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Effects of Gender and Sex Hormone Status on Intracellular Calcium and Contractility in the Rat Heart

Written by

Claire Louise Curl, B.Sc. (Hons.)

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Addendum

p2 line 23: Delete "(approximately 5-15 μm diameter and 50-100 μm long)" and read "(measurements of isolated rat cardiac myocytes indicate a length range of 75 – 175 μm and diameter of 10 –30 μm)"

p16 line 21: Delete "The energy for net Ca^{2+} transport across the sarcolemma by the Na^+/Ca^{2+} exchanger depends on the Na^+, Ca^{2+} and K^+ gradients along with the membrane potential" and read "The Na^+/Ca^{2+} exchanger does not require energy, and it's activity is dependent on the Na^+ and Ca^{2+} concentration gradients and the difference between the Na^+/Ca^{2+} exchanger reversal potential and the membrane potential."

p17 line 14: Delete "...which has a $2Na^+$:$1Ca^{2+}$ stoichiometry" and read "...and extrude this Ca^{2+} via a Na^+/Ca^{2+} exchanger with a $2Na^+$:$1Ca^{2+}$ stoichiometry (Langer, 1997)."

p54: Add at the end of para 2: "The temperature of solutions used for Langendorff perfusion was 37°C. All recordings from isolated myocytes were made at a solution temperature of 25°C."

p67 line 15: Add at the end of the sentence: "...with a temporal resolution of 16.7 msec."

p67 line 19: Replace "(Delbridge and Roos, 1997)" with "(Steadman *et al.*, 1998)."

p103 line 8: Add "It is important to realise that not significantly different does not mean that two values are the same. It may be that small differences are present but these differences are unable to be detected by statistical tests. Because excitation-contraction coupling depends very steeply on SR Ca^{2+} content, small changes can have a large impact on fractional SR Ca^{2+} release."

p105 end para 1: Add "It is, however, important to consider that the reduced Ca^{2+} transient amplitude in the female is essentially negatively inotropic."

p125 line 8: Replace "predicate" with "mitigate"

p128 line 26: Add "It has previously been demonstrated that these hormone replacement regimens normalise sex steroid hormone concentrations and actions in the rat (Coghill *et al.*, 1998)."

p147 line 2: Add "Trends in the time course of decay of the Ca^{2+} transient were similar to those reported in the amplitude of the Ca^{2+} transient. OVX female cardiac myocytes had similar time course of decay when compared with male cardiac myocytes."

p149 line 14: Replace "...higher intracellular Ca^{2+} levels..." with "...higher peak $[Ca^{2+}]_i$ and amplitude of the Ca^{2+} transient..."

p155 line 25: Add "This testosterone replacement regimen has been demonstrated previously to normalise sex steroid hormone concentrations and actions in the rat (O'Donnell *et al.*, 1994)."

p165: Comment: For Figures 6.9 to 6.14, animals in sham male and GDX male groups were implanted with a blank pellet of the same dimensions as the testosterone filled pellet.

p178 end para 1: A comparison of results in this manner raises an interesting dichotomy. Estrogen depresses Ca^{2+} transients whilst testosterone raises them. One might expect that in the absence of sex hormones that they should be the same. However, that is not the case, with OVX females displaying higher Ca^{2+} transients than GDX males. This suggests that there is something else that is different between the genders that is steroid hormone independent. However, the experiments reported in this thesis give no indication as what this might be.

Additional References:

Coghill E.K. et al. (1998). The effects of estradiol and progesterone on GnRH-induced calcium signals in gonadotrophs are unrelated to their feedback actions on LH concentrations. *Proc Endocrine Soc Aust*, 41:91.

O'Donnell L., McLachlan R.I., Wreford N.G. and Robertson D.M. (1994). Testosterone promotes the conversion of round spermatids between stages VII and VIII of the rat spermatogenic cycle. *Endocrinology,* 135:2608-2614.

Steadman B.W., Moore K.B., Spitzer K.W. and Bridge J.H. (1988). A video system for measuring motion in contracting heart cells. *IEEE Trans Biomed Eng,* 35:264-272.

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Declaration

This thesis does not include material which has been accepted for the award of any other degree or diploma in any university or institution. It contains no material previously published or written by another person except where due reference is made in the text. All experimental results presented in this thesis are the sole work of the author and have complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were awarded approval from the Monash University Standing Committee on Ethics in Animal Experimentation. I also declare this thesis is less than 100,000 words in length exclusive of tables, bibliographies, appendices and footnotes.

Parts of the work completed during my Ph.D. candidature have been published for the scientific community and are listed subsequently.

Full Papers

Curl C.L., Kotsanas G. and Wendt I.R. (2001). Effects of gender on intracellular [Ca^{2+}] in rat cardiac myocytes. *Pflügers Archiv – European Journal of Physiology.* 441:709-716.

Curl C.L., Wendt I.R., Canny B.J. and Kotsanas G. (2001). Effects of ovariectomy and 17β-estradiol replacement on $[Ca^{2+}]_i$ in female rat cardiac myocytes. *Journal of Molecular and Cellular Cardiology*. **Submitted**.

Abstracts

Holloway C.L., Kotsanas G. and Wendt I.R. (1999). Effects of gender on intracellular $[Ca^{2+}]$ in rat cardiac myocytes. *Proceedings of the Australian Physiological and Pharmacological Society.* 30(2):15P.

Curl C.L., Wendt I.R., Kotsanas G. and Canny B.J. (2000). Intracellular $[Ca^{2+}]$ in cardiac myocytes from male, female and ovariectomised female rats. *Journal of Muscle Research and Cell Motility*. 21(8): 804.

Curl C.L., Canny B.J., Wendt I.R. and Kotsanas G. (2001). Calcium homeostasis and the contractile properties of rat cardiac myocytes are differentially regulated by oestrogen and testosterone. *Proceedings of the Endocrine Society of Australia.* 44: 297.

Claire L. Curl (nee Holloway)

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Abstract

Gender based differences in the incidence of cardiovascular disease are now well recognised with pre-menopausal women displaying a reduced risk when compared with age-matched men. After menopause the risk of developing cardiovascular disease in women increases to similar levels to those seen in men unless estrogen replacement therapy is given. Epidemiological evidence such as this suggests that estrogen is a key factor in the reduced risk of cardiovascular disease which is present in pre-menopausal women. Originally it was thought that the beneficial actions of estrogen were solely due to its positive effects on the lipid profile and vasculature, however, more recent studies have suggested a Ca^{2+} inhibitory role for estrogen in the heart. The purpose of this thesis was to attempt to elucidate if gender differences were present in Ca^{2+} handling by the heart, and if differences were present, to determine if they were due to the sex steroid hormones estrogen and testosterone.

Cardiac myocytes were isolated from both male and female rats, with intracellular $[Ca^{2+}]$ measured with the Ca^{2+} fluorescent dye fura-2. Increasing extracellular $[Ca^{2+}]$ and stimulus frequency resulted in significant increases in peak $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} transient in male and female cardiac myocytes. However, as

extracellular [Ca^{2+}] was raised, peak [Ca^{2+}]$_i$ and the amplitude of the Ca^{2+} transient increased significantly more in male than in female cardiac myocytes. These results confirmed the existence of gender based differences in Ca^{2+} handling by cardiac myocytes. In addition the time course of decay of the Ca^{2+} transient was found to be significantly slower in female cardiac myocytes. Female cardiac myocytes also displayed a lesser extent of cell shortening and slower relaxation time than did male cardiac myocytes.

The gender-related differences observed in intracellular Ca^{2+} handling of single cardiac myocytes was reflected in differences in contractility of intact multicellular cardiac muscle preparations. Force recordings were taken from both male and female papillary muscle strips in response to varied extracellular [Ca^{2+}], the Ca^{2+} channel antagonist nifedipine and the Ca^{2+} channel agonist Bay K8644. Male papillary muscle strips consistently demonstrated significantly greater force in response to varied extracellular [Ca^{2+}], and in the presence of nifedipine and Bay K8644 when compared with female papillary muscle strips.

The effects of estrogen removal and subsequent replacement in female rats was then studied in an attempt to elucidate if estrogen was the key factor in these gender based differences. Ovariectomised (OVX) female cardiac myocytes consistently demonstrated significantly greater peak [Ca^{2+}]$_i$ and amplitude of the Ca^{2+} transient when compared with sham operated and 17β-estradiol replaced OVX female cardiac myocytes. OVX cardiac myocytes also displayed faster rates of decay of the Ca^{2+} transient along with a faster time to 50% relaxation, and greater cardiac myocyte shortening.

To determine if testosterone was also playing a role in the gender based differences in Ca^{2+} handling, the effects of its removal and exogenous replacement on male cardiac myocytes was also studied. Gonadectomised (GDX) male cardiac myocytes consistently demonstrated significantly smaller peak [Ca^{2+}]$_i$ and the amplitude of the Ca^{2+} transient when compared with both sham operated and testosterone replaced GDX male cardiac myocytes. In addition GDX males also displayed slower rates of decay of the Ca^{2+} transient along with slower time to 50% relaxation and reduced cardiac myocyte shortening.

In conclusion, these studies have demonstrated gender specific differences in both the Ca^{2+} handling properties of cardiac myocytes and indices of contractility in myocytes and papillary muscles. In addition, significant effects of the animal's sex hormone

status on intracellular Ca^{2+} movements were demonstrated, with estrogen appearing to favour reduced $[Ca^{2+}]_i$ while testosterone was associated with increased $[Ca^{2+}]_i$. Although the exact mechanisms by which the sex hormones may be influencing intracellular Ca^{2+} handling by the heart are yet to be elucidated, it is speculated that one pathway may involve regulation of L-type Ca^{2+} channel expression.

Abbreviations

AC	Adenylate cyclase
AM	Acetoxymethyl ester
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AV	Atrioventricular
B	The ratio of the fura-2 fluorescence emission intensity at 380nm excitation in the absence of Ca^{2+} to that in the presence of saturating Ca^{2+}
BDM	2,3- butanedione monoxime
BSA	Bovine serum albumin
$[Ca^{2+}]_i$	Intracellular calcium ion concentration
$[Ca^{2+}]_o$	Extracellular calcium ion concentration
cAMP	Cyclic 3',5'-adenosine monophosphate
cGMP	Cyclic 3',5' quanosine monophosphate
DHP	Dihydropyridine
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
EGTA	Ethyleneglycol-bis-(β-amino-ethyl ether) N,N,N',N'-tetra acetic acid
ER α	Estrogen receptor alpha
ER β	Estrogen receptor beta
ERA	Estrogen Replacement and Atherosclerosis
ERE	Estrogen Response Element
ERKO	Estrogen Receptor Knockout
F_{340}	Fluorescence at 340nm
F_{380}	Fluorescence at 380nm
Fura-2/AM	Fura-2 acetoxymethyl ester
GDX	Gonadectomised
GnRH	Gonadotropin-releasing hormone
HDL cholesterol	High density lipoprotein cholesterol
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]
HERS	Heart and Estrogen-progestin Replacement Study
i.d.	Inner diameter
i.m.	Intra muscular
IP_3	Inositol trisphosphate
IU	International Units
KD	Kilodalton
K_d	Dissociation constant
K-H	Krebs-Henseleit
LDL cholesterol	Low density lipoprotein cholesterol
mRNA	Messenger ribonucleic acid
Na^+/Ca^{2+} exchanger	Sodium/calcium ion exchanger
NCX	Sodium/calcium ion exchanger
Na^+/H^+ antiporter	Sodium/hydrogen antiporter
Na^+/K^+ ATPase	Sodium/potassium pump
o.d.	Outer diameter
OVX	Ovariectomised
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A (cyclic AMP dependent protein kinase)
PKC	Protein kinase C

PMCA	Plasma membrane Ca^{2+}-ATPase
R	$R_{340/380}$
$R_{340/380}$	Ratio of fluorescence at 340nm and 380nm excitation
R_{max}	Maximum fluorescence ratio
R_{min}	Minimum fluorescence ratio
RyR	Ryanodine receptor
SEM	Standard error of the mean
SERCA	SR Ca^{2+}-ATPase
SHBG	Sex Hormone Binding Globulin
SL	Sarcolemma
SR	Sarcoplasmic Reticulum
T	Testosterone
T_3	Triiodothyronine
TnC	Troponin C

Chapter 1

Chapter 1

Literature Review

The heart is an amazing and vital organ within the body, whose rhythmic contractile activity from before birth until death provides the means by which the blood is circulated through the body. It is composed predominantly of specialised muscle cells, known as cardiac muscle cells. It is the contraction of these cardiac muscle cells that generates the pressure within the chambers of the heart that ultimately provides the driving force for the circulation of blood through the body's vasculature. As such, the heart's function is essentially that of a pump. The amount of blood pumped from the heart during each contraction cycle is dependent upon the strength of the heart's contraction, which in turn is dependent on the end-diastolic volume and also on the contractility of the cardiac muscle cells. Changes in end-diastolic volume will cause changes in stroke volume (i.e. the volume of blood pumped by each ventricle during one heartbeat) as per the Frank Starling Law of the heart. The underlying mechanism relates to the fact that changes in end-diastolic volumes lead to changes in the length of the cardiac muscle cells prior to their subsequent contraction. Any changes in contraction strength that occur independently of changes in end-diastolic volume are referred to as contractility changes. A number of different agents can alter the contractility and hence the inotropic state of the heart. Generally these act by

influencing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) or the ability of the contractile proteins to respond to changes in $[Ca^{2+}]_i$. In most circumstances changes in Ca^{2+} movements into and out from the cardiac muscle cell cytoplasm are responsible for contractility changes and, consequently, cellular Ca^{2+} handling is of crucial importance to the heart's function. $[Ca^{2+}]_i$ is the key factor in the excitation-contraction coupling process which is essential for cardiac muscle contraction and, therefore, overall heart function. Changes in Ca^{2+} movements within the cardiac muscle cells can have profound effects on the functioning of the heart and its ability to adequately pump blood around the body. Whilst many factors within the heart co-ordinate to result in its normal functioning, it can be said that the Ca^{2+} ion concentration is one of the most important.

1.1 Cardiac Muscle: General Characteristics

The mammalian heart is functionally divided into a right and a left side. Each side has two chambers, an atrium and a ventricle. It is the contraction of the ventricles that is responsible for the ejection of blood from the heart. The walls of the heart are composed mostly of the myocardium which comprises predominantly cardiac muscle cells. The inner surface of the myocardium is lined by the epicardium, whilst the outer surface is lined by the endocardium. The myocardium of the left ventricle which ejects blood into the systemic circulation, is considerably thicker than that of the right ventricle which is responsible for the pumping of blood through the pulmonary circulation. The average pressure generated by the left ventricle is approximately five fold higher than that generated by the right ventricle.

1.1.1 Structure

Cardiac muscle cells are generally small in size (approximately 5-15 μm diameter and 50-100 μm long), and are attached to each other, generally end-to-end, although they can also be attached side-by-side. Some cells also branch such that the end of one cell connects to two other cells. The connection between the cells occurs via specialised structures called intercalated discs, which are very important in the function of the heart as a whole. In this region there is a strong mechanical connection as well as low resistance electrical coupling. The mechanical connection is present in the form of a cellular structure called a desmosome, or tight junction, whilst the electrical connection is made through a gap junction. The intercalated disc has been described as freely permeable to small charged particles and, therefore, represents a low-resistance pathway

between adjacent cells (Katz, 1977). The presence, and features, of the intercalated discs that link all of the cardiac muscles in the ventricles ensures that the cardiac muscle cells are functionally coupled. This allows the muscle cells in the heart to beat in synchrony which in turn allows the heart to function as one large entity (Bers, 1991). An important consequence of the electrical coupling between cardiac muscle cells is that unlike skeletal muscle, the heart is unable to use a recruitment-like mechanism to alter its contractile strength. If changes in the heart's contractile strength are to occur, they must be achieved at the level of each single cell.

1.1.1.1 Cellular Structure

An outer lipid bilayer membrane called the sarcolemma (SL) covers each cell. The sarcolemma extends inwards into the cell forming a region called the transverse tubule (t-tubule) which is an important structural component involved in the excitation-contraction coupling process (Sommer, 1982). The sarcolemma and t-tubule form the permeability barrier between the intracellular and extracellular space (Katz, 1977; Bers, 1991). These membranes contain ion pumps, exchangers and channels, which are responsible for the transfer of substances between the intra- and extracellular spaces (Katz, 1977). An important feature of the sarcolemma is its close coupling with the sarcoplasmic reticulum (SR) (Gathercole *et al.*, 2000). It appears that parts of the t-tubular membrane are involved in junctional complexes with the SR. This has been suggested to aid the process of excitation-contraction coupling.

Within each cell is a single nucleus, usually centrally located, along with other cellular components including golgi apparatus, lysosomes and mitochondria (Sommer, 1982). The mitochondria, which occupy approximately 35% of the volume of mammalian ventricular muscle cells, are usually cylindrical in shape (Bers, 1991) with the surface area of the inner membrane increased by a series of infoldings called cristae (Katz, 1977; Sommer, 1982). The mitochondria are the site of oxidative phosphorylation and are, therefore, largely responsible for the high level of aerobic metabolism needed for the continuous function of the heart.

