately observe electrophysiological changes associated with physiological stretch was also entertained. Replicating experiments in isolated Langendorff perfused guinea-pig hearts where the action potential (as recorded by MAPs) is a minimum of 60 % longer also failed to

adequately show changes in the electrophysiology of the left ventricle with physiological stretch.

It was demonstrated, however, that non-timed, extremely rapid inflations or deflations in EDLVP could induce ectopic beats and additional depolarisations in the MAP recording.

Chapter 7 – Epicardial and Endocardial MEF Responses:

Using transmural MAP techniques that enabled the simultaneous recording of sub-epicardial and endocardial MAPs during manipulations of EDLVP, it was observed that physiological levels of stretch (up to 20-25 mmHg) had no influence on sub-epicardial MAPs but shortened endocardial MAPs. Moreover, extreme stretch (50-55 mmHg) significantly shortened both sub-epicardial and endocardial MAPS, but the latter to a greater extent. These effects were blunted or abolished in both sub-epicardial and endocardial MAPs in the presence of the SAC modulators streptomycin (80 μM), Riluzole (30 μM) and chlorpromazine (10 μM).

Chapter 8 – Further Investigations into Transmural MEF:

A reduction in Langendorff perfusate temperature from 37 ^{o}C to 32 ^{o}C increased the durations of both sub-epicardial and endocardial MAP recordings but had no effect on extreme stretch-induced electrophysiological changes of the left ventricle. This is despite the temperature dependence of stretch-activated channels.

In Langendorff perfused guinea-pig hearts, transmural MAP electrodes were able to detect a an abbreviation in action potential durations (as recorded by MAPs) in the presence of physiological levels of stretch (EDLVP = 20-25 mmHg) and this effect was modulated in the presence of streptomycin (80 μM).

The K_{ATP} channel blocker glibenclamide (10 μM) was used to successfully block extreme stretch-induced action potential shortening observed with the contact MAP in isolated Langendorff perfused rat hearts.

Chapter 9 – MEF in Isolated Muscle Preparations

In isolated, bath perfused rat left ventricular papillary muscles, moderate (0.6 g) and extreme stretch (1.2 g+) whilst associated with increases in contractility did not significantly alter intracellular recordings of resting membrane potential, action potential amplitude or durations. In addition, SAC modulators streptomycin (80 μM), Riluzole (30 μM), chlorpromazine (10 μM) and mild inhibition of contractile movements with 1 mM 2,3-BDM did not influence this effect. Moreover, as with the Langendorff perfused heart, reducing perfusate and bathing temperature of isolated papillary muscles from 37 to 32 ^{o}C and performing stretch did not alter or enhance any electrophysiological response associated with stretch.

Chapter 10 – MEF in the Isolated, Stretched Left Atrium

In isolated, bath perfused rat left atrial appendage, stretch whilst inducing significant increases in contractility also failed to induce observable changes in atrial electrophysiology when studied with intracellular recording techniques.

Chapter 11 – Contribution of MEF to the Gregg Effect

In the rat Langendorff perfused heart, stretch-induced changes in coronary flow rate (8, 12 and 16 ml/min) whilst associated with changes in contractility, were unaccompanied by electrophysiological changes in either suction, surface contact or transmural (sub-epicardial, endocardial) MAP recordings.

Chapter 12 – MEF in the Isolated, Perfused Right Ventricle

In isolated, perfused rat right ventricular preparations, changes in coronary flow rate (4, 8, 12 and 16 ml/min) whilst associated with changes in coronary perfusion pressure, were not accompanied by changes in resting membrane potential, action potential amplitude or changes in action potential duration at 20, 50 or 80 % repolarisation as recorded using intracellular techniques.

Chapter 13 – MEF in response to rapid stretch

Rapid stretch-induced ectopic beats performed at specific points within the cardiac cycle through the use of a servo-controlled syringe injector were not modulated by low

$[Ca^{2+}]_o$ conditions or the NSAC modulator streptomycin (80 μM) nor the Na^+-Ca^{2+} exchanger inhibitor $NiCl_2$ (5-10 μM). Stretch-induced ectopic thresholds were, however, significantly increased by the L-type Ca^{2+} channel blocker verapamil (20 μM).

Likewise, *in vivo* occlusion of the aorta in rats evoked ectopic beats and a change in heart rate during and following the occlusion period. Moreover, when the heart was extrinsically paced, aortic occlusion was not associated with changes in action potential duration at 80% repolarisation during aortic occlusion.