The sarcoplasmic reticulum (SR) is an intracellular membrane bounded compartment, which is separate from the sarcolemma. Structurally it can be divided into two compartments, the longitudinal SR and the terminal cisternae. The longitudinal SR spans between adjacent t-tubules with the terminal cisternae coming into close

apposition with the t-tubules at either end. The membranes of the SR and the t-tubules never come into contact with each other, however, they do come into close proximity with only a narrow space remaining between the two. The function of the SR within the cell is to store and release Ca^{2+} during the excitation-contraction coupling process. As briefly mentioned previously, the SR forms specialised junctions with the sarcolemma (Sommer, 1982). Although the membranes of the SR and sarcolemma do not make direct contact with each other, the junction between the two is spanned by bridging structures called "feet" (Franzini-Armstrong, 1970; Katz, 1977; Langer, 1997; Bers and Perez-Reyes, 1999). These feet have been identified as the ryanodine receptor (because of their high affinity for the plant alkaloid ryanodine). Three genetically distinct isoforms of the ryanodine receptor (RyR) are known in mammals. RyR1 is the primary isoform in skeletal muscles and purkinje cells of the cerebellum. RyR2 is primarily found in cardiac muscle and in the brain and RyR3 has been detected in the epithelial cells of the lung, specific regions of the brain and in smooth muscle (Ogawa *et al.*, 1999). The ryanodine receptors are also known to be the Ca^{2+} release channels of the SR and are located on the terminal cisternae of the SR (Ogawa *et al.*, 1999). Block *et al.* (1988) have shown that the feet on the SR are arranged in a distinct pattern which corresponds to a similar array of particles on the t-tubular membrane. It has been suggested that this array of particles are actually L-type Ca^{2+} channels, which are aligned to the SR and which play a pivotal role in excitation-contraction coupling. In cardiac muscle there are many more ryanodine receptors on the terminal cisternae of the SR than there are L-type Ca^{2+} channels in the adjacent t-tubular membrane, suggesting that a single L-type Ca^{2+} channel may be associated with between four to ten ryanodine receptors (Bers and Perez-Reyes, 1999). Except for these feet on the terminal cisternae of the SR, the remainder of the SR membrane contains predominantly SR Ca^{2+}-ATPase pump proteins suggesting that the majority of the SR membrane is concerned with Ca^{2+} removal from the cytoplasm.

Within the junctional SR lumen is the low affinity but high capacity Ca^{2+} binding protein calsequestrin, whose purpose is to buffer the high levels of Ca^{2+} present within the SR (Bers and Perez-Reyes, 1999; MacLennan, 2000). Structurally it contains 109 acidic amino acids with an excess of 69 negatively charged residues, which contribute to calsequestrin's ability to bind a large amount of Ca^{2+} (Scott *et al.*, 1988). In a study using transgenic mice exhibiting 10-fold over expression of cardiac calsequestrin, Jones

et al. (1998) demonstrated Ca^{2+} release through the ryanodine receptor to be suppressed and caffeine-induced Ca^{2+} transients and subsequent Na^{+}/Ca^{2+} exchanger currents to increase proportionally to the degree of overexpression of calsequestrin, supporting the notion that calsequestrin is involved in the store of releasable Ca^{2+} within the SR. Other functions for calsequestrin have been suggested, including a possible involvement in ryanodine receptor gating (Bers and Perez-Reyes, 1999), with calsequestrin being shown to be anchored to the ryanodine receptor via junctional face proteins (Jones *et al.*, 1998). A complete understanding of its structure and function is, however, still lacking.

1.1.1.2 Myofilaments

The myofilaments represent the contractile machinery of the cell and are responsible for force generation and shortening of the myocyte. The myofilaments make up approximately half of the cell volume in mammalian ventricular myocardium (Bers, 1991) and are arranged in an overlapping array into structures called sarcomeres (Phillips and Petrofsky, 1983). The myofilaments themselves are of two types, a myosin containing thick filament, and an actin containing thin filament (Tonomura, 1973). Cardiac muscle myosin is hexameric containing 2 heavy chains (~ 200kD each) and 4 light chains (~ 20kD each) (Gordon *et al.*, 2000). Each myosin heavy chain has a long helical alpha tail and a globular head which forms the cross-bridge that interacts with the thin filament during shortening. The tail regions make up the backbone structure of the filament. The thin filament is composed predominantly of actin arranged in a two-stranded helix, however it also has the proteins tropomyosin and troponin situated on it (Gordon *et al.*, 2000). These two proteins together play a critical role in the initiation of the cross-bridge cycle and represent the main site of Ca^{2+} regulation of contraction. It has also been shown that the position of the tropomyosin on the thin filament determines the interaction of myosin with actin (Gordon *et al.*, 2000). This interaction between the thick and thin filaments must take place in order for contraction and muscle shortening to occur.

1.1.2 Contraction of Cardiac Muscle

Contraction of cardiac muscle occurs through the sliding filament mechanism (Huxley, 1954a; Huxley, 1954b). This involves attachment of the myosin head, or cross-bridge of the thick filament, to the thin filament. Rotation of the attached myosin head in a

rowing like motion produces relative sliding of the thin filament over the thick filament. This relative sliding of the thick and thin filaments over one another leads to shortening of the sarcomeres. The degree of overlap of the thick and thin filaments determines the number of cross-bridges that can potentially attach, and consequently this is an important determinant of the force of contraction (Calaghan and White, 1999). It underlies the alteration of the heart's contractile strength through changes in end-diastolic volume.

For contraction of cardiac muscle to occur, a series of co-ordinated ion movements and protein interactions must transpire. Crucial to the process is an increase in the intracellular Ca^{2+} concentration. During the relaxed state, when internal Ca^{2+} is low, interaction between the thick and thin filaments is blocked by the troponin-tropomyosin complex. It is only the increase of the intracellular Ca^{2+} concentration that can remove this block and cause cardiac muscle to contract. The tightly orchestrated sequence of events that leads from excitation of the cell to contraction is termed excitation-contraction coupling.

1.1.2.1 Excitation-Contraction Coupling

The process of excitation-contraction coupling transfers the resting relaxed myocyte, via membrane electric events, ion fluxes, and myofilament interaction to the actively contracting state and then returns the myocyte to its relaxed state (Barry and Bridge, 1993). Ringer (1883) recognised over 100 years ago that cardiac muscle requires Ca^{2+} in the extracellular space for contraction to occur. In the process of cardiac excitation-contraction coupling, Ca^{2+} in the extracellular space enters the cell and triggers a sequence of events that lead to activation of the myofilaments and contraction. Excitation-contraction coupling begins when the sarcolemma is depolarised by an action potential. This causes an influx of Ca^{2+} into the myocyte, and results in Ca^{2+} release from the SR and ultimately cardiac contraction. The removal of the Ca^{2+} from the cytosol by uptake back into the SR and by extrusion out of the cell results in relaxation of the cardiac myocyte and the end of the excitation-contraction coupling cycle (Calaghan and White, 1999).

1.1.2.1.1 The Cardiac Action Potential

The cardiac action potential is the initiating event in the excitation-contraction coupling process. The excitation of the heart normally originates in the primary pacemaker of the

heart, the sinoatrial node, where spontaneous action potentials are initiated that rapidly propagate throughout the entire heart via the specialised conducting system and the intercalated discs linking the cardiac muscle cells. In the ventricular cells the action potential is characterised by an initial fast upstroke (Phase 0), which is a result of a rapid influx of Na^+ through fast, voltage-gated Na^+ channels. This depolarises the cardiac sarcolemma from its resting voltage of –90 mV to around +20 mV. During this depolarisation, at approximately –45 to –20 mV, a second set of channels open to allow Ca^{2+} to enter the cardiac myocyte (Ikonomidis *et al.*, 1990). Phase 1 follows this initial rapid depolarisation and is characterised by a small amount of repolarisation as the high Na^+ permeability begins to decline. This is followed by a plateau phase (Phase 2) during which the membrane potential stays depolarised, due to the open Ca^{2+} channels that carry inward current and voltage gated K^+ channels that are carrying outward current. These two currents approximately balance, hence the plateau in the action potential. Repolarisation, or Phase 3, follows due primarily to inactivation of the Ca^{2+} channels and increasing K^+ permeability. The final phase (Phase 4) of the action potential then occurs, where the membrane potential falls to its original value and remains steady until the next beat. The level of $[Ca^{2+}]_i$ is increased greatly as a result of the action potential and the flux of Ca^{2+} into the cytoplasm of the cell, both from the extracellular space and from the SR itself. The Ca^{2+} which enters the cardiac myocyte from the extracellular space does so through voltage-dependent Ca^{2+} channels.

1.1.2.1.2 Voltage-Dependent Ca^{2+} Channels

Depolarisation of the plasma membrane, or sarcolemma, is associated with a change in fluxes of both Na^+ and Ca^{2+} into the cell. The Na^+ influx depolarises the cardiac sarcolemma and in conjunction with this voltage-dependent Ca^{2+} channels open and allow Ca^{2+} entry into the cell. Voltage-dependent Ca^{2+} channels are predominantly regulated by membrane potential but have also been shown to be influenced by hormones, protein kinases, neurotransmitters and a variety of drugs (Hofmann *et al.*, 1999; Kamp and Hell, 2000). These channels play an integral role in many excitable mammalian cells by regulating their intracellular Ca^{2+} concentration (Hofmann *et al.*, 1999).

Two different types of voltage-dependent Ca^{2+} channel have been recognised in the cardiac cell, namely the L-type Ca^{2+} channel and the T-type Ca^{2+} channel. Voltage-dependent Ca^{2+} channels consist of a pore-forming $\alpha 1$ subunit, which has four domains

each containing six putative transmembrane segments (Dolphin, 1998). The α1 subunit is the main functional component of the channel complex and contains the ion conducting pore, the selectivity filter of the pore and the voltage sensor, along with binding sites for Ca^{2+} channel blockers (Bers and Perez-Reyes, 1999; Kamp and Hell, 2000). Nine individual genes have been identified for the α1 subunit from which the class Cα1 gene has been found to be expressed in numerous parts of the body including smooth muscle, endocrine, neuronal and heart cells. In particular, the cardiac L-type Ca^{2+} channel is coded predominantly by the Cα1 gene (Hofmann *et al.*, 1999).

The L-type Ca^{2+} channel was so named because of its large conductance and long lasting openings (Hess *et al.*, 1984; Bean, 1985; Bers, 1991). It is also well known for its sensitivity to dihydropyridines (DHPs) such as nifedipine and Bay K8644 (Ikonomidis *et al.*, 1990; Bers and Perez-Reyes, 1999). It is thought to be the primary Ca^{2+} ion carrier into the cell during excitation-contraction coupling. It is now recognised that the L-type Ca^{2+} channels are located predominantly in the t-tubules adjacent to the SR, and occur in the peripheral sarcolemma only in discrete regions (Langer, 1997; Gathercole *et al.*, 2000). The abundance of L-type Ca^{2+} channels in the t-tubular region allows the influx of Ca^{2+} through these channels to have ready access to the ryanodine receptors on the SR and therefore enables Ca^{2+}-induced Ca^{2+} release to be instigated quickly (Katz, 1977; Ikonomidis *et al.*, 1990; Bers, 1991; Morad, 1995).

The L-type Ca^{2+} channel activates at membrane potentials from –40 to ~ +10 mV, and has been shown to inactivate in response to both changes in voltage and intracellular Ca^{2+} concentration (Bers and Perez-Reyes, 1999; Hobai and Levi, 1999; Anderson, 2001). Under normal physiological conditions, the initial component of inactivation is rapid and thought to be Ca^{2+} ion-dependent, whilst the second component is slower and voltage-dependent (Anderson, 2001). The Ca^{2+} that is released from the SR during the excitation-contraction coupling process contributes the majority of Ca^{2+} for Ca^{2+}-dependent inactivation of the L-type Ca^{2+} channel purely because of the close proximity of the ryanodine receptor on the SR to the L-type Ca^{2+} channel (Anderson, 2001).

Other factors are also able to modulate the activity of the L-type Ca^{2+} channel. β-adrenergic agonists such as isoprenaline increase $[Ca^{2+}]_i$ in a second messenger dependent fashion. β-adrenergic stimulation leads to G protein activation of adenylyl cyclase, increased production of cAMP and, therefore, stimulation of the cAMP-

dependent protein kinase A (PKA). PKA is known to be able to phosphorylate the L-type Ca^{2+} channel at multiple potential sites, including at the important Cα1 site, to influence changes in channel characteristics. PKA has been suggested to effect a shift in the voltage-dependence of activation and inactivation to more negative membrane potentials (Bers and Perez-Reyes, 1999; Kamp and Hell, 2000). It has also been suggested that PKA activation decreases the number of blank sweeps (or no openings) of channel gating and thereby increases the Ca^{2+} current into the cardiac cell (Kamp and Hell, 2000). In addition to the well known effects of PKA, protein kinase C (PKC) pathways have also been implicated in a role for influencing the L-type Ca^{2+} channel. Activation of PKC can both stimulate and inhibit L-type Ca^{2+} current depending on the cells studied and the conditions that experiments are undertaken (Cheng *et al.*, 1995; Thomas *et al.*, 1997; He *et al.*, 2000). It seems, however, that the effects of PKC activation are possibly dependent on the particular isoform of PKC that is activated by the signalling pathway. At present, the underlying molecular mechanism for PKC's regulation of the L-type Ca^{2+} channel remains largely unknown (Kamp and Hell, 2000).

In addition to the β-adrenergic agonists, the dihydropyridines, as mentioned briefly above are also able to modulate Ca^{2+} channel activity. These can act as both L-type Ca^{2+} channel agonists, such as Bay K8644, and antagonists, such as nifedipine. The dihydropyridine agonists act by increasing the open time of single Ca^{2+} channels, thereby greatly increasing the Ca^{2+} current into cardiac cells (Hess *et al.*, 1984). The majority of dihydropyridines, however, are antagonists which inhibit the Ca^{2+} current, thereby reducing the Ca^{2+} concentration inside cardiac cells (Bers and Perez-Reyes, 1999). The Ca^{2+} antagonist nifedipine inhibits the current entering through the L-type Ca^{2+} channel by favouring the mode of gating behaviour which is known as mode 0. This mode is characterised by reduced openings because of channel unavailability (Hess *et al.*, 1984).

The second Ca^{2+} channel in cardiac muscle cells is the T-type channel which demonstrates small conductance and brief, transient openings (Bers, 1991). It has been shown to have a low threshold of activation along with a slow activation and fast inactivation (Nargeot, 2000). Just as the Cα1 gene codes for the L-type Ca^{2+} channel, the T-type Ca^{2+} channel is also coded by α1 genes, but rather than the Cα1 subunit, it is coded by the Gα1 and the Hα1 subunits. Both of these genes are also expressed elsewhere in the body including the kidney and the brain (Hofmann *et al.*, 1999). The T-

type Ca^{2+} current has been speculated to play a role in pacemaker activity (Hagiwara *et al.*, 1988) but is thought not to contribute, or at least to contribute negligible amounts of Ca^{2+} to excitation-contraction coupling under normal circumstances in the ventricular myocyte. There is some evidence that the T-type current may play a more prominent role during development and hypertrophy (Bers and Perez-Reyes, 1999).

1.1.2.1.3 Ca^{2+}-Induced Ca^{2+} Release

Originally it was thought that cardiac muscle contraction occurred via direct activation of the myofilaments by the Ca^{2+} that entered through L-type Ca^{2+} channels (Langer and Frank, 1972; Langer, 1976). It was not until the classic experiments of Fabiato (1983) that it was recognised that the Ca^{2+} that enters the cell through the L-type Ca^{2+} channel after depolarisation can bind to the Ca^{2+} release channel, or ryanodine receptor, of the SR to cause release of Ca^{2+} from this internal store. The ryanodine receptor was so named due to its high affinity for the plant alkaloid ryanodine. Interestingly, however, ryanodine administered at low concentrations (<10 μmol/L) induces an open, low conductance configuration of the channel, whilst higher concentrations of ryanodine completely block Ca^{2+} release from the channel. Ryanodine has proven to be a most useful tool for studying the Ca^{2+} release mechanism from the SR during the Ca^{2+}-induced Ca^{2+} release process (Barry and Bridge, 1993). Previous experiments have shown that the probability of opening of the SR Ca^{2+} release channel is greatly increased when exposed to micromolar concentrations of Ca^{2+} (Rousseau *et al.*, 1987; Anderson *et al.*, 1989; Barry and Bridge, 1993) supporting the idea of the Ca^{2+}-induced Ca^{2+} release mechanism. In addition, the $[Ca^{2+}]$ that activates the ryanodine receptor is in the physiological range (1-100 μm) suggesting its relevance in excitation-contraction coupling (Bers and Perez-Reyes, 1999). It is interesting to note that when the SR Ca^{2+} content decreases to below approximately 50% of normal, SR Ca^{2+} release cannot be activated in a normal manner. This is thought to be a protective mechanism helping the SR to reload with Ca^{2+} and replenish to the point where enough Ca^{2+} is stored again for normal functioning to resume (Bers, 2000). In the intact heart, there would be an increased Ca^{2+} influx to support contraction, possibly due to removal of the inactivation of the L-type Ca^{2+} channels by Ca^{2+} released from the SR, and therefore contribute to a greater Ca^{2+} current and hence make available a greater amount of Ca^{2+} for sequestration by the SR.