DISCUSSION

Left ventricular stretch is manifest as an increase in contractile force, an effect that is observed in the whole heart as well as isolated tissue preparations. The present results confirm that this effect, or at least the rapid component, is independent of changes in the cardiac action potential. This confirms later suggestions that stretch-induced changes in the sensitivity of TnC may explain the fast increase in contractile force associated with stretch and the Frank-Starling mechanism and thus is unlikely to involve SACs (McDonald *et al.*, 1995; Smith & Fuchs, 2000; Martyn & Gordon, 2001).

By contrast, the slow increase in contractility (not readily seen in the present experiments) otherwise described by the Anrepp effect is accompanied by an increase in Ca^{2+} transient amplitude. One of the possible mechanisms to explain this rise in Ca^{2+} transient amplitude is diastolic Ca^{2+} influx through stretch-activated non-selective cation channels and increased SR Ca^{2+} loading (Ruknudin *et al.*, 1993; Zhang *et al.*, 2000; Zou *et al.*, 2002; Kamkin *et al.*, 2003b) However, others have found that diastolic $[Ca^{2+}]_i$ does not change during stretch, and thus cannot contribute to additional SR Ca^{2+} loading. (Kentish & Wrzosek, 1998). Instead, it is suggested that stretch-induced changes in the Ca^{2+} transient occur during the action potential, possibly through enhancement of the reverse mode of the Na^+-Ca^{2+} exchange mechanism (Kentish & Wrzosek, 1998; Perez *et al.*, 2001; von Lewinski *et al.*, 2003). The present results are consistent with the absence of NSAC-induced changes in SR Ca^{2+} loading which would be expected to alter action potential morphology through changes in diastolic $[Na^+]_i$ and $[Ca^{2+}]_i$. Whilst this would appear controversial at first, others have demonstrated different mechanisms whereby enhancement of the Ca^{2+} transient is possible via other mechanisms that do not necessarily initiate changes in diastolic $[Ca^{2+}]_i$ nor action potential morphology. Of the proposed mechanisms, a more recent suggestion is an auto-paracrine effect involving angiotensin II and

endothelin release (Alvarez *et al.*, 1999; Cingolani *et al.*, 2003; Cingolani *et al.*, 2005). Activation of the sodium-hydrogen exchanger has also been implicated (Cingolani *et al.*, 2001; Calaghan & White, 2004)

Involvement of SACs

Although the presence of SACs appears plausible at first in altering contractility and action potential morphology, the sensitivity of these channels and their ability to respond to physiological levels of stretch in the whole heart remain largely unexplored. NSACs are readily activated in cell-attached patches by applying either positive or negative pipette pressure (TREK-1, only the later) to the patch membrane (Sigurdson *et al.*, 1992; Kim, 1993, 2003) or even hypotonic swelling (Kim, 1993). Whilst others have performed axial stretch of isolated cardiomyocytes using glass or carbon styli (White *et al.*, 1993; Kamkin *et al.*, 2000a; Isenberg *et al.*, 2003; Kamkin *et al.*, 2003b; Kondratev & Gallitelli, 2003; Kondratev *et al.*, 2005), there is no direct means of extrapolating the mechanical stressors of these effects back to the whole heart or *vice versa*. As such, it remains plausible that SACs of either type are only activated by extreme levels of stretch or stress. Others have reported that stretch-activated currents in isolated adult rat ventricular myocytes are not easily stimulated and require a continuous mechanical deformation of at least 5 minutes in duration before a whole cell mechanosensitive current is observed (Bett & Sachs, 2000b). Other difficulties associated with the NSAC is the inability to demonstrate the current (I_{NSAC}) at the single channel level (Zeng *et al.*, 2000; Niu & Sachs, 2003). In keeping with the pathological nature of the stretch reported here, larger cell deformations did not elicit whole cell mechanosensitive currents, instead they resulted in Ca^{2+} overload, spontaneous contractions and cell death (Bett & Sachs, 2000b). This observation is likely explained by stretch-induced degredation of the patch pipette seal or possibly the point of contact used to initiate stretch in isolated cells (White *et al.*, 1993; Tavi *et al.*, 1998).