Ca^{2+}-induced Ca^{2+} release results in increased cytoplasmic [Ca^{2+}] which ultimately activates the myofilaments and results in cardiac muscle contraction. The idea of Ca^{2+}-induced Ca^{2+} release first came from a study by Fabiato and Fabiato (1975) which demonstrated that external Ca^{2+} could trigger Ca^{2+} release from the SR in skinned ventricular myocytes. They subsequently showed that in mammalian cardiac muscle no influx of Ca^{2+} of any magnitude could activate the myofilaments directly without first triggering Ca^{2+} release from the SR (Fabiato and Fabiato, 1977; Fabiato and Fabiato, 1978; Fabiato, 1982). In addition a subsequent series of experiments demonstrated that the release of Ca^{2+} from the SR was graded by the cytoplasmic [Ca^{2+}] and that high concentrations of cytoplasmic Ca^{2+} led to inactivation or inhibition of the Ca^{2+}- induced Ca^{2+} release mechanism (Fabiato, 1985). Further studies supporting the idea of a Ca^{2+}-induced Ca^{2+} release mechanism followed, especially from the observation of Ca^{2+} current "tail transients". These occur when Ca^{2+} channels are open but no Ca^{2+} current is flowing, for example when a cell which is voltage clamped near the reversal potential for Ca^{2+} is then clamped back to a negative potential where Ca^{2+} channels deactivate. As the Ca^{2+} channels are closing, a large but brief Ca^{2+} current tail activates a Ca^{2+} transient and contraction (Cannell *et al.*, 1987; Beuckelmann and Wier, 1988). This supports the idea of Ca^{2+}-induced Ca^{2+} release (Bers and Perez-Reyes, 1999). It is interesting to note that the amount of Ca^{2+} that enters the cell determines the extent of Ca^{2+} release from the SR (Bassani *et al.*, 1995), along with the content of Ca^{2+} in the SR at that time. It can, therefore, be assumed that the extracellular Ca^{2+} concentration is a very important determinant in the control of myocardial contractility (Bers and Langer, 1979; Bers *et al.*, 1981; Ikonomidis *et al.*, 1990). In support of this is the evidence that not all Ca^{2+} within the SR is released with each beat (Moravec and Bond, 1991) but rather is controlled by the influx of Ca^{2+} into the cell in each beat (Barry and Bridge, 1993).

In addition to the L-type Ca^{2+} channel, the Na^{+}/Ca^{2+} exchanger has also been suggested to be involved in the elevation of intracellular Ca^{2+} to initiate contraction. It is thought that the Na^{+} influx at the beginning of depolarisation could be large enough to send the Na^{+}/Ca^{2+} exchanger into reverse mode, thus bringing Ca^{2+} into the cell to contribute to the trigger for Ca^{2+}-induced Ca^{2+} release and hence contraction (Leblanc and Hume, 1990; Sham *et al.*, 1992; Lopez-Lopez *et al.*, 1995; Blaustein and Lederer, 1999; Wier and Balke, 1999). It is thought, however, that the Na^{+}/Ca^{2+} exchanger is less efficient at

triggering Ca^{2+} from the SR than the L-type Ca^{2+} channel, due solely to the fact that the L-type Ca^{2+} channel is situated in such close proximity to the SR (Trafford and Eisner, 1998). Another way in which the Na^{+}/Ca^{2+} exchanger can be switched into reverse mode and could, therefore, contribute to the Ca^{2+}-induced Ca^{2+} release mechanism is by membrane potential activation. The rapid increase in membrane potential during the upstroke of the action potential is enough to send the Na^{+}/Ca^{2+} exchanger into Ca^{2+} 'in' mode. Once the L-type Ca^{2+} channels open, however, the large increase in $[Ca^{2+}]_i$ would cause the Na^{+}/Ca^{2+} exchanger to alter direction and work in forward mode to remove Ca^{2+} from the cytosol (Bers and Perez-Reyes, 1999). It has been suggested that Ca^{2+} entry through the Na^{+}/Ca^{2+} exchanger, whilst not being the predominant pathway of Ca^{2+} entry during the excitation-contraction coupling process, may provide a back-up system for activation of SR Ca^{2+} release (Bers and Perez-Reyes, 1999).

1.1.2.1.4 Myofilament Activation

The Ca^{2+} that is released from the SR, combined with the Ca^{2+} that enters from the extracellular space, initiates contraction by activating the myofilaments (Bers, 1991; Barry and Bridge, 1993). At rest the troponin-tropomyosin complex inhibits the interaction between the thin actin filament and the thick myosin filament (Ebashi, 1984; Solaro, 1991; Farah and Reinach, 1995). When the cytoplasmic Ca^{2+} level rises, Ca^{2+} binds to troponin C causing a conformational change which breaks the inhibition of the troponin-tropomyosin complex allowing interaction of actin and myosin and initiation of the contractile process (Katz, 1977; Ikonomidis *et al.*, 1990; Bers, 1991; Barry and Bridge, 1993). This involves transduction of the chemical energy of ATP, which allows a shift in the position of the attached cross-bridge to produce force and a relative sliding of the thick and thin filaments (Katz, 1977). The force that is generated during a cardiac contraction is an indication of the number of cross-bridges that attach. This will be related to the availability of myosin binding sites on actin, which in turn will be determined by the number of troponin C regulatory units that bind Ca^{2+} (Bers, 1991). Ultimately, therefore, the amount of Ca^{2+} influx will determine the force of contraction, since it will determine the amount released from the SR and hence the number of troponin C units that bind Ca^{2+} and the number of cross-bridges that attach.

1.1.2.2 Relaxation of Cardiac Muscle

Relaxation of cardiac muscle requires removal of Ca^{2+} from the cytosol such that the free intracellular Ca^{2+} concentration drops to below the threshold for activation of the myofilaments (Vaughan-Jones, 1986). As the cytosolic Ca^{2+} concentration falls Ca^{2+} dissociates from troponin C and the troponin-tropomyosin complex returns to its original inhibitory position on the thin filament (Katz, 1977). There are three main transport proteins which compete for the cytosolic calcium, namely the SR Ca^{2+}-ATPase, the sarcolemmal Ca^{2+}-ATPase and the Na^{+}/Ca^{2+} exchanger. The mitochondria have also been implicated in Ca^{2+} removal from the cytosol during relaxation, however, their role is less well understood.

The SR Ca^{2+}-ATPase transports Ca^{2+} from the cytosol into the SR where, after being returned to the Ca^{2+} release sites within the terminal cisternae of the SR it is once again available for release. The Na^{+}/Ca^{2+} exchanger and SL Ca^{2+}-ATPase on the other hand extrude Ca^{2+} from the cell into the extracellular space. It is important to understand that the competition between the SR Ca^{2+}-ATPase and the Na^{+}/Ca^{2+} exchanger and SL Ca^{2+}-ATPase will influence the amount of Ca^{2+} retained within the cell (and within the SR) and the amount extruded. It is also important to realise that since some Ca^{2+} enters the cell from the extracellular space with each excitation some must subsequently be extruded to prevent a continuous accumulation of intracellular Ca^{2+}. Under steady-state conditions, the amount of Ca^{2+} extruded from the cell during one cycle of the excitation-contraction coupling process will exactly balance that which entered from the extracellular space. Under this condition the SR Ca^{2+} load would be constant from contraction to contraction as would be the Ca^{2+} release and the contractile response. If there is a shift in balance between the Ca^{2+} removal processes this will affect the SR Ca^{2+} load and the subsequent Ca^{2+} release. For example, if more Ca^{2+} were to be extruded from the cell during one cardiac cycle when compared with the previous one, then the Ca^{2+} load of the SR would be reduced. This would in turn lead to reduced Ca^{2+} release and reduced force production. Conversely, if less Ca^{2+} is extruded to the extracellular space than entered from it, the SR Ca^{2+} load would increase giving the potential for increased Ca^{2+} release and force production.

1.1.2.2.1 SR Ca^{2+}-ATPase

The SR Ca^{2+}-ATPase (SERCA) pump is found in the longitudinal component of the SR (Jorgensen *et al.*, 1982), is a 100-115 kD protein and has been shown to operate

optimally in the range of physiological Ca^{2+} concentrations seen during normal contraction and relaxation (Barry and Bridge, 1993). It works efficiently by using the energy of ATP to actively pump Ca^{2+} (2 Ca^{2+} ions per ATP consumed) from the cytosol into the SR to refill it before the next contraction (Bers, 2000). The SR Ca^{2+} -ATPase is the Ca^{2+} transport protein responsible for the majority of Ca^{2+} uptake from the cytosol.

Six distinct SERCA isoforms encoded by three different genes have been described. The SERCA1 gene encodes two alternatively spliced transcripts, SERCA1a which is found in adult fast skeletal muscle and SERCA1b present in neonatal fast skeletal muscle. The SERCA2 gene also encodes two alternatively spliced isoforms, SERCA2a which is present in heart, slow skeletal muscles and some smooth muscles and SERCA2b which is present in all cell types and is regarded as a housekeeping isoform. SERCA3 also encodes two alternatively spliced isoforms, SERCA3a and SERCA3b which are coexpressed in many tissues (Barry and Bridge, 1993; Langer, 1997; Lompre, 1999). The affinity for Ca^{2+} of the different enzymes varies in the following order: 2b>2a=1≥3a>3b (Lompre, 1999). It is the SERCA2a which is of interest to this discussion as this isoform represents the Ca^{2+}-ATPase pump in the SR of the heart.

Experiments conducted by Bers and Bridge (1989) demonstrated the SR Ca^{2+}-ATPase to be the main pathway for reduction of $[Ca^{2+}]_i$ during relaxation of rabbit cardiac muscle, showing that blocking of the Na^+/Ca^{2+} exchanger slowed relaxation by ~29%, whereas blockage of SR Ca^{2+} accumulation by addition of caffeine slowed relaxation by ~1000%. It is important to note that the capacity of the SR for Ca^{2+} has been estimated by numerous groups, and although a large variance was reported (values vary from around 57 to approximately 300 μmol Ca^{2+}/kg wet wt) (Dani *et al.*, 1979; Fabiato, 1983), it can be assumed that there is enough Ca^{2+} stored within the SR to activate the myofilaments and initiate contraction (Bers, 1991).

The activity of the SR Ca^{2+}-ATPase is regulated by phosphorylation of phospholamban (Ikonomidis *et al.*, 1990; Barry and Bridge, 1993; Brittsan and Kranias, 2000). Phospholamban is a protein that is thought to bind to and inhibit Ca^{2+} transport by the SR Ca^{2+}-ATPase (Fujii *et al.*, 1987; Barry and Bridge, 1993). Phosphorylation of phospholamban by either cAMP-dependent protein kinase, Ca^{2+}-calmodulin dependent protein kinase or protein kinase C removes this inhibitory effect (Tada *et al.*, 1974). It has been shown that phospholamban interacts with the SR Ca^{2+}-ATPase near the phosphorylation site only when phospholamban is dephosphorylated (James *et al.*,

1989) supporting the idea of an inhibitory action for phospholamban. It is thought that the phosphorylation of phospholamban stimulates the SR Ca^{2+}-ATPase pump, increasing both the Ca^{2+} affinity and rate of Ca^{2+} transport. For example, phosphorylation of phospholamban occurs during β-adrenergic stimulation which results in an increase in the affinity of SR Ca^{2+}-ATPase for Ca^{2+} and enhanced rates of relaxation in the heart (McIvor *et al.*, 1988; Brittsan and Kranias, 2000). Whilst the role of phospholamban in the cardiac SR is reasonably well established, the molecular mechanism by which phospholamban exerts its regulatory effects are still unclear (Brittsan and Kranias, 2000).

1.1.2.2.2 Sarcolemmal Ca^{2+}-ATPase

The SL Ca^{2+}-ATPase pump uses the energy of ATP to remove Ca^{2+} from the cytosol into the extracellular space against both a concentration and an electrical gradient. It has a molecular mass of 134,000 kD and displays a much higher affinity for Ca^{2+} than does the Na^{+}/Ca^{2+} exchanger (Carafoli, 1992; Langer, 1997). At least four separate genes code for the SL Ca^{2+}-ATPase pumps, namely PMCA1 (Plasma Membrane Ca^{2+}-ATPase), PMCA2, PMCA3 and PMCA4 (Carafoli, 1992). Each of these four isoforms also undergoes alternative splicing. PMCA1 and PMCA4 have been found to be expressed in all tissues including the heart, whilst PMCA2 and PMCA3 are only found in neuronal tissues (Stauffer *et al.*, 1995).

The SL Ca^{2+}-ATPase is stimulated by the Ca^{2+} calmodulin complex and is also thought to be activated by phosphorylation (Carafoli, 1992; Barry and Bridge, 1993). Calmodulin is thought to bind to the pump in a Ca^{2+}-dependent fashion with high affinity. It has been found that the calmodulin binding site is an autoinhibitory portion of the pump protein. When calmodulin is not bound, this region interacts with another part of the protein to cause inhibition. Binding of calmodulin relieves this inhibition and stimulates pump function (Falchetto *et al.*, 1991; Falchetto *et al.*, 1992). The SL Ca^{2+}-ATPase pump is also activated by PKA phosphorylation. It is thought that PKA phosphorylation carries out its stimulatory actions on the pump in a similar way to calmodulin, with suggestions that it exerts a stimulatory effect on the pump by decreasing the potency of the autoinhibitory domain (Langer, 1997).

The SL Ca^{2+}-ATPase pump transports one Ca^{2+} ion per ATP split, but has been shown to transport Ca^{2+} at a rate too slow to be of major importance in determining Ca^{2+} fluxes

during a single cardiac contraction (Bers, 1991). In a study conducted by Barry *et al.* (1985) using cultured cardiac myocytes, the rate at which the SL Ca^{2+}-ATPase could remove Ca^{2+} from the myocyte was shown to be a nd 1/10 of the rate of the Na^+/Ca^{2+} exchanger. This comparison was made over a ra.,e of physiological cytosolic Ca^{2+} concentrations. It has therefore been postulated that the main purpose of the SL Ca^{2+}-ATPase pump is to maintain the level of Ca^{2+} within the cell low enough to prevent the release of Ca^{2+} from the SR in between contractions (Fabiato, 1983; Barry and Bridge, 1993). It may also be present to counteract any slow leak of Ca^{2+} into the cell during rest (Barry and Bridge, 1993).

1.1.2.2.3 Na^+/Ca^{2+} Exchanger

The Na^+/Ca^{2+} exchanger is an ion transport protein which is found not only in cardiac tissue but also in skeletal and smooth muscle and in neural tissue (Yashar *et al.*, 1998). In cardiac muscle the Na^+/Ca^{2+} exchanger is responsible for the majority of Ca^{2+} extrusion from the cytosol to the extracellular space. It is a protein containing 938 amino acids with a molecular mass of 110 kD (Barry and Bridge, 1993; Reeves, 1998; Shigekawa and Iwamoto, 2001). The Na^+/Ca^{2+} exchanger has a reasonably high affinity Ca^{2+} transport site at the intracellular membrane surface and a low affinity Ca^{2+} transport site at the extracellular surface. This allows the transporter to be reversible, as it allows Ca^{2+} extrusion from the cell during relaxation (forward mode) but also allows the Na^+/Ca^{2+} exchanger to work in reverse mode to bring Ca^{2+} into the cell during excitation (Yashar *et al.*, 1998). The energy for net Ca^{2+} transport across the sarcolemma by the Na^+/Ca^{2+} exchanger depends on the Na^+, Ca^{2+} and K^+ gradients along with the membrane potential (Yashar *et al.*, 1998; Blaustein and Lederer, 1999). The Na^+/Ca^{2+} exchanger has a $3Na^+$:$1Ca^{2+}$ stoichiometry (Boyett *et al.*, 1992; Blaustein and Lederer, 1999; Calaghan and White, 1999) and is essential for maintaining Ca^{2+} homeostasis in cardiac myocytes.

At least three separate genes code for the Na^+/Ca^{2+} exchanger, namely NCX1, NCX2 and NCX3. Genes homologous to NCX1 have been characterised in many different mammalian species including human, rabbit, guinea pig and rat (Blaustein and Lederer, 1999). NCX1 has at least 8 isoforms (Matsuda *et al.*, 1997). It was the first Na^+/Ca^{2+} exchanger to be cloned and is highly expressed in cardiac muscle and brain, and to a lesser extent in many other tissues (Shigekawa and Iwamoto, 2001). NCX2 and NCX3

are expressed in the brain and skeletal muscle but are not found in the adult heart (Matsuda *et al.*, 1997).

The Na^+/Ca^{2+} exchanger, when operating in forward mode, is the main site for Ca^{2+} extrusion from the cardiac myocyte. It has a lower affinity for Ca^{2+} when compared with the sarcolemmal Ca^{2+}-ATPase pump, but it has a higher calcium transport activity (Shigekawa and Iwamoto, 2001). The Na^+/Ca^{2+} exchanger has also been shown to have a housekeeping role within the cardiac myocyte by maintaining a low intracellular Ca^{2+} concentration in between contractions (Yashar *et al.*, 1998). The activity of the Na^+/Ca^{2+} exchanger is stimulated by $[Ca^{2+}]_i$, protein phosphorylation and PKC activators, and is inhibited by Na^+, Ni^{2+}, La^{3+} and Cd^{2+}.

1.1.2.2.4 Mitochondria

In cardiac muscle cells, as in other cells, the mitochondria represent a significant potential intracellular Ca^{2+} sink. Mitochondria are able to take up large quantities of Ca^{2+} from the cytosol via the Ca^{2+} uniporter which has a $2Na^+$:$1Ca^{2+}$ stoichiometry (Langer, 1997). The primary role of Ca^{2+} within the mitochondria is likely to be the regulation of oxidative phosphorylation (Duchen *et al.*, 1998), however it has been postulated that the mitochondria may also have a role in excitation-contraction coupling (Katz, 1977). Recent studies have shown that the distance between mitochondria and Ca^{2+} release sites on the SR is quite small, suggesting the possibility of $[Ca^{2+}]$ communication between these two compartments (Duchen *et al.*, 1998; Szalai *et al.*, 2000). This raises the possibility that the mitochondria may be contributing to cardiac muscle relaxation by taking up some Ca^{2+} during this phase of excitation-contraction coupling (Bassani *et al.*, 1994). The exact role of the mitochondria, if any, in excitation-contraction coupling has not been elucidated, however, the mitochondrial Ca^{2+} pump has been shown to have low Ca^{2+} affinity and the rate of Ca^{2+} transport at physiological Ca^{2+} levels is slow (Katz, 1977; Langer, 1997; Szalai *et al.*, 2000), suggesting that mitochondrial contribution to excitation-contraction coupling on a beat-to-beat basis would be, if present, modest at best.