Effect of Experimental Conditions

The main problem with the current body of literature regarding the involvement of NSACs is the lack of congruency between the application of SAC modulators such as streptomycin, Gd^{3+} etc and the recording of electrophysiological changes in complex models. A number of studies have published that inhibition of SACs with these modulators influence various physiological and pathophysiological parameters but

without supporting electrophysiological evidence (Lab *et al.*, 1994; Slinker & Tobias, 1996; Nicolosi *et al.*, 2001; Lamberts *et al.*, 2002a; Yeung *et al.*, 2003). It was hoped that the current series of experiments would address both the electrophysiological changes associated with various methods of stretch, the possible involvement of NSACs and the effects of their modulation on these responses. In addition to these difficulties, all of the cellular studies investigating the contribution of SACs to electrophysiological phenomena have been performed at non-physiological temperatures. The main concern of this practice is the possibility that mechanosensitivity and temperature sensitivity may contribute directly to one another. This was hinted in the study on TREK-1 channels by (Maingret *et al.*, 2000a; Kang *et al.*, 2005) and demonstrates that the ability of these channels to respond to stretch is greatly diminished by their already high probability of being in the open state at physiological temperatures. Since the NSAC is still to be demonstrated at the single channel level, it is reasonable to confer the temperature-mechanosensitive properties demonstrated for TREK-1 channels to NSACs. Thus, it would be reasonable to assume that if temperature dependence and mechanosensitivity are directly related, as is the case for TREK-1 channels, that NSACs are also likely to play a background role since their open probability would be significantly high at physiological temperatures. TREK-1 channels display little activity at room temperature, and hence during stretch are able to display a far greater dynamic change in open probability than if recordings were made at physiological temperatures where the basal open probability is already much higher.

Regulation and Modulation of SAC Activity

Other possibilities include the intracellular regulation of SACs. TREK-1 is known to be inhibited by both PKC and PKA activation (Patel *et al.*, 1998; Koh *et al.*, 2001; Patel & Honore, 2001; Murbartian *et al.*, 2005) in addition to forming a "leak" or background K^+ channel. Moreover, the cytoplasmic C-terminal domain of TREK-1 important to TREK-1 modulation by stretch includes phosphorylation sites for PKA (Murbartian *et al.*, 2005) and thus phosphorylation may play a role in TREK-1 inactivation and hence the lack of stretch-induced activity in the present experiments. Given the high open probability of KSACs like TREK-1 under physiological conditions, and the imminent likelihood of finding specific blockers of these channels, it may be reasonable to assume that inhibition of these channels whilst in their "leak" or "background" state

may lead to cellular depolarisations as opposed to restoring resting membrane potentials. The usefulness of such drugs is not overly evident and will primarily depend on their selectiveness for these channels.

Stretch and the Gregg Effect

Whilst attempts to replicate the Gregg Effect in chapters 11 and 12 did not result in the suggested SAC-induced electrophysiological changes reported elsewhere (Lamberts *et al.*, 2002b), the theory and current literature retains implications for many aspects in the isovolumically contracting Langendorff perfused heart used throughout this study. Of particular importance and previously discussed is the anatomical differences between the epicardial and endocardial perfusion systems (Westerhof *et al.*, 2006). As discussed earlier, the sub-endocardial layers have a 10% higher coronary flow rate during diastole than the corresponding sub-epicardial layers. Whilst this appeared to be of little importance in the Gregg effect of the present results, it is likely to explain why extreme stretch induced a larger reduction in action potential duration of the endocardial MAPs when compared the reduction (if any) observed in epicardial MAP recordings. At EDLVPs of 0-25 mmHg, which are relatively smaller than the coronary perfusion pressure, the difference of mean flow through the endo and epicardial layers would have been reasonably small and thus resulted in little change in the action potential durations of either layer. However, is some cases, the pressure may have been large enough to induce a slight restriction to sub-endocardial perfusion and thus result in small changes to endocardial action potential durations. As the diastolic pressure in these experiments increased to 50-55 mmHg (approaching the coronary perfusion pressure), there would have been a greater impediment to the coronary flow through the sub-endocardial layers compared with the sub-epicardial layers. As a result, there is likely to have been a greater reduction in coronary flow through the endocardial tissue contributing to regional ischaemia and the expected reduction in action potential duration (Westerhof *et al.*, 2006). This analysis is supported by the observation that glibenclamide (10 μM), a known K_{ATP} channel blocker inhibited stretch-related shortening of action potential duration of the present experiments. Indeed, changes in cardiac contractility are known to inhibit coronary flow (Allaart & Westerhof, 1996) which is likely to induce mild global ischaemia, activation of K_{ATP} channels and action potential shortening which is readily ablosihed by glibenclamide (Hamada *et al.*, 1998). In the present study, it is reasonable to expect additional

changes in intra-left ventricular pressure would add to this impediment in coronary flow and facilitate the activation of K_{ATP} channels and hence action potential shortening. In fact, others have in the past noted the link between ischaemic induced reductions in action potential duration and those reductions seen with mechanical peturbations (Lerman *et al.*, 1985).

Thus as the heart contracts against the isovolume of the intraventricular balloon, the luminal pressure of the left ventricle increases and imposes an ever increasing pressure on the ventricular wall. This pressure is proportional to the ventricular pressure recorded and exceeds the coronary perfusion pressure supplying the heart. As a result, the coronary vessels become increasingly occluded with increasing EDLVP and completely occluded during systolic contractions. (Westerhof *et al.*, 2006) Thus, under physiological conditions (low EDLVP and a coronary perfusion pressure of 60-70mmHg), there is little impediment of coronary perfusion, thus little regional ischaemia and hence little or no reduction in action potential duration. However, at pathological and extreme levels of intraventricular stretch, the diastolic pressure alone occludes adequate coronary flow to the endocardial layers inducing an ischamemic state that likely dissipates across the myocardial wall, resulting in greater reductions in endocardial action potential duration compared to the less ischameic epicardial layers. This is suggested to explain why most infarcts are found in the sub-endocardium compared to other layers in the heart. (Westerhof *et al.*, 2006)

Other Sources of MEF

Cardiac fibroblasts that form the extracellular matrix may also explain the observations of MEF in the whole heart model. Like cardiomyocytes, fibroblast are also subject to the mechanical loading and mechano-stimulation has been shown to result in cellular changes in these cells (Gudi *et al.*, 1998). Due to their electrical coupling with cardiomyocytes, fibroblasts may play a sensory role and affect cardiac electrophysiology via MEF (Kohl *et al.*, 2005).

Sensitivity of Stretch Responses

As with this study and many other studies, stretch-induced changes were not observed at physiological levels of stretch, but rather extreme or pathological levels of stretch. As such, the thought that stretch-induced changes in electrophysiology of the heart are

only relevant in pathological conditions should be entertained. Such conditions may include infarcts, ischaemia and the well researched effects of an acute impact to the chest – *commotio cordis*.

Given the reduction in action potential duration associated with extreme, pathological levels of stretch and the distinct possibility of its inhibitory effects on coronary flow, it remains possible that physiological saline perfused heart models are not adequate for observing MEF responses. The isolated blood-perfused heart is likely to be more advantageous in this respect due to the greater oxygen-carrying capacity of blood compared to bicarbonate or HEPES buffered saline solutions (Clements-Jewery *et al.*, 2002). Assuming that extreme stretch is adding to the hypoxia of the saline buffer-perfused hearts, it is possible that action potential shortening may be reduced in blood perfused hearts if due to ischaemia resulting from the occluding pressure of EDLVP on coronary flow. Likewise, if the reduction in action potential duration is due specifically to SACs, perfusion with blood is also likely to increase action potential duration under basal (no stretch) conditions, enabling a larger reduction in APD to be seen with stretch – assuming it is the result of SAC-induced ion currents.

The theory of MEF itself developed based on observations made on previous existing data. Since the early conception of the idea that mechanical perbutations of the heart may elicit electrical responses, numerous studies have been conducted with many demonstrating conflicting and incongruent results despite many similarities in preparations. Differences in ion conductance, the ions conducted, adaptability of channels to stretch through to the sensitivity to various proposed pharmacological modulators of stretch-activated ion channels makes interpretation and comparisons extremely difficult. Moreover, there is no consistent conclusion regarding the effects of stretch on the cardiac action potential either in Langendorff perfused hearts or in isolated cardiac preparations. Explanations of differences in outcomes have been related to differences in recording techniques and basic experimental differences are not entirely satisfactory and inturn demonstrate that MEF within physiological limits is not a robust cardiac response. One possible reason for discrepancies between published outcomes may be the presence of other mechanosensitive ion channels (see literature review regarding NSACs and KSACs). More so, given the specificity in function of various cardiac ion channels, the observation that a range of established ion channels are sensitive to mechanical perbutations of the cell membrane may

suggest that mechanosensitivity is not an inbult mechanism or a specific function but rather a technical or experimental anomaly brought about by pathological stretch.