1.1.2.2.5 Species Differences in the Fractional Contribution of Each Relaxation Pathway

Under most circumstances the majority of the Ca^{2+} removed from the cytosol during relaxation is achieved by the SR Ca^{2+}-ATPase. There are, however, known to be species

differences in the fractional contribution of each pathway. In rat cardiac myocytes it has been shown that SR Ca^{2+}-ATPase activity accounts for approximately 92% of the Ca^{2+} removal from the cytosol, whilst the Na^{+}/Ca^{2+} exchanger and sarcolemmal Ca^{2+}-ATPase pump account for 7 and 1% respectively (Bassani *et al.*, 1994; Bers, 2000). In contrast, in rabbit cardiac myocytes the SR Ca^{2+}-ATPase removes approximately 70% of the activator Ca^{2+} from the cytosol. The Na^{+}/Ca^{2+} exchanger removes 28% and the sarcolemmal Ca^{2+}-ATPase is responsible for only approximately 1% of the Ca^{2+} removal from the cytosol during relaxation (Bassani *et al.*, 1994; Bers, 2000). In any particular species the balance could also be altered through regulatory actions on particular pathways. For example, stimulation of SR Ca^{2+} uptake through phospholamban regulation as might occur during β-adrenergic stimulation may increase the proportion of the cytosolic Ca^{2+} sequestered into the SR which in turn would increase the SR Ca^{2+} store and give the capacity for greater subsequent Ca^{2+} release.

1.1.3 Summary of the Excitation-Contraction Coupling Process

The process by which cardiac contraction occurs is one which involves a complex chain of ionic movements and protein interactions. The Ca^{2+} ion is undoubtedly the most important factor in these events, with each step of the excitation-contraction coupling process designed to deliver Ca^{2+} in large enough amounts to initiate contraction and then remove this Ca^{2+} to allow relaxation to occur. An overall schematic diagram of the excitation-contraction coupling process is illustrated in Figure 1.1, which demonstrates the movements of Ca^{2+} during a single cardiac cycle.

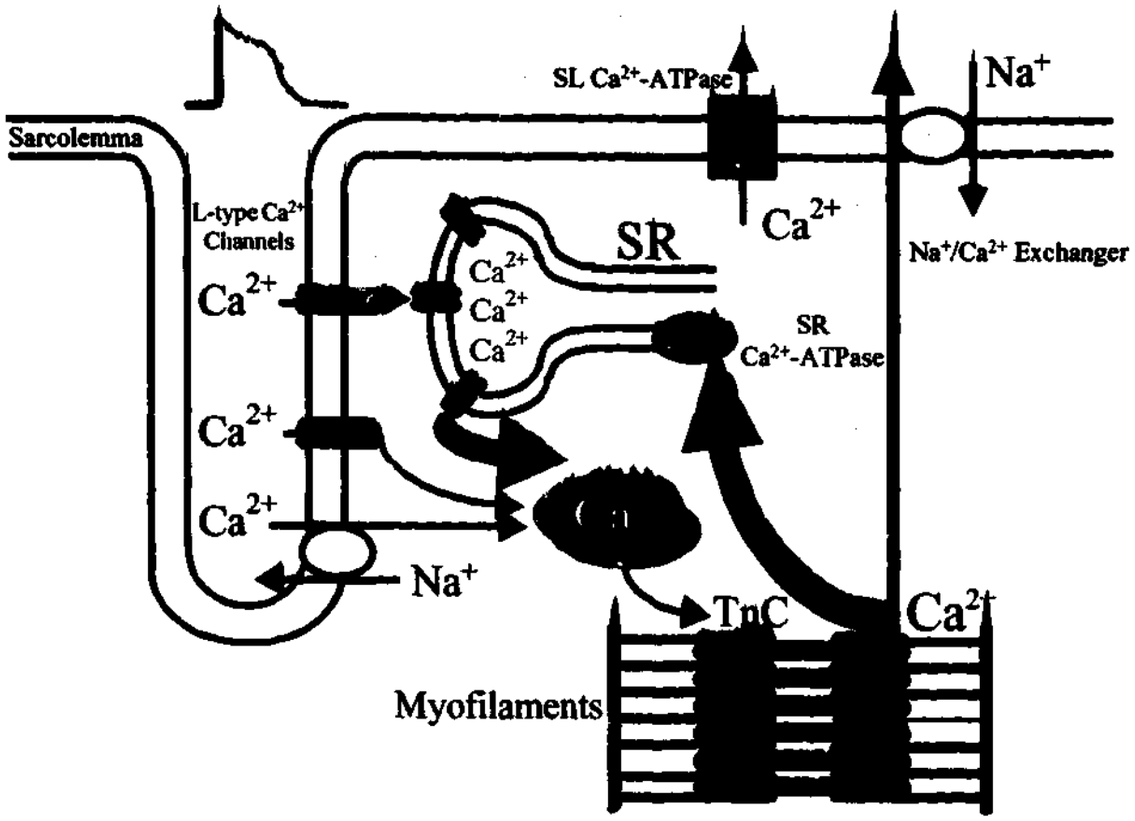


Figure 1.1: Schematic diagram of the excitation contraction coupling process in cardiac muscle. Calcium enters the cell through the sacolemmal (SL) L-type Ca^{2+} channels and binds to its receptor on the sarcoplasmic reticulum (SR) causing the release of a greater quantity of Ca^{2+} and an increase in the cytosolic Ca^{2+} concentration. Ca^{2+} from both the extracellular space and the SR binds to troponin C (TnC) on the thin filaments to initiate cross-bridge cycling and contraction. Most of the cytosolic Ca^{2+} is sequestered back into the SR via the SR Ca^{2+}-ATPase, while a smaller fraction is extruded from the cell by the Na^+/Ca^{2+} exchanger. The decrease in cytosolic [Ca^{2+}] allows the muscle to relax. The activity of the SL Ca^{2+}-ATPase is considered to be too low to contribute significantly to Ca^{2+} extrusion and relaxation during the cardiac cycle, but is important in maintaining low diastolic Ca^{2+} levels.

1.2 Altered Ca^{2+} Movements – Effects on Cardiac Function

It is clear that the Ca^{2+} ion is the crucial and fundamental factor in regulating cardiac muscle contraction. It can, therefore, be assumed that changes to the Ca^{2+} levels and movements within the heart would have profound effects on cardiac function. Many different factors can affect the movements of Ca^{2+} within the heart. Some circumstances which result in changes to Ca^{2+} movements within the heart are detailed as follows.

1.2.1.1.1 Adrenergic Agents

β-adrenergic agonists such as isoproterenol affect cardiac inotropy by producing large increases in the amplitude of the Ca^{2+} transient and cardiac contraction. Isoproterenol has three main effects within the heart which include increased Ca^{2+} current, increased SR Ca^{2+} uptake and decreased myofilament Ca^{2+} sensitivity (Tada and Katz, 1982; Skeberdis *et al.*, 1997). β-adrenergic agonists act by stimulating adenylate cyclase (AC) which results in an increase in cAMP levels, activation of PKA and eventual phosphorylation at a number of different sites including the sarcolemmal Ca^{2+} channels, troponin and phospholamban. The phosphorylation of these sites results in much faster and larger Ca^{2+} transients (Bers, 1991).

In addition to the β-adrenergic agonists are the α-adrenergic agonists, which also have been shown to increase the amplitude of the Ca^{2+} transient, although they do so via different mechanisms to the β-adrenergic agonists. α-adrenergic agonists stimulate cAMP phosphodiesterase activity and reduce cAMP. They also increase hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol. IP_3 has been shown to modulate SR Ca^{2+} release and is thought to play a role in the inotropic effects of α-adrenergic agonists (Bers, 1991). It important to note that the inotropic effects of α-adrenergic agonists are less potent than those exerted by the β-adrenergic agonists.

1.2.1.1.2 Heart Rate – Staircase Response

The relationship between heart rate and contractile force has been studied extensively with the positive force-frequency or 'positive staircase effect' being the most described. Increases in stimulation frequency or heart rate lead to increases in contractile force. Factors contributing to this increase in contractile force include increased Ca^{2+} current entering the cell from the extracellular space per unit time, higher diastolic $[Ca^{2+}]_i$ and also increased SR Ca^{2+} available for release (Bers, 1991). The higher diastolic $[Ca^{2+}]_i$ would come about as a result of the increased entry of Ca^{2+} from the extracellular space coupled with the fact that there is less time between contractions for Ca^{2+} to be extruded from the cell. An increase in intracellular $[Na^+]$ would reduce the Na^+ concentration gradient resulting in a decline in Ca^{2+} efflux via the Na^+/Ca^{2+} exchanger. Ca^{2+} efflux through the Na^+/Ca^{2+} exchanger would be less than Ca^{2+} influx through the L-type Ca^{2+} channel, therefore $[Ca^{2+}]_i$ would increase until Ca^{2+} efflux equals Ca^{2+} influx resulting

in increased force of contraction (Boyett *et al.*, 1992). The increased SR Ca^{2+} content would occur as a consequence of the greater amount of $[Ca^{2+}]$ available within the cytosol of the cell. Overall this leads to greater cardiac contraction and therefore greater cardiac contractile force produced (Lewartowski and Pytkowski, 1987; Boyett *et al.*, 1992).

1.2.1.1.3 Pharmacological Interventions

Glycosides

Cardiac glycosides, such as ouabain, have a positive inotropic effect on the heart and have been shown to enhance myocardial contractility through an action affecting Ca^{2+} fluxes across the sarcolemma (Satoh *et al.*, 2000). It has been found that cardiac glycosides inhibit the Na^+/K^+ ATPase pump of the sarcolemma (Reeves, 1998). This pump which is ATP dependent exchanges sodium which enters the cell during the action potential for extracellular potassium. Increased intracellular Na^+ can have a large impact on the balance of Ca^{2+} fluxes mediated by the Na^+/Ca^{2+} exchanger. The increase in intracellular Na^+ increases Ca^{2+} influx through the Na^+/Ca^{2+} exchanger and also limits Ca^{2+} extrusion via this same mechanism (Satoh *et al.*, 2000). This leads to a higher diastolic $[Ca^{2+}]_i$ level. In conjunction with this increase is a rise in SR Ca^{2+} content as a result in a shift in competition between the SR Ca^{2+}-ATPase and the Na^+/Ca^{2+} exchanger during relaxation resulting in increased uptake of Ca^{2+} into the SR (Satoh *et al.*, 2000).

L-type Ca^{2+} Channel Blockers

L-type Ca^{2+} channel blockers act by directly binding to the channel itself and therefore progressively block it relative to the dosage of Ca^{2+} channel blocker administered (Langer, 1997). Three classes of these compounds have been identified: (1) benzeneacetonitriles (e.g. verapamil), (2) dihydropyridines (e.g. nifedipine) and (3) benzothiazepines (e.g. dilitazem). All of these drugs have been shown to possess an ability to block the slow inward Ca^{2+} current and therefore inhibit the trigger Ca^{2+} entry through the L-type Ca^{2+} channel which is needed for the initiation of Ca^{2+}-induced Ca^{2+} release and contraction. The actions of nifedipine are thought to be relatively specific and it is thought that nifedipine acts by actually plugging the Ca^{2+} channels, whereas verapamil and dilitazem are thought to block Ca^{2+} channels in a less specific and possibly stimulus dependent fashion (Braunwald, 1982; Sun and Triggle, 1995). The

Na^+/Ca^{2+} exchanger has been found to be able to provide the trigger Ca^{2+} entry for excitation-contraction coupling when the L-type Ca^{2+} channel is not functioning (Langer, 1997).

1.2.1.1.4 Pathophysiological Conditions

Abnormalities of Ca^{2+} homeostasis have also been reported in several pathophysiological conditions of the heart supporting the notion that changes in intracellular Ca^{2+} movements may underlie the dysfunction of the heart seen in these circumstances (Ikonomidis *et al.*, 1990). Some examples of heart dysfunctions which are thought to arise from Ca^{2+} handling abnormalities are briefly discussed below. Each of these conditions are complex with numerous factors undoubtedly contributing to their development and the functional consequences for the heart. Detailed coverage of the literature pertaining to these pathophysiological conditions is beyond the scope of this thesis, however, attention has been drawn to them to highlight the possible involvement of disturbances in cellular Ca^{2+} handling pathways in the myocardial dysfunction that is observed.

Ischemia

Ischemia has a detrimental effect on cardiac function and results from a lack of, or reduced, blood flow to a region of the heart due to functional constriction or obstruction of a coronary blood vessel (Katz, 1977). This blockage of an artery can be caused by numerous different events such as atherosclerotic plaque formation, thrombosis, narrowed lumen or coronary artery spasm. Ischemia is also common among patients undergoing cardiac surgery. It has been recognised that ischemia and reperfusion can result in disordered myocardial cellular function, with evidence gathering which links changes in Ca^{2+} movements to the myocardial dysfunction induced by ischemia and reperfusion (Ylitalo *et al.*, 2000). It is thought that an intracellular Ca^{2+} overload is the major cause of cellular injury related to cardiac ischemia (Shigematsu and Arita, 1999). The mechanisms responsible for the increase in $[Ca^{2+}]_i$ during ischemia/reperfusion are not fully understood although both the SR and the Na^+/Ca^{2+} exchanger have been suggested as possible sites for the cause of this Ca^{2+} overload (Shigematsu and Arita, 1999; Bourdillon and Poole-Wilson, 2000). A slowing of myocardial relaxation has been shown to be a major feature of abnormal cardiac function during ischemia which suggests that impaired Ca^{2+} removal from the cytosol is a contributing factor during ischemia (Halow *et al.*, 1999). Both the SR and Na^+/Ca^{2+} exchanger are likely to be

implicated in this. The SR Ca^{2+} uptake and release activities have been reported to be altered in ischemic/reperfused hearts (Temsah *et al.*, 1999), but it is thought that the Na^{+}/Ca^{2+} exchanger could play the central role in Ca^{2+} accumulation during ischemia/reperfusion. It is thought that a rise in intracellular Na^{+} during ischemia/reperfusion reduces the ability of the exchanger to remove Ca^{2+} and eventually causes the exchanger to operate in reverse mode bringing Ca^{2+} into the cell rather than extruding it (Goldhaber, 1999; Shigematsu and Arita, 1999). This would result in an increased Ca^{2+} load within the cell.

Hypertrophy

Cardiac hypertrophy refers to an increase of muscle cell size and protein content in the heart. It can be as a result of a disease which places an abnormal load on the heart causing enlargement of the chambers of the heart in an attempt by the organ to compensate to normalise wall stress. It is the single most important contributor to cardiovascular morbidity and mortality in the western world (Balke and Shorofsky, 1998). Cardiac hypertrophy results in contractile abnormalities many of which are associated with increases in the amplitude $[Ca^{2+}]_i$ (Shorofsky *et al.*, 1999). Peak active tension has been shown to increase (Bing *et al.*, 1988; Conrad *et al.*, 1991), but the rate of contraction and relaxation have been shown to be slowed (Brooksby *et al.*, 1992). The exact mechanisms for this increase are yet to be elucidated, however, both the SR and the L-type Ca^{2+} channel have been suggested as possible sites of disruption. It has been suggested that an increase in SR Ca^{2+} release is responsible for the increased $[Ca^{2+}]_i$, but the mechanisms responsible for this increase remain to be defined (Shorofsky *et al.*, 1999). One theory, although controversial, suggests an increase in L-type Ca^{2+} current which would trigger an increased Ca^{2+} release from the SR. This increase in L-type Ca^{2+} current is thought to be as a result of increased numbers of L-type Ca^{2+} channels in the hypertrophic heart (Keung, 1989; Mukherjee and Spinale, 1998). If hypertrophy is not controlled, it can progress to heart failure.

The Failing Myocardium

In the failing myocardium, the ability of the heart to sustain a level of acceptable output in response to increased demand is reduced (Sipido *et al.*, 1998). As hypertrophy progresses to heart failure, the amplitude of the $[Ca^{2+}]_i$ transient actually decreases (Balke and Shorofsky, 1998). In fact contractile dysfunction in end-stage human heart failure has been connected to depressed myofilament Ca^{2+} sensitivity, reduced Ca^{2+}

transients and also reduced SR Ca^{2+}-ATPase activity. It is believed that the negative force-frequency relationship that is apparent in end-stage human heart failure, and which results in a decrease in contractile performance at higher heart rates, is a result of depressed SR Ca^{2+} uptake (Phillips *et al.*, 1998; Sipido *et al.*, 1998) and increased Na^{+}/Ca^{2+} exchange (Bers *et al.*, 1996; Mattiello *et al.*, 1998) which lead to an underloaded SR (Pieske *et al.*, 1999). The rate and extent of decline of $[Ca^{2+}]_i$ during relaxation in the cardiac myocyte is also thought to be diminished in failing myocardium (Beuckelmann *et al.*, 1992; Balke and Shorofsky, 1998; Movsesian and Schwinger, 1998) again suggesting a role for depressed SR Ca^{2+}-ATPase activity in the failing heart. The L-type Ca^{2+} current has also been suggested to be altered in failing myocardium, although the exact mechanisms behind this are unclear (Balke and Shorofsky, 1998; Sipido *et al.*, 1998; Shorofsky *et al.*, 1999).