Of interest to the present studies is that it has been proposed that the same mechanism that results in the Frank-Starling response during moderate stretch can also induced pathological changes in cardiac myocytes when they are subjected to a more intense stretch (Tavi *et al.*, 1998). However, in the present study, there were no observations made of action potential lengthening or depolarisation of resting membrane potential with stretch. However, it was clearly observed that the mechanism during rapid stretch was able to induce ectopic activity. As such, the current body of data presented (both physiological and electrophysiological) are consistent with the notion that MEF plays no role in the beat-to-beat regulation of the heart (Slinker & Tobias, 1996).

Other Mechanisms of MEF

A final possibility is presented by (Cingolani *et al.*, 2005) where stretch has been implicated in a chain of autocrine/paracrine events in the heart. In this model, the Na^+/H^+ exchanger, angiotensin II and endothelin form crucial steps leading to intracellular Na^+ accumulation and increased Ca^{2+} influx through consequential reverse mode Na^+-Ca^{2+} exchange activity.

In addition to this proposed mechanism, small reductions in intracellular pH (in the order of 0.2 units) are able to change the response of rat atria to stretch. Most noticeably, intracellular acidosis of this magnitude inhibited stretch-induced changes in action potential duration, possibly by inhibition of Ca^{2+} extrusion mechanisms (Tavi *et al.*, 1999).

The present study is not the first to report an absence of electrophysiological changes with stretch except under extreme conditions. This was first reported in cat papillary muscles (Lab, 1980), and later reported in a comprehensive study on 57 frog ventricular cells where only 4 robust cases of mechanically induced stimulation out of more than 250 stretches resulted in electrophysiological changes (Riemer & Tung, 2003). This alone is inconsistent with earlier studies on intact frog ventricular experiments (Lab, 1978b). These observations combined with conflicting results between numerous studies in both atria and ventricle (Nazir & Lab, 1996; Satoh & Zipes, 1996; Tavi *et al.*, 1996; Ravelli & Allessie, 1997; Babuty & Lab, 2001) suggests

that MEF alone is not a robust and necessarily repeatable phenomenon, especially between preparations and animal models. Whilst it could be suggested the conflicts arise from the different environments (isolated cells, isolated papillary muscles and Langendorff perfused hearts) or even inter-species differences, this can only partially explain the results since most MEF studies have been done on the same animal models and across all 3 preparation methods. As with the current study, the discrepancy in results of extreme stretch across the Langendorff perfused rat heart and isolated papillary muscle preparations cannot be easily dismissed by these considerations. Furthermore, differences in temperature were tested and found not to influence the result. Thus the discrepancy between findings in the present series of studies and by other research groups remain to be explained.

A distinct possibility is that stretch-induced responses in cardiac electrophysiology (especially regarding the induction of ectopics) may be brought about by junctions between cardiomyocytes and other cell types. Fibroblasts that are electrically coupled with cardiomyocytes (Kamkin *et al.*, 2005; Kohl *et al.*, 2005) have been demonstrated to respond to stretch (Stockbridge & French, 1988; Glogauer *et al.*, 1995; Kiseleva *et al.*, 1996; Kamkin *et al.*, 2003a; Kamkin *et al.*, 2003d). Moreover, the cellular junction between the pulmonary veins and cardiomyocytes are a known emanating region of cardiac arrhytmias during atrial stretch (Gerstenfeld *et al.*, 2003; Kalifa *et al.*, 2003; Perez-Lugones *et al.*, 2003). As such, MEF may originate by mechanisms other than cardiomyocytes and alter cardiomyocyte electrophysiology through the normal conduction pathways. It is possible that junctions between cell types may result in different strain levels and distribution on connecting cardiomyocytes leading to a population of "hypersensitive" cells.

Despite the present findings in the physiological normal rat heart, others have successfully demonstrated the involvement of SACs and beneficial effects of modulating them in pathological conditions such as dilated cardiomyopathy (Nicolosi *et al.*, 2004). Such observations appear promising since similar results were obtained with gadolinium (10 μM) and very modest concentrations of streptomycin (40 μM). As such, SACs remain a popular mechanistic tool for explaining various pathological conditions and electrophysiological changes that may accompany the heart when under increased load.

Suitibility of MAP Recordings for Observing MEF

The question remains how MAP recordings can be generated by the presence of NSACs (Tranquillo *et al.*, 2004) and yet remain unaffected by concentrations of NSAC blockers that otherwise inhibit MEF responses (Eckardt *et al.*, 2000). These inconsistencies in the present theory behind both mechanisms provide some insight into the infancy of MEF theory. In addition, given the variability in reported findings of MEF in various cardiac preparation and the distinct inability to adequately disclose the function and contribution of SACs to various electrophysiological phenomena such as *commotio cordis*, the inhibition of SACs with pharmacological agents would appear a futile method for avoiding pathological consequences of severe stretch. By contrast, inhibition of SACs may have an important place in slower, more progressive cell signalling such as those involved in hypertrophy and other chronic stretch conditions associated with muscle dysfunction (Nicolosi *et al.*, 2004).

SACs and Ischaemia

The notion that SACs are important in ischaemic conditions has also been postulated, shown and disproved (Link *et al.*, 1999; Kamkin *et al.*, 2000b; Garan *et al.*, 2005; Barrabes *et al.*, 2006). It is suggested that border zones between normal, functional cardiac tissue and ischaemic zones is likely under considerable stress during contraction due to refuced contractility of ischaemic regions (Lab, 1996; Lab *et al.*, 1996; Lab, 1999). Here, NSACs may play an important role in cellular depolarisation or propagation of intracellular messages. However, it is unlikely under these conditions that TREK-1 channels are likely to contribute to MEF or electrophysiological changes since their ability to be activated by stretch is strongly inhibited under hypoxic conditions (Miller *et al.*, 2003). Although this has been specifically demonstrated using stretch, others have shown that hypoxia does not diminish TREK-1 activation by arachidonic acid (Buckler & Honore, 2005; Caley *et al.*, 2005). More recently it was demonstrated that Gd^{3+} (the NSAC blocker) infused into the ischaemic region of swine hearts was unable to suppress ventricular arrhythmias after coronary occlusion (Barrabes *et al.*, 2006) further abating the major role of SACs and theory of stretch-induced arrhythmias. This is despite the fact that Gd^{3+} has been demonstrated to inhibit stretch responses in vivo previously (Ovize *et al.*, 1994; Takano & Glantz, 1995).

Methods of Demonstrating SAC Responses

Also of interest to the patching of stretch-activated currents at both the whole cell and single channel level is the possibility that small leak currents or noise introduced by the techniques may in fact facilitate the very observation of these mechano-sensitive events via stochastic resonance (Douglass *et al.*, 1993; Levin & Miller, 1996). These observations, whilst having a possible physiological importance in neurones would only act to complicate the presence of SACs in isolated cardiomyocytes and their application to MEF at the level of the whole heart. More importantly, currently no experiments have investigated the correlation between activation of single mechanosensitive currents and changes in relative whole cell currents. This is an area that needs to be addressed since others have found that the two are not correlated and that the process of patch-clamping cells artificially sensitises channels to mechanical stimuli (Morris & Horn, 1991). This may help explain why differences have been observed in stretch-activated currents between researchers as well as the presence and absence of MEF which may be facilitated under specific experimental conditions. Thus, it remains unclear whether the detailed properties of SACs in patches or whole cells accurately reflect their properties in tissues or the whole heart. Patch clamping by virtue disrupts the cortical cytoskeleton and cellular membrane which will likely modify the forces sensed by the channels and membrane disruption itself is likely to introduce some level of stochastic resonance, especially during membrane or patch stretches. This process of sensitisation may explain the vast differences in the amount of suction reported to fully activate NSACs (4 mmHg vs 20-30 mmHg) (Kim, 1993; Ruknudin *et al.*, 1993). Likewise, it may be reasonable to suggest that if SACs found in cardiomyocytes do not display the stochastic resonant nature of other stretch-sensitive channels such as those found in the neural/auditory systems, then this may suggest that mechanosensitivity is not the primary function but a property inherited from another function and that these channels are in fact more likely to be background channels involved in membrane stabilisation.