Diabetic Cardiomyopathy

Diabetic cardiomyopathy is characterised by depressed contractile reserve, slowed contraction and slowed relaxation (Lopaschuk *et al.*, 1983; Russ *et al.*, 1991; Davidoff and Ren, 1997) and there is now substantial evidence that these impaired mechanical properties of the diabetic heart stem predominantly from disturbances in Ca^{2+} handling with consequent effects on excitation-contraction coupling (Tomlinson *et al.*, 1992; Davidoff and Ren, 1997). A change in the level of intracellular Ca^{2+} has been suggested in the diabetic heart, however the direction of this change has been the subject of some debate with both increased (Levy *et al.*, 1994), and decreased diastolic Ca^{2+} levels (Bouchard and Bose, 1991; Ren and Davidoff, 1997) being reported. Depressed SR Ca^{2+}-ATPase (Holloway *et al.*, 1999; Kotsanas *et al.*, 2000) and sarcolemmal Ca^{2+}-ATPase activities, as well as altered Na^{+}/Ca^{2+} exchange have been reported to underlie the alterations in Ca^{2+} handling present in the diabetic heart. Decreased SR Ca^{2+} release has also been reported in the diabetic heart which may account for the depression in contractility associated with diabetes. This has been speculated to be due to a diminished number of, or defective, SR Ca^{2+} release channels (Ren and Davidoff, 1997). Alternatively the reduction in SR Ca^{2+} release could be due to a decrease in the level of Ca^{2+} within the SR. This would be expected to arise as a consequence of the depressed SR Ca^{2+}-ATPase activity, which would also be partly responsible for the slowed relaxation present in the diabetic heart (Penpargkul *et al.*, 1981; Makino *et al.*, 1987). The well documented decrease in sarcolemmal Ca^{2+}-ATPase activity along with

altered Na^+/Ca^{2+} exchange could also be implicated in the slowed relaxation (Pierce *et al.*, 1990).

1.3 Altered Ca^{2+} Movements – Effects of Hormones

The Ca^{2+} handling pathways within the heart are also thought to be modified by a number of different hormones within the body. These hormones exert their effects primarily via genomic actions on the heart. Some examples of hormones that affect Ca^{2+} movements in the heart are briefly discussed below, followed by a more detailed review of the literature pertaining specifically to the possible influences of the sex steroid hormones on the heart.

Renin/Angiotensin

Increasing evidence suggests that there is a local renin-angiotensin system in the heart (Dzau, 1988). In particular, it has been suggested that L-type Ca^{2+} current and myocardial contractility are regulated by the cardiac renin-angiotensin system (De Mello, 1998). Genes of angiotensinogen and renin are coexpressed in isolated cardiac myocytes (Ohkubo *et al.*, 1986), suggesting a role for them in modulating cardiac function. In addition, angiotensin II receptors have been located in cardiac muscle cells (Baker *et al.*, 1984). The direct inotropic effect of angiotensin II on the myocardium is controversial. Several studies have been undertaken to elucidate the effect of angiotensin II on $[Ca^{2+}]_i$ in the heart. In a study by De Mello (1998) whole-cell voltage clamp techniques were used to measure L-type Ca^{2+} current. The L-type Ca^{2+} current was found to decrease significantly with the addition of angiotensin II. Meissner *et al.* (1998) also showed a reduction in peak systolic $[Ca^{2+}]_i$ with the addition of angiotensin II. Other studies, however, have shown angiotensin II to exert positive inotropic actions within the heart (Skolnick *et al.*, 1998; Aiello and Cingolani, 2001), with significant increases in the amplitude of the Ca^{2+} transient reported (Fujita and Endoh, 1999) It is believed that the effects of angiotensin II on the L-type Ca^{2+} current are related to an intracellular mechanism, possibly through a cGMP mediated pathway, which would suggest that the cardiac renin-angiotensin system could play an important role in the regulation of heart contractility (De Mello, 1998).

Aldosterone

In conjunction with the previous findings, the mineralocorticosteroid aldosterone is also thought to modulate Ca^{2+} movements within the heart. Aldosterone is classically known to regulate sodium reabsorption in the kidney and to increase the excretion of potassium and hydrogen ions. It has also been suggested, however, to have an effect on cardiovascular function through direct actions on myocytes (Benitah and Vassort, 1999). An agonist-specific mineralocorticoid receptor has been shown in cardiomyocytes (Lombes *et al.*, 1992), along with direct production of aldosterone within cardiac tissue itself (Silvestre *et al.*, 1998). Benitah and Vassort (1999) demonstrated that aldosterone upregulates the L-type Ca^{2+} current in rat ventricular myocytes through genomic regulation. It is also thought that aldosterone may regulate the expression of the major cardiac Na^+/K^+-ATPase isoform as well as having possible effects on the Na^+/H^+ antiporter and Cl^-/HCO_3^- exchanger (Benitah and Vassort, 1999).

Thyroid Hormones

The thyroid state is believed to be an important determinant of cardiac function. Thyroid hormones have been shown to bind directly to receptors in cardiomyocytes and are thought to affect various sarcolemmal proteins, the myosin heavy chain proteins of the contractile apparatus, and proteins related to the SR (Holt *et al.*, 1999). Increases in thyroid hormone levels have been shown to enhance myocardial contractility, cardiac output and heart rate (Jiang *et al.*, 2000). Holt *et al.* (1999) demonstrated an increased SR Ca^{2+}-ATPase/phospholamban ratio and a decrease in phospholamban protein content in long-term triiodothyronine (T_3) treated cells which resulted in a parallel increase of contraction and Ca^{2+} transients and more rapid Ca^{2+} uptake in cardiomyocytes (Holt *et al.*, 1999). Overexpression of functional ryanodine receptors has also been demonstrated in thyroid hormone-induced cardiac hypertrophy which may be responsible, in part, for changes in SR Ca^{2+} release and the Ca^{2+} transient (Jiang *et al.*, 2000).

1.4 Gender Differences in Cardiovascular Disease

It has been recognised from epidemiological studies that pre-menopausal women have a reduced risk of cardiovascular disease when compared with men of the same age (Barrett-Connor and Bush, 1991; Farhat *et al.*, 1996). When women reach menopause, however, their risk of cardiovascular complications increases to the same, or even

greater, levels than are present in comparably aged men. This increased risk in post-menopausal women is reduced if hormone replacement therapy is received, suggesting a role for female sex hormones in protection against cardiovascular diseases (Bush, 1990; Gerhard and Ganz, 1995; Hayward *et al.*, 2000; Gray *et al.*, 2001).

1.4.1 Epidemiological Studies

The putative cardioprotective effect of female hormones has received a great deal of attention. Many epidemiological studies have been undertaken, however, these have often produced mixed and complex results. In studies of this type it is difficult to come to clear and sound conclusions, as many factors and outside variables are involved in each study. Pre-existing cardiovascular problems, genetic predisposition, and behavioural factors such as cigarette smoking and diet are among the many factors that contribute to the mix of results.

A great many observational studies have been undertaken, and with the exception of only a few (Wilson *et al.*, 1985; Hulley *et al.*, 1998), these have concluded that female sex hormones, and in particular estrogen, have a beneficial effect in the female heart (Bush *et al.*, 1987; Bush, 1990; Barrett-Connor and Bush, 1991; Stampfer *et al.*, 1991; Grady *et al.*, 1992; Pelzer *et al.*, 1997). There is a large body of evidence that has been gathered in conjunction with these studies which suggests that estrogen replacement in post-menopausal women reduces the risk for coronary heart disease by about 50% as compared to post-menopausal women not receiving such hormone replacement (Barrett-Connor and Bush, 1991). It has also been suggested that the reduction in risk may be greater for women who have already had previous cardiovascular problems (Sullivan *et al.*, 1988; Grady *et al.*, 1992). Concern regarding the cohort of women participating in these studies was raised, in particular regarding their selection process and background medical histories. It was suggested that a bias may be involved, based on the notion that women who choose to take hormones could initially be healthier and have a more favourable coronary heart disease profile than those that do not (Hulley *et al.*, 1998). Hulley *et al.* (1998) decided that the only way to determine the unbiased effects of estrogen on the post-menopausal heart was to conduct their own randomised, double-blind placebo-controlled trial, the likes of which had never been attempted before. The Heart and Estrogen-progestin Replacement Study (HERS) was subsequently conducted and involved the administration of oral conjugated equine

estrogens plus medroxyprogesterone acetate in a group of women with established coronary heart disease. The follow-up with these women averaged 4.1 years, and showed that there were no significant differences in the overall incidence of coronary heart disease in post-menopausal women either with hormone replacement or without. This was a surprising outcome, with the study since receiving some criticism. The combination of estrogen and progestin was firstly questioned, as it is thought that progestins oppose the main beneficial effects of estrogen within the vasculature and the heart, thus counteracting any favourable cardiovascular outcome caused by the estrogen replacement (Sherwin and Gelfand, 1989; Grady *et al.*, 1992). Secondly, the use of women who already had established coronary heart disease was questioned (Wenger, 1998). The study also did not evaluate unopposed estrogen treatment which is most commonly used in women who have undergone hysterectomy (Barrett-Connor and Bush, 1991; Hulley *et al.*, 1998; Wenger, 1998). In addition to this study are early reports from the Estrogen Replacement and Artherosclerosis (ERA) trial which demonstrated similar findings to that of the HERS study. However, this study has also received similar criticism to the HERS study, as older women with pre-existing heart conditions were again represented in the study cohort (Herrington *et al.*, 2000).

Clearly there is still some uncertainty about the benefits of estrogen replacement in post-menopausal women in terms of reducing the risk of cardiovascular disease. Although there is a large body of observational evidence proclaiming the benefits of estrogen replacement therapy in reducing cardiovascular risk, specific trials directed at testing this have provided somewhat inconclusive results. Since the development of cardiovascular disease is likely, in many cases, to have a multifactorial basis it is clearly difficult to select study populations that will allow conclusive assessment of the impact of a single factor, such as estrogen administration. At the present time, this leaves unanswered the question as to the values of estrogen administration in reducing the risk of cardiovascular disease in post-menopausal women. Nonetheless the relatively low risk of cardiovascular disease in pre-menopausal women together with the increase following menopause and the possibility that estrogen replacement may mediate against this, highlight the need for an improved understanding of the actions of estrogen within the cardiovascular system.

1.4.1.1 Mechanism of Estrogen's Beneficial Actions

Originally it was thought that estrogen's beneficial actions within the cardiovascular system were due solely to effects on the blood lipid profile. After menopause, women develop a more atherogenic profile with low-density lipoprotein (LDL) levels increasing and high-density lipoprotein (HDL) levels decreasing (Bush *et al.*, 1987; Sacks and Walsh, 1990; Barrett-Connor and Bush, 1991; Grady *et al.*, 1992; Gerhard and Ganz, 1995; Farhat, 1996). The high-density lipoprotein has functions within the body which include lipid transport, carrying fats to cells for use in energy metabolism and membrane construction. They are also believed to prevent cholesterol from settling in the walls of arteries. The low-density lipoproteins, on the other hand, carry cholesterol and fats to tissues for use in manufacturing steroid hormones, but also favour the deposition of cholesterol in arterial walls (Sullivan, 1999; Stevenson, 2000). The ratio of low-density lipoprotein to high-density lipoprotein is very important and has been suggested to influence the risk levels of coronary heart disease. A predominance of low-density lipoprotein is therefore associated with a greater risk of atherosclerotic progression in the vasculature.

It has been well documented that oral estrogen received as part of a hormone replacement therapy scheme increases high-density lipoproteins between 10 and 15%, whilst reducing the levels of low-density lipoproteins (Bush, 1990). Estrogen is thought to suppress hepatic lipase activity, which will cause a rise in high-density lipoprotein levels (Barrett-Connor and Bush, 1991). The Lipid Research Clinic Study found that women receiving estrogen replacement therapy had a 65% reduced risk of coronary heart disease than women not on hormone treatment (Bush *et al.*, 1987; Bush, 1990; Barrett-Connor and Bush, 1991). Two studies since, have indirectly measured the changes in lipoproteins as a function of estrogen's beneficial effects and found that between 25% and 50% of the beneficial effect of estrogen on coronary heart disease risk is through changes in the lipid profile (Bush *et al.*, 1987; Gruchow *et al.*, 1988). This implies that at least 50% of estrogen's beneficial effect is not accounted for by the improved lipid profile (Barrett-Connor and Bush, 1991).

Direct effects of estrogen on the vasculature have also been considered to underlie its cardiovascular protective action with it being thought to modify the functions of the endothelium and vascular smooth muscle. These modifying actions have also been suggested to have protective effects in terms of plaque formation and, therefore,

atherosclerosis (Bush *et al.*, 1987; Gerhard and Ganz, 1995). Estrogen is thought to promote vasodilation in part by stimulating prostacyclin and nitric oxide synthesis; (Farhat *et al.*, 1996; Sullivan, 1999), in addition to which it may protect against vascular injury by inhibiting smooth muscle cell migration and proliferation (Gray *et al.*, 2001). It has also been suggested to have rapid actions which affect membrane events and could contribute to the modulation of intracellular signalling pathways in smooth muscle, particularly those involving calcium (Farhat *et al.*, 1996). Estrogen's protective effects on endothelial function have also been attributed to its antioxidant properties. An increased production of oxygen-derived free radicals in atherosclerosis causes low density lipoprotein oxidation and inactivation of nitric oxide. Estrogen replacement decreases low density lipoprotein oxidation resulting in a beneficial outcome (Farhat *et al.*, 1996; Christ and Wehling, 1998; Sullivan, 1999).

1.4.2 Estrogen Receptors in the Heart

The profound effects of estrogen on the lipid profile and its potential actions within the vascular system and, in particular, the coronary vasculature can explain many of estrogen's beneficial effects in relation to the heart. However, it was the discovery of estrogen receptors within the heart itself by Stumpf *et al.* (1977) which created new possibilities and provided an alternative site of action for estrogen within the cardiovascular system. Stumpf *et al.* (1977) used autoradiographic techniques in rats to demonstrate a nuclear concentration of 17β-[^{3}H] estradiol in atrial cells of the heart, indicating the presence of target cells for estradiol. The notion that the heart contains estrogen receptors was confirmed in a follow-up study, in which McGill Jr *et al.* (1981) demonstrated similar findings in baboons. Their study showed nuclear uptake of 17β-[^{3}H] estradiol in atrial and ventricular myocardial fibers, cardiac interstitial cells and arterial endothelial cells. Although these studies demonstrated radiolabelled binding of estrogen to cardiac tissue, the presence of functional estrogen receptors was still not established. In 1997, the presence of functional estrogen receptors in both cardiac myocytes and fibroblasts was confirmed in an immunoblot and immunofluorescent staining study by Grohe *et al.* (1997). This study demonstrated both the presence of estrogen receptor protein in male and female cardiac myocytes, and nuclear translocation of the estrogen receptor after the addition of 17β-estradiol. In addition to this, it was shown that application of 17β-estradiol resulted in activation of cardiac

specific genes with a significant increase in the expression of the estrogen receptors α and β in cardiac myocytes (Grohe *et al.*, 1997).

Until recently it was thought that the genomic actions of estrogen were carried out through a single high-affinity receptor, α (ERα) which is the common form of the estrogen receptor and is present in many tissues including the gonads, reproductive tract, mammary gland, cardiovascular system and brain (Gray *et al.*, 2001). Expression of a second estrogen receptor subtype, β (ERβ) was found in the rat prostate and ovary (Kuiper *et al.*, 1996). In the adult rat, ERβ has been found in brain, prostate, ovary, lung, bladder and epididymis (Enmark and Gustafsson, 1999). This estrogen receptor sub-type has also been found in adult rat heart highlighting the potential importance of estrogen mediated effects within the heart (Saunders *et al.*, 1997; Gray *et al.*, 2001). The distribution of ERβ is slightly different in humans where the most striking dissimilarity is in the prostate, with very high expression in the rat but very low expression found in the human. Estrogen receptors α and β have approximately 60% homology in their receptor ligand binding domains and are different in many respects including the fact that they are distinct proteins and are encoded by separate genes (Enmark and Gustafsson, 1999; Gustafsson, 1999; Gray *et al.*, 2001). The ERβ protein is able to stimulate transcription of an estrogen receptor target gene in a similar manner to ERα, however in many cell types the degree of activation is lower than that of ERα. It is interesting to note, however, that most of the existing estrogen receptor ligands cannot discriminate between the subtypes, including the physiological estrogen, 17β-estradiol (Gray *et al.*, 2001).

The discovery of the estrogen receptor in the heart is clearly an important breakthrough in determining the way in which this hormone may influence the cardiovascular system. It raises the possibility of a direct effect of estrogen on cardiac muscle which may contribute to gender-based differences that are present in cardiovascular diseases.

1.4.3 Estrogen and Ca^{2+} in the Heart

As has been discussed previously, the gender-based differences in cardiovascular disease have been attributed to the effects of estrogen on several different pathways within the cardiovascular system. The discovery of the estrogen receptor in the heart has led to speculation about direct actions of estrogen on the myocardium, however, the precise nature of these direct actions are yet to be elucidated. Estrogen has also been

suggested to possess calcium antagonistic properties, which have to date been demonstrated in cardiac myocytes and vascular smooth muscle cells (Collins *et al.*, 1996; Rosano and Panina, 1999). These and other studies involving estrogen and its effects on Ca^{2+} movements have led researchers to postulate that estrogen can have both genomic, long-term effects within the heart, as well as short-term, non-genomic effects.

1.4.3.1 Classical Genomic Action

The most common and well accepted mode of action for estrogen within the body is via a genomic pathway. Steroid hormones, such as estrogen, are lipophilic and bind reversibly to the hormone binding domain of highly specific cytoplasmic protein receptors (Gerhard and Ganz, 1995; Pelzer *et al.*, 1997; Gray *et al.*, 2001). The receptor ligand complex translocates into the nucleus of the cell where the estrogen receptor binds to the estrogen response element (ERE) to regulate transcriptional activity of the target gene (Pelzer *et al.*, 1996; Meyer *et al.*, 1998). This changes the behaviour of the gene, activating, suppressing or altering the transcription of mRNA. These interactions lead to changes in protein expression and hence cellular function (Christ and Wehling, 1998; Sullivan, 1999). Because of this complex pathway, the genomic action of estrogen may take up to several hours for target and protein gene expression before the effects can be observed (Pelzer *et al.*, 1997).

Perhaps the most convincing study investigating the genomic effects of estrogen within the heart was that by Johnson *et al.* (1997) which involved the use of the estrogen receptor knockout (ERKO) mouse. The development of this mouse strain was first reported by Lubahn *et al.* (1993), and was used by Johnson *et al.* (1997) to examine the consequences of estrogen receptor removal on the cardiac L-type Ca^{2+} channel. Interestingly, their study was conducted in male ERKO mice rather than female mice. This was because the circulating levels of estradiol and progesterone have shown quite large variation in female ERKO mice, but remain reasonably constant in male ERKO mice. The level of circulating hormones was important to this study because of possible pharmacological effects of estradiol on Ca^{2+} and K^{+} channels. Consequently the stable hormone levels in the male ERKO mouse were felt to give a better indication of genomic effects on Ca^{2+} channels and currents.

Johnson *et al.* (1997) used radioligand binding techniques to demonstrate a 46% increase in binding of the Ca^{2+} channel antagonist isradipine to cardiac membranes in

ERKO mice when compared with control mice, suggesting that the lack of estrogen receptors in the ERKO mice led to increased numbers of Ca^{2+} channels. Measurement of Ca^{2+} channel current by whole-cell patch clamping techniques revealed an increase in Ca^{2+} channel current by 49% in ERKO mice. In addition, the kinetics of activation and inactivation of the Ca^{2+} channel current were not changed, suggesting that the increased Ca^{2+} current in ERKO ventricular myocytes could not be attributed to a reduction in Ca^{2+}-dependent or voltage-dependent inactivation or acceleration of activation (Johnson *et al.*, 1997). Ventricular action potential duration was also measured in an attempt to support earlier Ca^{2+} current findings. Action potential duration measured at half repolarisation was increased 75% in the ERKO mice when compared with control mice. This increase in duration of the action potential was consistent with the observed increase in Ca^{2+} current (Johnson *et al.*, 1997). These findings strongly suggest that estrogen regulates the expression of the cardiac L-type Ca^{2+} channel and acts within the cardiac myocyte to reduce the number of L-type Ca^{2+} channels which, in turn, may limit Ca^{2+} entry into the cell. It has been suggested that the limiting of Ca^{2+} entry could underlie part of the cardioprotective effects of estrogen (Grohe *et al.*, 1996).

In a more recent preliminary study, Mendelsohn (2000) used an ERβ knockout model in mice to investigate the role of estrogen receptors in physiological cardiac function. ERKO male mice were found to have significantly smaller left and right ventricles when compared with wild-type mice. Hemodynamic and echocardiographic measures of cardiac contractility were also investigated with both peak pressure and fractional shortening measured. Both of these measurements demonstrate that the male ERKO mice are hypercontractile when compared with wild-type mice. They concluded an important physiological role for estrogen receptors in the heart (Mendelsohn, 2000).

1.4.3.2 Non-Genomic Action

Recently it has been recognised that some of the actions of estrogen within the heart cannot be attributed to effects of receptor mediated gene expression as they occur in a time-frame that is too rapid for this (Farhat *et al.*, 1996; Pelzer *et al.*, 1997; Christ and Wehling, 1998; Sullivan, 1999; Mendelsohn, 2000). Such non-genomic actions of estrogen do not require *de novo* protein synthesis and can, therefore, occur within seconds of the application of the hormone (Pelzer *et al.*, 1996; Pelzer *et al.*, 1997). The way in which estrogen carries out these non-genomic effects on the myocardium is still unclear, however a small body of evidence suggests that estrogen may be causing rapid

effects in cardiac muscle via a second messenger system involving cAMP and PKA (Buitrago *et al.*, 2000). In addition, it has been suggested that steroids can bind to cell membranes and induce rapid cellular events (Schumacher, 1990; Farhat *et al.*, 1996). The rapid non-genomic membrane effects of steroid hormones are also thought possibly to interact with genomic actions. It has been suggested that the rapid actions of circulating hormones on target neurons are dependent on the long-lasting effects of previous hormone exposures. For example in the brain, the rapid effects of acutely applied 17β-estradiol on preoptic and septal neurons are dependent on prior hormone priming (Schumacher, 1990).

There is a growing body of evidence to support a non-genomic, calcium antagonistic action of estrogen within the cardiovascular system. Electrophysiological and contractility studies provide the main evidence within the heart, with these studies demonstrating that acute application of 17β-estradiol has a negative inotropic effect (Raddino *et al.*, 1986; Sitzler *et al.*, 1996) and inhibits L-type Ca^{2+} current (Jiang *et al.*, 1992; Nakajima, 1995; Grohe *et al.*, 1996; Berger *et al.*, 1997; Meyer *et al.*, 1998) in isolated cardiac myocytes and muscle preparations from rat, guinea-pig and human hearts. The inhibition of the inward Ca^{2+} current would result in a reduced intracellular Ca^{2+} concentration and hence less Ca^{2+} available to initiate contraction. In addition to estrogen's actions on the heart, its calcium antagonistic properties have also been demonstrated in other tissues including vascular smooth muscle (Zhang *et al.*, 1994; Nakajima, 1995; Sheldon and Argentieri, 1995) where estrogen has been shown to inhibit contraction of coronary vascular smooth muscle by inhibiting calcium influx without affecting the calcium sensitivity of the contractile elements (Collins *et al.*, 1996). Similarly in rat neostriatal neurons acute membrane-receptor-mediated inhibition of L-type Ca^{2+} current by estrogen has been shown (Mermelstein *et al.*, 1996).

It is important to point out that the majority of these studies have only shown the calcium antagonistic properties of estrogen using acute application of supraphysiological concentrations of estrogen, usually in the micromolar range. The levels of circulating estrogens within men is approximately 40-60 pM (Contoreggi *et al.*, 1990) and in women the levels are higher at approximately 90 pM to 8 nM (Collins, 1993) but definitely not in the micromolar range. Several reasons have been offered to explain the Ca^{2+} antagonistic effects of 17β-estradiol at pharmacological concentrations. It has been suggested that outside membrane receptors for estrogen may

be damaged during the isolation procedure of cells. In addition it has been speculated that 17β-estradiol needs a cofactor such as a sex hormone binding globulin which is present in blood to allow an effect at lower concentrations. Lastly it is possible that the Ca^{2+} antagonistic actions of 17β-estradiol are due to non-specific binding to a receptor for molecules other than estrogens (Meyer *et al.*, 1998). It is, however, also important to note that the high concentrations of estrogen that have been measured and used may not adequately reflect the concentration of the steroid at the site of action. As estrogen is highly lipophilic it will be more soluble in the phospholipid bilayer than in the aqueous phase, which may result in high concentrations of intracellular estrogen. Therefore even though pharmacological concentrations of estrogen are required to bring about a response in isolated tissues, without complete mechanistic information, estrogen's acute actions should not be considered physiologically irrelevant at this time (Austin, 2000).

The potential for clinical relevance of the reported calcium antagonistic properties of estrogen is highlighted in studies on cardiac disease states. In particular L-type calcium channel antagonists such as nifedipine have been shown to alter the progression of atherosclerosis in animals fed a cholesterol-rich diet (Henry and Bentley, 1981) along with clinical studies which show similar results (Lichtlen *et al.*, 1990; Waters *et al.*, 1990). Estrogen has been shown to possess a similar capacity to nifedipine for inhibiting the progression of atherosclerosis in the coronary arteries of monkeys (Adams *et al.*, 1990). It has been suggested that the calcium antagonistic effects of estrogen, if in existence in the human, may provide a major protective mechanism against the progression of atherosclerotic disease in the coronary arteries of women (Collins *et al.*, 1993).

1.5 Gender Differences in Cardiac Function

The overwhelming epidemiological evidence supporting the existence of gender related differences in the risk of cardiovascular disease led to large interest and subsequent studies using animal models. These animal studies were undertaken in an attempt to elucidate the underlying mechanisms behind the apparent gender based difference, however, they seem to have only added considerable confusion to the body of information as they have resulted in contradictory outcomes. Schaible and Scheuer, (1984) used the isolated working rat heart to show that intrinsic cardiac function is moderately greater in male rats than in female rats. They completed a series of studies

measuring coronary flow, end diastolic volume, stroke work, ejection fraction and fractional shortening in male and female hearts at controlled levels of end-diastolic pressure and aortic pressure. In accordance with this study was that of Leblanc *et al.* (1998) who demonstrated that papillary muscles from female rats older than 6 months showed smaller isometric and isotonic contractions than age-matched males. In opposition to these findings, however, are those of Capasso *et al.* (1983) who reported very different findings, showing that isolated papillary muscles from female rats had greater contractile performance than those from male rats. Wang *et al.* (1998) also reported that female rat atrial and papillary muscles developed greater tension with increasing extracellular Ca^{2+} concentrations than did similar preparations from male rats. In contradiction to all foregoing studies, Brown *et al.* (1996) demonstrated no difference in intrinsic contractile performance between male and female rat hearts. Instead of providing a clear answer as to the possible existence of gender-specific differences in cardiac function these studies have increased the confusion by contributing to the pool of unanswered questions.

1.5.1 Is Estrogen Solely Responsible?

The gender related differences in the incidence of cardiovascular disease have been inextricably linked to estrogen and its effects on the blood lipid profile, the vasculature and the heart. Estrogen appears clearly to be acting cardioprotectively in the female but it is also possible that the sex steroid testosterone may be involved in the apparent gender related differences. It has been shown that there is a decrease in cardiac contractile function in gonadectomised male rats when compared with control rats, with a reduction in myosin ATPase activity associated with a shift from the V1 myosin isoenzyme to the less active V3 isoform (Schaible *et al.*, 1984). In a follow up to this study, Scheuer *et al.* (1987) demonstrated similar findings to those of Schaible *et al.* (1984), however in this later study a testosterone replaced group was added in which increased contractile function back to, or in some cases, slightly above the control groups was observed (Scheuer *et al.*, 1987). Clearly, further investigation of the effects of testosterone on cardiac function is warranted.

1.6 Testosterone and Cardiovascular Disease

The effects of testosterone withdrawal and its replacement in relation to influencing the incidence of cardiovascular disease are not well defined at this point. Whilst a great

deal of attention has been placed on the beneficial effects of estrogen in the cardiovascular system, in contrast a relatively small amount of information regarding testosterone and its effects in the heart exists. The studies that have been undertaken to try to elucidate the effects of testosterone in the heart have resulted in outcomes that are unclear or controversial.

1.6.1 Epidemiological Studies

The evidence that men have a greater incidence of cardiovascular disease when compared with women of a similar age, coupled with the fact that men have an androgenic body fat distribution which is linked to coronary artery disease, led to the belief that high levels of testosterone indicated a predisposition for developing cardiovascular problems (Rosano, 2000). Apart from this circumstantial evidence, no direct proof which links testosterone to coronary artery disease is available (Rosano and Panina, 1999). Originally it was felt that testosterone replacement in male patients was beneficial with early studies reporting positive outcomes in the treatment of angina pectoris in patients with coronary artery disease (Hamm, 1942; Stigler *et al.*, 1943). Given the observation that men with coronary artery disease have lower serum testosterone concentrations than healthy men of similar age and weight (White *et al.*, 1998), the reasoning for giving replacement of testosterone to patients seemed validated. It is well known that testosterone levels decrease with increasing age (Bhasin *et al.*, 1998), suggesting that the increase in the incidence of cardiovascular disease as men get older may be attributable, in part, to decreasing testosterone levels. In accordance with this it appears that low testosterone levels have been linked to many cardiac risk factors (Webb *et al.*, 1999) including hypertension, with an increase in both systolic and diastolic blood pressures in men with low testosterone levels (Phillips *et al.*, 1994), high fibrinogen levels which have been found to be an independent risk factor for myocardial infarction (Caron *et al.*, 1988; Glueck *et al.*, 1993) and obesity (Zumoff *et al.*, 1990). Low levels of testosterone have also been linked to thrombosis and an increased risk of acute myocardial infarction and thrombotic stroke (English *et al.*, 1997). However, although men suffering from cardiovascular disease often have low testosterone levels, studies investigating the link between low testosterone levels and coronary artery disease have been inconclusive with investigators finding no significant association between testosterone levels and the development of cardiovascular disease (Barrett-Connor and Khaw, 1988).

1.6.1.1 Mechanisms of Action

Just as the effects of testosterone in the cardiovascular system are controversial so too are the mechanisms of action for testosterone. The discovery of the importance of the lipid profile in the development of atherosclerosis, coupled with the suggestion that testosterone did not have a favourable influence on the lipid profile in both men and women, soon raised many questions as to the efficacy of testosterone replacement (Jones *et al.*, 1989; Asscheman *et al.*, 1994; Lovejoy *et al.*, 1996; Shapiro *et al.*, 1999). The changes in the lipid profile that are associated with changes in testosterone levels are not fully defined, with conflicting results reported. It seems that men with hypotestosteronaemia have a pro-atherogenic lipid profile with a higher level of LDL cholesterol and a lower level of HDL cholesterol (Rosano, 2000). This would seem to fit with previous findings regarding low testosterone levels and increased incidences of cardiovascular disease. In contrast, however, it has been shown in cynomolgus monkeys treated with testosterone that total and LDL cholesterol increased whilst HDL cholesterol decreased (Weyrich *et al.*, 1992). Cross-sectional and interventional studies looking at the association between androgens and the lipid profile have reported different outcomes. The majority of cross-sectional studies focussing on androgen levels and HDL cholesterol show that testosterone has a positive effect on levels of HDL cholesterol and a negative correlation with LDL cholesterol (Barrett-Connor, 1992; English *et al.*, 1997; Simon *et al.*, 1997; Rosano, 2000). In addition the majority of cross-sectional studies have reported an association between hypotestosteronaemia and cardiovascular morbidity (Rosano, 2000). In contrast studies involving testosterone administration in young or hypogonadal men show a decrease in HDL cholesterol (Bagatell and Bremner, 1995). The changes in HDL and LDL profiles are also dependent on the type of testosterone used, with non-aromatisable androgens causing profound decreases in HDL levels and an increase in LDL levels (Bagatell and Bremner, 1995). It is important to recognise in the controversy that surrounds the positive or negative effects of testosterone on the heart that the use of physiological concentrations of testosterone versus supraphysiological concentrations and the type and route of administration of androgens can profoundly affect the outcome of each study.

Testosterone has also been shown to have acute effects on the vascular system, where it can induce relaxation in the aorta and coronary arteries (Yue *et al.*, 1995; Webb *et al.*,

1999), but has also been shown to facilitate vasoconstriction (Masuda *et al.*, 1991; Schror *et al.*, 1994; Rubio *et al.*, 1998; Ceballos *et al.*, 1999). In association with this it has been suggested that androgens may affect the release of nitric oxide from the vascular endothelium (Green *et al.*, 1993). Effects on the fibrinolytic system have also been suggested, with low levels of testosterone associated with high fibrinogen levels, and administration of exogenous testosterone causing these levels to fall (Anderson *et al.*, 1995; English *et al.*, 1997). Several studies investigating the role of testosterone in hypertension have found a relationship between low levels of testosterone and raised systolic and diastolic blood pressures (Cauley *et al.*, 1987; Barrett-Connor and Khaw, 1988; English *et al.*, 1997; Simon *et al.*, 1997).

It has been suggested that replacement in males of normal physiological levels of testosterone (12 to 35 nmol/L) may protect against cardiovascular disease, whilst supraphysiologic levels may, however be associated with undesirable changes to lipid profiles and increased risk of myocardial infarction (Shapiro *et al.*, 1999). A normal physiological level of testosterone may protect men against hyperlipidemia, hypertension, obesity and other risk factors associated with coronary artery disease (English *et al.*, 1997). This idea is, however, purely speculative with a larger body of evidence needed before firm conclusions can be drawn.

1.6.2 Androgen Receptors and the Heart

The effects of androgens on cardiovascular disease have been mainly attributed to actions on the lipid profile and changes to the vasculature, however, the discovery that androgen receptors are present within the heart suggests that androgens may also affect cardiac function directly. Krieg *et al.* (1978) found specific androgen receptor protein which was physicochemically the same as the receptor protein found in various androgenic target organs. Two years later, McGill Jr *et al.* (1980) demonstrated androgen receptors in atrial and ventricular myocardial cells from baboons by both autoradiography and biochemical analyses. More recently, Marsh *et al.* (1998) has reiterated and extended this finding to conclude that androgen receptors are present in cardiac myocytes from many different species (including the human) and that these receptors can modulate the cardiac phenotype and are, therefore, functionally active. They further showed that they could produce hypertrophy by direct receptor-specific mechanisms. The androgen receptor antagonist cyproterone was used in this study and,

when added, abolished the hypertrophic response which showed that the hypertrophic effects of testosterone are mediated specifically by the hormone binding to the androgen receptor. It seems the presence of a functional receptor for androgenic steroids would indicate a role for testosterone within the heart. The exact nature of this role has not been elucidated and requires further investigation.