Modulation of SAC-mediated MEF Responses in the Heart

The field of MEF consists of many incomplete studies. For example, rapid stretch-induced ectopic beats attributed to NSACs over 10 yrs ago (Zabel *et al.*, 1996; Bode *et al.*, 2006) are yet to be modulated with known NSAC blockers. It remains unclear if this suggests that previous attempts to show ectopic modulation with SAC blockers in

the whole heart has failed and remained unreported except in a few cases previously mentioned (Garan *et al.*, 2005). On the other hand, MEF research has covered other avenues including the disruption of the cytoskeletal matrix (Sadoshima *et al.*, 1992; Parker *et al.*, 2001; Isenberg *et al.*, 2003; Calaghan *et al.*, 2004). As such, with the current lack of congruency amongst studies, one must wonder if the complete story of MEF has indeed been told.

A number of studies have shown "diastolic-type" depolarisations in both Langendorff hearts and isolated cells (Stacy *et al.*, 1992; Hongo *et al.*, 1996; Hu & Sachs, 1996; Tavi *et al.*, 1996; Zabel *et al.*, 1996; Zeng *et al.*, 2000; Kamkin *et al.*, 2003c), whilst others have reported hyperpolarisations (Nakagawa *et al.*, 1988), little replication or follow-up has been applied to the modulation of these effects with the various known SAC modulators. Examples would include streptomycin or GsmTX4 modulation of the responses to stretch observed in the isolated beating heart (Stacy *et al.*, 1992; Zabel *et al.*, 1996; Dick & Lab, 1998). Likewise at the cellular level, novel experimental methods have been utilised where follow-up experiments would be a helpful addition to MEF theory (Riemer & Tung, 2000; Iravanian *et al.*, 2003; Riemer & Tung, 2003; Kong *et al.*, 2005) , subsequent data remain to be published, possibly due to variable results as reported by others (Nakagawa *et al.*, 1988; Babuty & Lab, 2001; Riemer & Tung, 2003). At the cellular level, reports of initial rapid inward currents may suggests that some structure within the cell either relaxes or is broken, exposing the mechanosensitive element(s) to stress (Bett & Sachs, 2000b), which may reflect damage to the cellular seal and provide "SAC-like" ion currents which are really leak currents associated with failing impalements (Tavi *et al.*, 1998).

This possibility could explain inconsistencies between authors and individual responses which have been reported as markedly variable. Sustained stretch in the atria has been reported to decrease in MAP APD_{50} and $MAPD_{90}$ in 50 % of cases, induce no change or an increase in duration in 25 % of cases (Babuty & Lab, 2001). Streptomycin where used as a SAC blocker has also reportedly had insignificant effects (Babuty & Lab, 2001; Garan *et al.*, 2005). These results suggest the participation of ionic channels other than specific stretch-activated channels in the response to stretch. This could possibly include Ca^{2+} release from the contractile apparatus as mentioned previously and readily demonstrated in isolated cardiomyocytes (Miura *et al.*, 1993; Han *et al.*, 1999; Wakayama *et al.*, 2001; Spencer & Sham, 2003). It doesn't seem to matter the method of application, stretch-related responses at the cellular or tissue level are

variable at best. In addition to these responses with acute stretch in the atrium, others have found variable MEF responses in response to fluid jet stimulation in monolayers of cultured cardiac myocytes. Here (Kong *et al.*, 2005) demonstrate out of 62 sites in 25 monolayers that only 16 % could not be mechanically excited and 84 % only once. That is, less than 21 % of the sample population could be reproducibly excited and displayed true mechanosensitive properties and these effects were inhibited by 50 μM streptomycin but only partly abolished by 10 μM Gd^{3+}. Likewise, fewer stretch-related changes have been observed in isolated cells by others (Riemer & Tung, 2003).

CONCLUDING REMARKS

Throughout this thesis, a series of detailed experiments has provided information on various aspects of MEF in the rat and guinea pig heart. Of the major observations are the following:

- Stretch-induced changes in electrophysiology are not readily observable in rat epicardial recordings from isolated, Langendorff perfused hearts at EDLVPs associated with increasing contractility by the Frank-Starling mechanism.

- Extreme or elevated stretch (EDLVP of 50-55 mmHg) induces an observable and consistent reduction in action potential duration as recorded by MAPs in the isolated Langendorff perfused heart

- Pharmacological agents that manipulate SACs have little or no effect on stretch-induced action potential shortening associated with extreme stretch.

- Extreme-stretch induced action potential shortening is likely to involve a mechanism other than SACs and may result from the crushing effect that left ventricular pressure is likely to have on coronary pressure and coronary flow.