1.6.3 Testosterone and Calcium in the Heart

The discovery of androgen receptors in the heart has lead to speculation that testosterone may be having direct actions on the heart itself. Several studies have mentioned some similarities between estrogen and testosterone including the idea that they both seem to be able to act via genomic and non-genomic pathways in the heart and also both possess Ca^{2+} antagonistic properties (Zhang *et al.*, 1998; Reckelhoff *et al.*, 1999). In particular, testosterone has been shown to have Ca^{2+} antagonistic effects on coronary vasoconstriction. In coronary arteries of castrated male pigs and male rats, testosterone was shown to cause coronary relaxation by inhibiting Ca^{2+} entry, but also by inhibiting other mechanisms independent of this pathway (Crews and Khalil, 1999a; Crews and Khalil, 1999b). This effect of testosterone has also been shown in isolated coronary vascular smooth muscle cells. The testosterone-induced relaxation of these cells and decrease of $[Ca^{2+}]_i$ was mainly attributed to inhibition of Ca^{2+} entry from the extracellular space, but not inhibition of Ca^{2+} release from intracellular stores (Murphy and Khalil, 1999). Testosterone has also been shown to have a negative inotropic action in guinea-pig papillary muscle. But this negative action was only demonstrated at supraphysiological levels of testosterone (Zhang *et al.*, 1998). Rubio *et al.* (1998) also demonstrated effects of testosterone in isolated, perfused rat heart which they attributed to a non-genomic pathway. They showed testosterone blockade of adenosine-induced vasodilatation as well as partial testosterone blockade of bradykinin-induced increases in $[Ca^{2+}]_i$. As they perfused testosterone in periods no longer than one hour they concluded that the effects that they were seeing were of non-genomic origin and the testosterone might be interacting with, or modulating membrane receptors.

Apart from the effects mentioned above in relation to the possible genomic modulation of Ca^{2+} channels in the heart by testosterone and estrogen, both estrogen and testosterone can modulate ion channels in other tissues as well. Estradiol has been shown to increase the expression of Ca^{2+} channels in several different tissues including

uterine smooth muscle (Heyward and Clarke, 1995), pituitary cells (Ritchie, 1993) and ovine gonadotrophs (Batra, 1987). Testosterone has been shown to regulate gonadotropin-releasing hormone (GnRH)-induced calcium signals in male rat pituitary gonadotrophs (Tobin and Canny, 1998). Findings from this study suggest that the effects of testosterone on GnRH-induced Ca^{2+} signalling in intact male rats are mediated preferentially by the metabolite of testosterone, dihydrotestosterone. In addition this study also suggested that in the absence of androgens, estradiol may regulate GnRH-induced Ca^{2+} signalling in the male rat pituitary (Tobin and Canny, 1998).

1.7 The Sex Steroid Hormones

The sex steroid hormones estrogen and testosterone are derived from cholesterol and are produced in the ovary and the testis respectively (Brook, 1996). The majority of testosterone synthesis occurs in the interstitial Leydig cells and the hormone is produced by a common pathway of steroidogenesis via several different steps involving numerous intermediates including cholesterol and pregnenolone. A small amount of testosterone is also produced in the adrenal cortex (Rommerts, 1998). The Leydig cells do not store testosterone, but do store large amounts of the precursor (androstenedione) in the form of lipid droplets. Generally testosterone requires further metabolism to dihydrotestosterone by the microsomal enzyme 5α-reductase before it acts at its other target sites. It is, therefore, generally dihydrotestosterone that binds to the hormone binding domain of the androgen receptor to elicit the response (Brook, 1996).

Synthesis of estradiol occurs in the ovary and its production is cyclical. The precursors of estradiol are androstenedione and testosterone which are aromatised to yield estrone and estradiol (Dubey and Jackson, 2001). Estradiol can be converted into a large number of different metabolites via diverse pathways. Some of these metabolites include catecholestradiols and methoxyestradiols, and have been shown to be less active estrogens, although still biologically active. These metabolites are water soluble and able to be excreted in the urine (Dubey and Jackson, 2001). 17β-estradiol is the most biologically active form of estradiol and it is primarily this form that binds to the hormone binding domain of the estrogen receptor to elicit a response.

As mentioned above, estrogen is produced cyclically as part of the ovarian cycle. The cycle varies in length between species, with humans having a 24 -32 day menstrual

cycle, sheep a 16-17 day estrous cycle, whilst rats have a 4-5 day estrous cycle (Johnson *et al.*, 1995). The main differences between the human menstrual cycle and the rat ovarian cycle is the timing of events and the key events regulating ovarian function (Driancourt *et al.*, 1993). There are four different phases of the estrous cycle in the rat. These are estrous (when ovulation occurs), metestrous, diestrous and pro-estrous. Pro-estrous is the preparatory phase and is usually around 12 hours in duration and is characterised by surges in progesterone, prolactin and estradiol. Estrous follows and the high surge of hormones seen in pro-estrous drop back to almost basal levels. Metestrous follows estrous and is characterised by a slow build-up of estradiol and progesterone and usually lasts approximately 6 hours. Diestrous shows slightly elevated estradiol but is generally characterised as a non-hormonal phase which lasts approximately 60 hours (Driancourt *et al.*, 1993).

1.7.1 The Role of Sex Steroids Within the Body

Sex steroids are transported around the body in the circulatory system both freely and attached to binding proteins such as the sex hormone binding globulin (SHBG) and albumin. For example it has been reported that more than 90% of plasma estrogen is bound to either plasma albumin or sex hormone binding globulin (Kurata *et al.*, 2001). Protein-bound hormone is in equilibrium with unbound hormone. The free hormone can diffuse to tissues more readily and so the physiological state usually corresponds more closely with the concentration of the free hormone (Brook, 1996). The free steroid hormones are relatively lipophilic and therefore have direct access to the cell interior via the plasma membrane. Once the free steroid diffuses across the target-cell membrane it binds to the steroid hormone-receptor complex which can occur in the cell cytoplasm or in the cell nucleus. This complex binds to a receptor site on the DNA strand of a particular gene which sets off a cascade of events which ends in modification of a protein to give the final gene product (Evans, 1988). One thing to consider with this process is that there is a relatively long time period of 30-60 minutes, or longer, between the time of exposure of the target cell to the hormone and the onset of a biological response.

The receptors for estrogen and testosterone are present in a wide variety of cells and organs. Apart from the obvious sites in the reproductive tissues, these hormones have been shown to target the central nervous system, liver, bone, gastrointestinal tract and

the cardiovascular system (Gustafsson, 1999). The diversity of the interactions that these hormones have within the body highlights their importance, and also shows their potential in terms of beneficial or negative effects within the cardiovascular system.

1.8 Gender, Calcium Movements and the Heart

The reduced incidence of cardiovascular disease in pre-menopausal women when compared with age-matched men, coupled with the fact that the reduction in risk is lost when menopause is reached, suggests an influence of sex hormones on the development of cardiovascular disease. A large body of evidence exists which suggests that estrogen is a major factor in the reduced risk in pre-menopausal women, with effects on the systemic system and the vasculature. The discovery of the estrogen receptor within the heart has led to speculation that estrogen may also be acting directly on the heart itself to bring about its beneficial actions. Estrogen's Ca^{2+} antagonistic properties have also been highlighted as a possible mechanism by which estrogen has a beneficial effect. In addition to this, a small body of work has suggested that the gender difference in the incidence of cardiovascular disease may result from a combination of the effects of both estrogen and testosterone. Although a much smaller and conflicting body of evidence exists regarding testosterone's effects within the heart, it has been suggested that it too possesses Ca^{2+} antagonistic actions, and also could be beneficial in regards to the development of cardiovascular disease.

Clearly Ca^{2+} is a major factor in the normal day to day functioning of the heart. If estrogen and or testosterone can positively influence this ion, they have the potential to play a major role in the development or prevention of cardiovascular disease.

It was, therefore, the broad aims of this body of work to:

1. Establish if a gender specific difference is apparent in the Ca^{2+} handling of cardiac myocytes, and
2. Ascertain if this difference is due to the sex steroids estrogen and testosterone, by surgically removing the source of these hormones from the animals in question, and also exogenously replacing these hormones.

Chapter 2

Chapter 2

Methodology

The main experimental protocols and solutions that were used to complete the majority of experiments for this thesis are detailed within this chapter. Separate protocols which relate only to specific sections of the thesis will be detailed in the relevant chapter. All experiments undertaken to complete this thesis complied with the guidelines of the National Health and Medical Research Council of Australia on the care and use of animals in research and had the approval of the Monash University Animal Ethics Committee (Ethics approval number 99/032).

2.1 Experimental Model

All studies reported within this thesis were conducted using male and female rats (*Rattus norvegicus*) of the Wistar strain. All rats were in-bred by Monash University Animal Services. Male and female Wistar rats were housed separately and given free access to food (standard laboratory rat chow) and water. At the time of the experiments they were aged between 12 and 18 weeks. Experiments were performed randomly with female rats used at random times during their estrous cycle.

The experimental preparation that was predominantly utilised throughout this thesis was the freshly isolated single cardiac myocyte. Isolated adult cardiac myocytes have been a useful tool for investigators for several decades. Since the early work of Cavanaugh (1955) who used trypsin digestion of embryonic heart fragments to isolate single viable cardiac myocytes, dramatic improvements in isolation techniques have been developed. Initially successful isolation of myocytes could only be achieved in newborn animals, with the yield and viability of myocytes falling rapidly if taken from hearts of animals beyond five days of age (Harary and Farley, 1960). The first reports of isolation of myocytes from adult animals were by Kono (1969) and Berry *et al.* (1970) however, these early preparations were not tolerant to physiological concentrations of Ca^{2+}. Many modifications to the protocol used for cell isolation were undertaken to try to establish a technique that would yield Ca^{2+} tolerant cardiac myocytes. The understanding of the Ca^{2+} paradox was one of the crucial factors in establishing techniques for isolating Ca^{2+} tolerant myocytes. Clark *et al.* (1978) and Kao *et al.* (1980) along with Farmer *et al.* (1983) were some of the first groups to define a protocol to isolate Ca^{2+} tolerant myocytes. In the study of Farmer *et al.* (1983), they provided solid evidence for the Ca^{2+} tolerance of isolated myocytes by showing cells with structure that closely resembled myocytes of intact tissue, along with scanning electron micrographs showing intact internal structures within the myocyte. Since this discovery others have shown that single isolated myocytes from both rat and rabbit retain the systolic and diastolic contractile properties of intact muscle (Capogrossi *et al.*, 1986).

The advantages of the isolated myocyte preparation are firstly, that a high yield of cells during one isolation allows for multiple protocols to be performed on the one tissue with internal controls. Secondly, the membranes, hormone receptors, enzymes and contractile proteins are all intact and functioning in the same manner as in the intact tissue. Isolated cardiac myocytes are ideal for biochemical, *in vitro* pharmacological studies, electrophysiological studies as well as for morphological studies such as examination with scanning electron microscopy (Farmer *et al.*, 1983). Isolated myocytes have been used in a variety of experimental techniques and have proven to be an exceptionally useful tool for examining individual cellular processes.

2.1.1 Cell Isolation Procedure

Rats were killed by decapitation under deep chloroform anaesthesia. The heart was rapidly excised and washed in HEPES-buffered Krebs-Henseleit (K-H) solution (composition given in Section 2.2) containing heparin at 37°C. Following removal of the pericardium and other adhering tissue, the heart was cannulated via the aorta onto a Langendorff perfusion apparatus. Once the heart was secured on the perfusion apparatus, it was perfused at constant pressure with HEPES-buffered Krebs-Henseleit solution containing 1.5 mM Ca^{2+}. The solution was oxygenated with 100% O_2 and maintained at 37°C. The heart was perfused with the Ca^{2+} containing solution until a constant heart rate was attained. This helped to wash out any residual blood remaining in the coronary blood vessels. Once a steady heart beat was attained, this solution was replaced with a nominally Ca^{2+}-free HEPES-buffered Krebs-Henseleit solution to arrest mechanical activity. Once the heart had stopped beating, perfusion with the Ca^{2+}-free medium was continued for approximately three minutes. The Ca^{2+}-free perfusion washes out residual extracellular Ca^{2+} and begins to loosen structural connections within the extracellular matrix and weakens the intercellular connections of the intercalated disks between the myocytes (Stemmer *et al.*, 1992).

Following this initial period of Ca^{2+}-free perfusion, collagenase (Worthington; Type II) was added to the perfusate to a concentration of 120-150 IU/ml, and the Ca^{2+} concentration was increased to 50 μM. Cardiac myocytes are bonded together via a strong connective tissue network comprised mainly of Type I and II collagen fibers, as well as unknown extracellular matrix components (Borg *et al.*, 1984). Collagenase from *Clostridium histolyticum* is commonly used in myocyte isolation because of its ability to digest connective tissue and break down the collagen network connecting the cells (Stemmer *et al.*, 1992).

After 10-20 minutes of collagenase perfusion the heart was taken down from the perfusion apparatus and the atria and right ventricular free wall were removed. The remaining left ventricular tissue was teased apart with forceps and placed in a 50 ml conical flask to which 10 ml of the collagenase solution was added. The tissue mixture was placed in a shaking water bath (37°C) where myocytes were dispersed by gentle agitation for 5 minutes. Collagenase solution containing dispersed cells was decanted off and another 10 ml of collagenase solution was added to the remaining tissue and agitated for a further 7 minutes. This process was repeated 6-7 times.

As the supernatant from each fraction was collected, a small drop was placed onto a coverslip and viewed under a microscope to determine cell viability. Once the fractions with the highest proportion of viable cells were determined, they were filtered through a thin nylon gauze (~ 200 μm mesh) and then centrifuged for 2 minutes at ~ 60 × g. The supernatant was pipetted off and the cell pellet resuspended in 2-3 ml of HEPES-buffered Krebs-Henseleit solution containing 1 mM Ca^{2+} and 2.5% bovine serum albumin (BSA). They were gently shaken at room temperature in this solution until used. The cells were used within 1-6 hours following digestion of the heart.

2.1.1.1 Cell Viability

The ability to select viable cells is an integral step in the success of an experiment. The capacity to be able to select a few cells visually for an experiment means that the whole population of cells does not need to be viable, only a proportion of them need to be acceptable. It has been suggested that if the dispersed cells are greater than 45% rod-shaped before viable cell selection, then the preparation is deemed a good one (Stemmer *et al.*, 1992). Viable cells were considered to be those that were rod-shaped and elongated, with regular sarcomere spacing, and not spontaneously contracting. Cells which are rounded in appearance or contract spontaneously are considered to have membrane damage allowing excess Ca^{2+} into the cell and hence resulting in Ca^{2+} overload. Membrane damage generally occurs as a result of over digestion by collagenase, as well as damage at the region of the intercalated discs. This damage increases the sarcolemmal permeability to ions, especially Ca^{2+}, and results in Ca^{2+} entry, not linked to depolarisation by an action potential, into the cardiac myocyte. This excess Ca^{2+} can trigger focal release of Ca^{2+} from the SR which propagates through the cell as a contractile wave. Therefore these outward visible signs give a good indication of a compromised myocyte. Figure 2.1 shows examples of cardiac myocytes in three different stages of viability. The top panel shows an example of a viable cell which would be selected to be used for experimental recordings. On each end are the edge detector markers used to track the length changes of the cell (see Section 2.4.3). The middle panel shows a cell that is in the first stage of Ca^{2+} overload. This is apparent from its grainy appearance and also the rounded ends. The bottom panel illustrates a Ca^{2+} overloaded cell, which is dead. A full grainy appearance is now apparent along with a complete lack of structural definition.

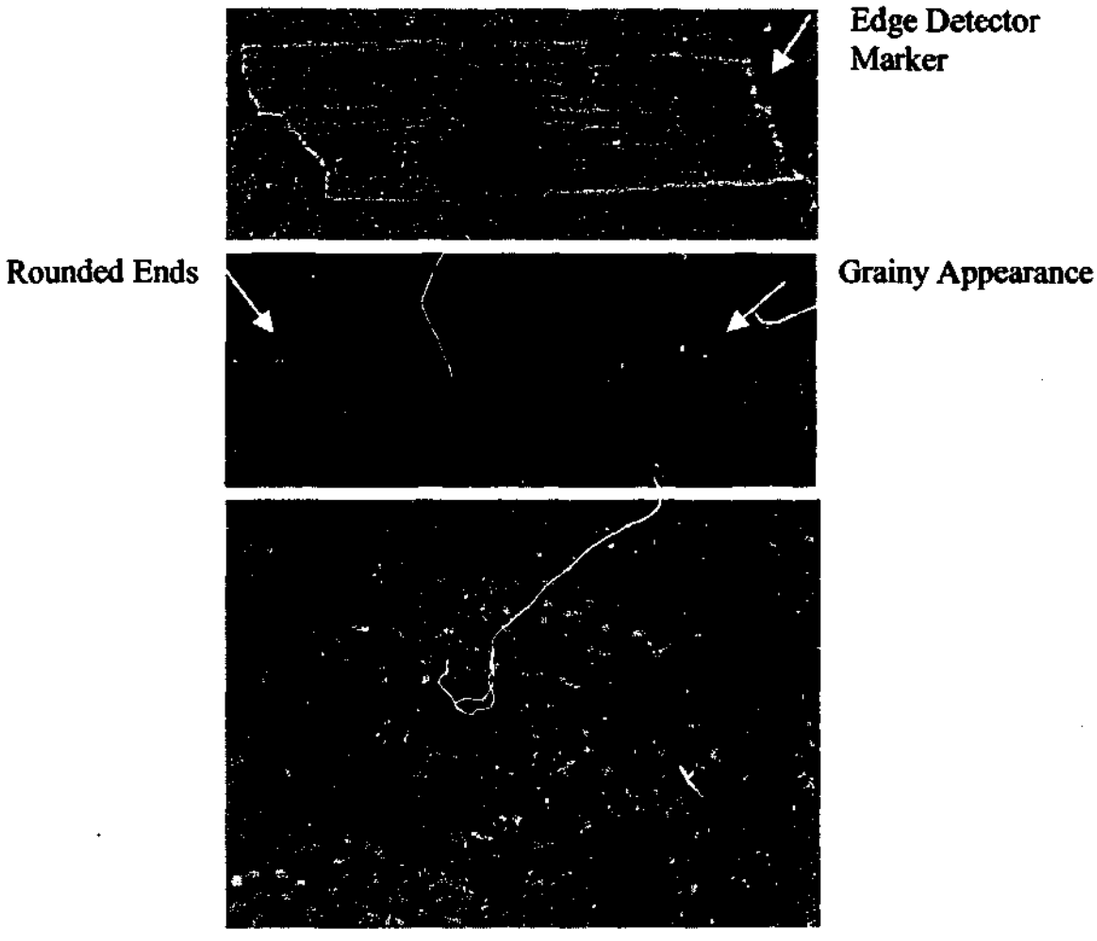


Figure 2.1: Examples of different levels of viability of cardiac myocytes. The top panel shows a viable healthy myocyte, the middle panel shows a myocyte which has the signs of initial stage Ca^{2+} overload, and the bottom panel shows a Ca^{2+} overloaded dead myocyte.