- Isolated preparations that rely on passive oxygen diffusion rather than perfusion for cellular metabolic activity show no reduction in action potential duration with extreme stretch, nor do they readily demonstrate diastolic depolarisations

- Rapid inflations of the heart show great promise in explaining the ability of blunt impacts to the chest wall in developing life threatening cardiac

arrhythmia. However, the present study showed that these effects were unable to be modulated through the expected mechanisms.

As it can be seen, mechanosensitive ion channels in the heart, although can be studied in isolation, fail to accurately define and explain MEF in the whole heart. Mechano-sensitivity depends on a complex arrangement of cellular ion channels and tissue architecture that cannot be overlooked when extrapolating from the isolated cell.

FUTURE DIRECTIONS:

Involvement of SACs in MAP Generation

The activation of NSACs by MAP electrodes has been postulated (Knollmann *et al.*, 2002; Tranquillo *et al.*, 2004) which would ultimately bring into question the suitability of MAP electrodes for observing MEF in the heart. This is especially true given the amount of force required to elicit an observable MEF result in the current experiments and the effect this would have on normal cardiac parameters including the electrophysiology. Despite the suggested involvement of NSACs in the generation of the MAP, local tissue damage may still be a prerequisite for obtaining a MAP signal (Kondo *et al.*, 2004; Okamoto *et al.*, 2006). It is possible that the force required against the left ventricular free wall when using contact MAP electrodes is adequate to induce localised ischaemia or even damage and hence support the theory that all MAP recordings are in fact "injury potentials" (Okamoto *et al.*, 2006). Whilst it is possible that localised ischaemia may induce the cellular depolarisation required to obtain a MAP recording without permanent physical damage (Franz, 1999, 2005), it is also possible that any damage induced is too small to be readily seen without the aid of histology. Ethics applications for the present thesis included a series of studies on "generation of the monophasic action potential" in which it was proposed that all three electrode types used in the present study were to be used on the same heart followed by perfusion with various histological markers of cellular apoptosis. The heart could then be fixed and the areas of interest marked for slicing and later analysis of slides for cell damage. In a second series of experiments it was suggested that a larger concentration of streptomycin or glibenclamide could be perfused between MAP recordings in an attempt to abolish the MAP signal through complete inactivation of NSACs or ischemic induced K_{ATP} currents. Thus, the experiments would indicate following histological and pharmacological analysis if (a) cellular damage is a

prerequisite, (b) if NSACs are involved, or (c) of cellular inactivation is required in generating MAP signals.

Involvement of TREK-1 in MEF

The open probability of TREK-1 channels is considerably high at physiological temperatures (Maingret *et al.*, 2000a; Kang *et al.*, 2005) and increases with membrane stretch at room temperature (Maingret *et al.*, 2000b; Patel *et al.*, 2001; Tan *et al.*, 2004; Lauritzen *et al.*, 2005). However, currently it is not known how Po of TREK-1 changes in response to membrane stretch at near physiological temperatures given its already highly active status. A series of experiments were proposed whereby the stretch sensitivity of TREK-1 was to be compared at different ambient temperatures to further validate Langendorff experiments where temperature was decreased in order to enhance the stretch-response of TREK-1 channels. These experiments would also provide direct evidence for the ability of TREK-1 channels to contribute to electrophysiological changes associated with MEF at physiological temperatures.

Involvement of SACs in Pathological Conditions

Recent research suggests that whilst SACs may not participate in normal physiological conditions, they may serve a pathological function in muscular dystrophy and dilated cardiomyopathy (Yeung et al., 2003; Nicolosi et al., 2004; Yeung et al., 2005; Whitehead et al., 2006). Likewise, high concentrations of streptomycin have been demonstrated to inhibit ectopic activity associated with myocardial infarction (Lu et al., 2007). Passive constraint of atrial dilation has also been demonstrated to inhibit mechano-electrical responses in the Langendorff perfused rabbit heart (Ninio & Saint, 2006). Likewise, it could be that SACs only partly contribute to the overall stretch phenomenon and other mechanosensitive mechanisms may play an important role (Linke, 2007). It may be that SACs are up-regulated during specific pathological conditions or sensitised by some means facilitating their contribution to electrophysiological changes during pathological myocardial changes.

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AEROPLANE Unbreakable Records of Melbourne, Australia
Starting production in May 1928; the company collapsed two months later due to poor sales. There were only ever 18 songs issued on these flexible records