The success of the isolation procedure, as evidenced by the proportion of viable cells that did not become Ca^{2+} overloaded, was a major factor in carrying out the studies reported in this thesis. Excessive permeability to Ca^{2+} is believed to underlie the Ca^{2+} overload seen in cells that do not survive the isolation procedure and this involves, in part, the Ca^{2+} paradox. The Ca^{2+} paradox comes about as a result of exposure of the heart to a period of Ca^{2+} free perfusion. During this time the sarcolemmal permeability to Ca^{2+} increases and when physiological concentrations of Ca^{2+} are reintroduced to the preparation, following the Ca^{2+} free period, a large influx of Ca^{2+} occurs. If the cell's Ca^{2+} uptake and extrusion mechanisms are unable to compensate for this and maintain a sufficiently low cytoplasmic Ca^{2+} concentration the cell will go into contracture, and ultimately die (Stemmer *et al.,* 1992). As mentioned above, a period of Ca^{2+}-free perfusion is necessary to loosen intercellular bonds at the intercalated disks. This could make the cells susceptible to the Ca^{2+} paradox and, together with possible compromise

of membrane integrity as a result of the collagenase digestion, lead to subsequent Ca^{2+} overload when the cells are resuspended in 1.0 mM Ca^{2+} containing solution.

In the myocytes that went into full Ca^{2+} overload, the time over which this occurred varied greatly from cell to cell, but could occur in a very short time. An example of this is shown in Figure 2.2 which illustrates a cell progressing to Ca^{2+} overload in a period of less than 15 seconds. The numbers in the top right corner of each panel indicate the date and time, with the two digits on the rightmost side referring to changes in seconds. In the top left panel, the cell has normal appearance and is not displaying any signs of Ca^{2+} overload. In the top right panel, a small amount of rounding and curling of the ends of the cell is apparent. Following this it is clear that the cell is Ca^{2+} overloaded and it takes only a matter of seconds for it to hyper-contract and die.

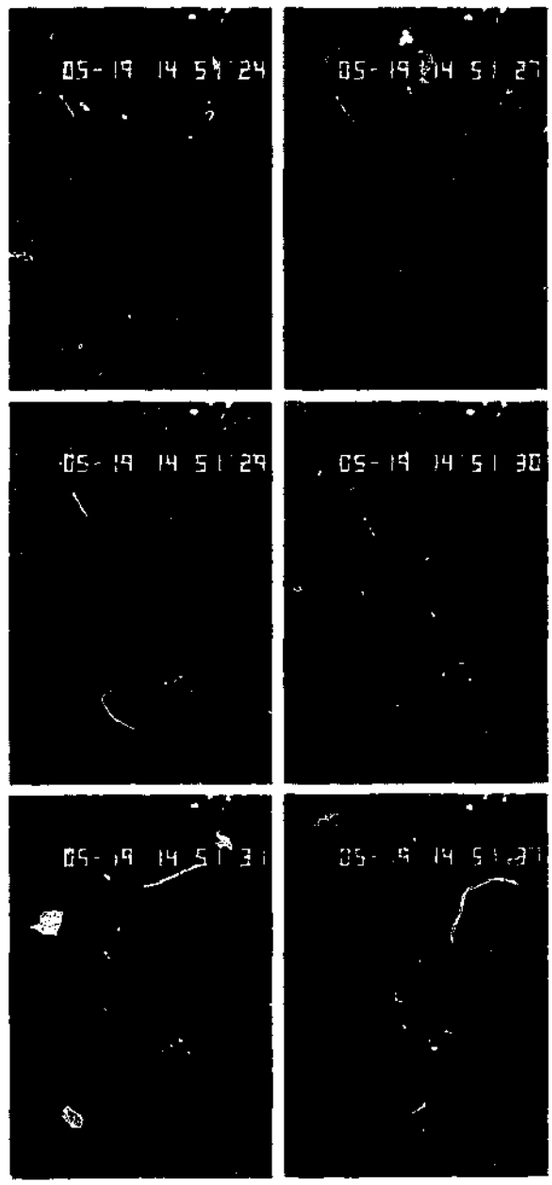


Figure 2.2: Example of a cell during transition to Ca^{2+} overload and subsequent hypercontracture.

2.1.1.2 Collagenase

Collagenase, Type II (Worthington) was used for isolating heart cells. This collagenase is not highly purified and concentrations of contaminating enzymes can vary quite considerably from batch to batch. It therefore follows that different batches of collagenase yielded different success rates in the cell isolation procedure, in that different levels of viable cells were achieved with each different batch used. In order to maximise the yield of viable cells, a sampling program was undertaken to ascertain the most appropriate batch of collagenase for experiments. When undertaking a round of the sampling program, 4-5 different batches of Type II collagenase would be tested. In some cases none of the batches were particularly successful and a new round of the sampling program would be undertaken. During the course of the thesis, the sampling program was undertaken 3 separate times in an attempt to find the most suitable batches of collagenase.

Collagenase with a slightly different composition to that used for male rats had to be utilised for female rats in order to obtain a similar number of viable cells. In addition, the batches of collagenase had to be changed for both the ovariectomy and the gonadectomy studies to maintain a reasonable level of viable cells. The composition of the different batches of collagenase are detailed in Table 2.1 as follows:

Table 2.1: Collagenase, caseinase, clostripan and tryptic activities, shown in units per mg dry weight, of the different batches of collagenase used throughout these studies.

Type of Collagenase	Collagenase Lot Number	U/mg dry	Yield (g)	U/mg dry	U/mg dry
Type II	M7P1445	228	817	4.4	0.13
Type II	M7S1551	255	605	5.2	0.12
Type II	S6M597	219	890	4.4	0.21
Type II	49K3232	305	766	5.3	0.26

When isolation of cells from female rats was attempted initially, with the collagenase that was successfully used for male rats, the yield of viable cells was low. A similar circumstance was apparent in the ovariectomised female groups and in the

gonadectomised male groups. It has been reported that female hearts have a significantly higher level of mRNA for collagenase Type I when compared with the male heart and that these differences are due to regulatory effects of male and female specific hormones (Rosenkranz-Weiss *et al.*, 1994). These differences may underlie the apparent variation in the effectiveness of particular batches of collagenase in the two genders, in particular in response to changes in hormonal status of the animals.

2.1.1.3 Modifications to Isolation Procedure

At various times throughout the course of this investigation modifications to the isolation procedure were necessary to maintain a reasonable yield of viable cells. For reasons that were not clear and were very frustrating, it was found that at different times throughout the course of experiments variable yields of cells would become apparent. As mentioned earlier, one of the greatest problems when isolating cells is the occurrence of Ca^{2+} overload. Because it is necessary to subject cells to a Ca^{2+}-free perfusion period in order to get proper breakdown of the material between the cells, there is a great risk that cells will undergo the Ca^{2+} paradox, and will be at risk of becoming excessively permeable to Ca^{2+}. In effect one of the key features of a good cell isolation procedure is to maintain a protocol which minimises the likelihood of the cells undergoing the Ca^{2+} paradox.

In an attempt to improve the yield of viable cells, the amino acid taurine was firstly added to the perfusate in an attempt to protect the cells against Ca^{2+} overload. Taurine is a naturally occurring amino acid which has been shown to have a normalising influence on contractility in the heart, by affecting various ion transport pathways across both the sarcolemma and the SR (Holloway *et al.*, 1999). It has also been shown by several groups that taurine may help protect against the Ca^{2+} paradox (Clark *et al.*, 1978; Kramer *et al.*, 1981; Chapman *et al.*, 1993). Kramer *et al.* (1981) showed that the presence of taurine protected against the loss of mechanical function resulting from the Ca^{2+} paradox in hearts from rats. Taurine was added to the perfusate at a concentration of 20 mM in some of the experiments presented in this thesis, however, this resulted in only limited success in terms of improving the yield of viable cells.

2,3- butanedione monoxime (BDM) which is known to arrest mechanical activity and protect against tissue cutting injury in the heart (Schwinger *et al.*, 1994; Kiriazis and Gibbs, 1995) was also employed in an attempt to increase the yield of successfully

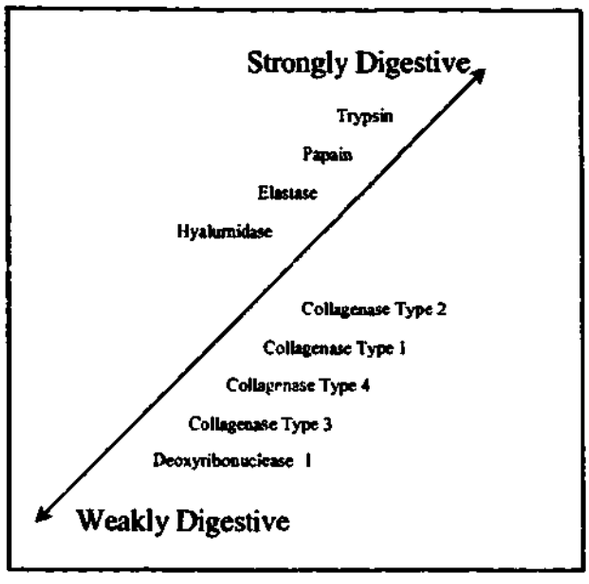
isolated cardiac myocytes. BDM has been shown to have cardioprotective properties and has been suggested to protect against the calcium paradox (Sellin and McArdle, 1994). It is also thought to reduce the contracture that is normally a consequence of damaging muscle cell membranes (Mulieri *et al.*, 1989). The mechanisms by which BDM exerts its protective actions are still unclear, however, it has been associated with a reduction in intracellular calcium and the metabolic demands of the cell (Kivisto *et al.*, 1995). Since BDM readily washes out and leaves no permanent or adverse effects on the cardiomyocytes, it is potentially an effective tool in the protection of cardiac myocytes against calcium overload, spontaneous behaviour, and ultimately cell death. When BDM was used in the cell isolation procedure it was present at a concentration of 25 mM in each of the Ca^{2+}-free, collagenase, shaking and incubation solutions. Prior to making recordings, BDM was fully washed out. At times where the yield of viable cells had become low when using the standard isolation procedure, the addition of BDM described above generally led to an increase in the yield of viable cells.

In addition to using BDM, trypsin inhibitor was added to the collagenase perfusion to increase the yield of cells. Trypsin is a relatively non-specific and active protease which has been shown to be detrimental to many cell functions (Stemmer *et al.*, 1992). Included amongst its detrimental effects is the fact that trypsin has been shown to destroy hormone receptors in other cell types (Ives *et al.*, 1978). In addition, trypsin is one of the most strongly digestive enzymes, with activity far above that of any type of collagenase, as shown in Figure 2.3. Trypsin inhibitor from soybean inactivates trypsin on an equimolar basis and was, therefore, employed to help counteract the negative effects of the trypsin which occurred in the collagenase mixture.

A general overview of the modifications to the cell isolation protocol is shown in Table 2.2. In addition, the different groups of animals have been listed and the modifications utilised with each group have been identified.

Table 2.2: Description of the different modifications to the cell isolation procedure. The ✓and × refer to the presence or absence of this modification in the protocol.

	Initial Perfusion	Ca^{2+}-free Perfusion	Collagenase Perfusion	Tissue Dissociation Solution	Cell Storage Solution	Experimental Groups Utilised
Standard Protocol	1.5 mM Ca^{2+} HEPES buffered K-H Solution 10 - 15 mins	Ca^{2+}-free HEPES buffered K-H Solution 3 - 5 mins	50 µM Ca^{2+} HEPES buffered K-H Solution & 120 IU/ml Collagenase 10 - 20 mins	50 µM Ca^{2+} HEPES buffered K-H solution & 120 IU/ml Collagenase 7 mins × 7	1 mM Ca^{2+} HEPES buffered K-H & 2.5% BSA 2-3 ml 4 - 7 hours	Intact male and intact female animals
+ Taurine	✓	✓	✓	✓	✓	Intact male and intact female animals
+ Trypsin Inhibitor	×	×	✓	✓	×	Sham female and OVX female animals
+ BDM	×	✓ Only after heart had stopped beating	✓	✓	✓	Sham female and OVX female animals
+ BDM & Trypsin Inhibitor	×	✓ No Trypsin Inhibitor. BDM: Only after heart had stopped beating	✓	✓	✓ BDM only	Sham female, 17β-estradiol replaced female, OVX female, sham male, testosterone replaced male and GDX male animals



Modified from Worthington Tissue Dissociation Guide.

Figure 2.3: Enzyme Digestion Scale. Trypsin is the most strongly digestive enzyme on the scale. Thus employing trypsin inhibitor reduces the risk of over digestion of cells.

2.2 Solutions and Reagents

The standard physiological saline solution used during these experiments was a HEPES-buffered Krebs-Henseleit (K-H) solution. It had the following composition (mM): NaCl, 130; KCl, 4.75; KH_2PO_4, 1.18; $CaCl_2$, 1.5; HEPES (N-[-2-Hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]), 10; glucose, 10. The solution was initially prepared Ca^{2+}-free by simply omitting the $CaCl_2$ and, when required, solutions with different Ca^{2+} concentrations were prepared by addition of the appropriate amount of $CaCl_2$. The solutions were equilibrated with 100% O_2 and the pH was adjusted to 7.4 with HCl. Solutions were prepared on the day prior to each experiment and stored overnight at 4°C. The solutions used for the Langendorff perfusion of the heart were filtered through a 0.2 μm Millipore filter prior to their use. This ensured the removal of any colloidal material that may have led to occlusion of small vessels and subsequent inadequate tissue perfusion.

All chemicals used in the preparation of the HEPES-buffered K-H solution were supplied from Sigma (St. Louis, MO, USA). In addition, both taurine and BDM were also from Sigma. Collagenase Type II and soybean trypsin inhibitor were produced by Worthington Biochemical (New Jersey, USA). Bovine Serum Albumin was purchased from CSL (Vic, Australia).

2.3 Measurement of Intracellular [Ca^{2+}]

2.3.1 Ca^{2+} Detection with Fura-2

Intracellular [Ca^{2+}] was monitored using the Ca^{2+}-sensitive fluorescent dye fura-2. Fura-2 is widely employed for measurements of intracellular Ca^{2+} and a number of detailed reviews describing the technique have appeared (Wier *et al.*, 1988; Moore *et al.*, 1990; Bers, 1996; Silver, 1998). Fura-2 is a tetra-carboxylate dye with a single Ca^{2+} binding site similar to that of the high affinity Ca^{2+} chelator EGTA (ethyleneglycol-bis(βaminoethylether) N,N,N',N'-tetra acetic acid). This site allows binding of a single Ca^{2+} ion (1:1 stoichiometry), and with this binding comes an alteration in the spectral characteristics of the molecule (Williams, 1987). The Ca^{2+}-free, unbound form of fura-2 has an excitation maximum at 363 nm whilst the Ca^{2+}-bound form has an excitation maximum at 335 nm. The emission maximum of both forms is at approximately 510 nm. Fura-2 is highly specific for Ca^{2+}over other divalent cations and its Ca^{2+} binding characteristics allow for the accurate detection of Ca^{2+} concentrations in the physiological range of 1 nM to approximately 2 μM.

Two distinct excitation wavelengths are used to measure Ca^{2+} concentrations using fura-2. 340 nm and 380 nm are the wavelengths most commonly used (Williams, 1987). When excited with light at 340 nm, the fluorescence emission of fura-2 increases with bound Ca^{2+}, whilst the fluorescence at 380 nm excitation decreases. The increase and decrease in fluorescence intensity at the respective excitation wavelengths is proportional to the amount of Ca^{2+} bound to the fura-2. It is the ratio of the fluorescence at 340 nm excitation to that at 380 nm excitation ($R_{340/380}$) which is an indicator of the free Ca^{2+} concentration. An example of the recordings of emitted fluorescence from a fura-2 loaded cardiac myocyte at the excitation wavelengths of 340 and 380 nm is shown in Figure 2.4, together with the calculated ratio of these signals. This ratiometric method of monitoring $[Ca^{2+}]_i$ has the advantage of minimising the effects of artifacts that are unrelated to changes in Ca^{2+} concentration that might influence the individual fluorescence signals (Kao, 1994). It also makes possible the calibration of the fluorescence signals into actual Ca^{2+} concentrations without the need to know exact concentrations of the dye.

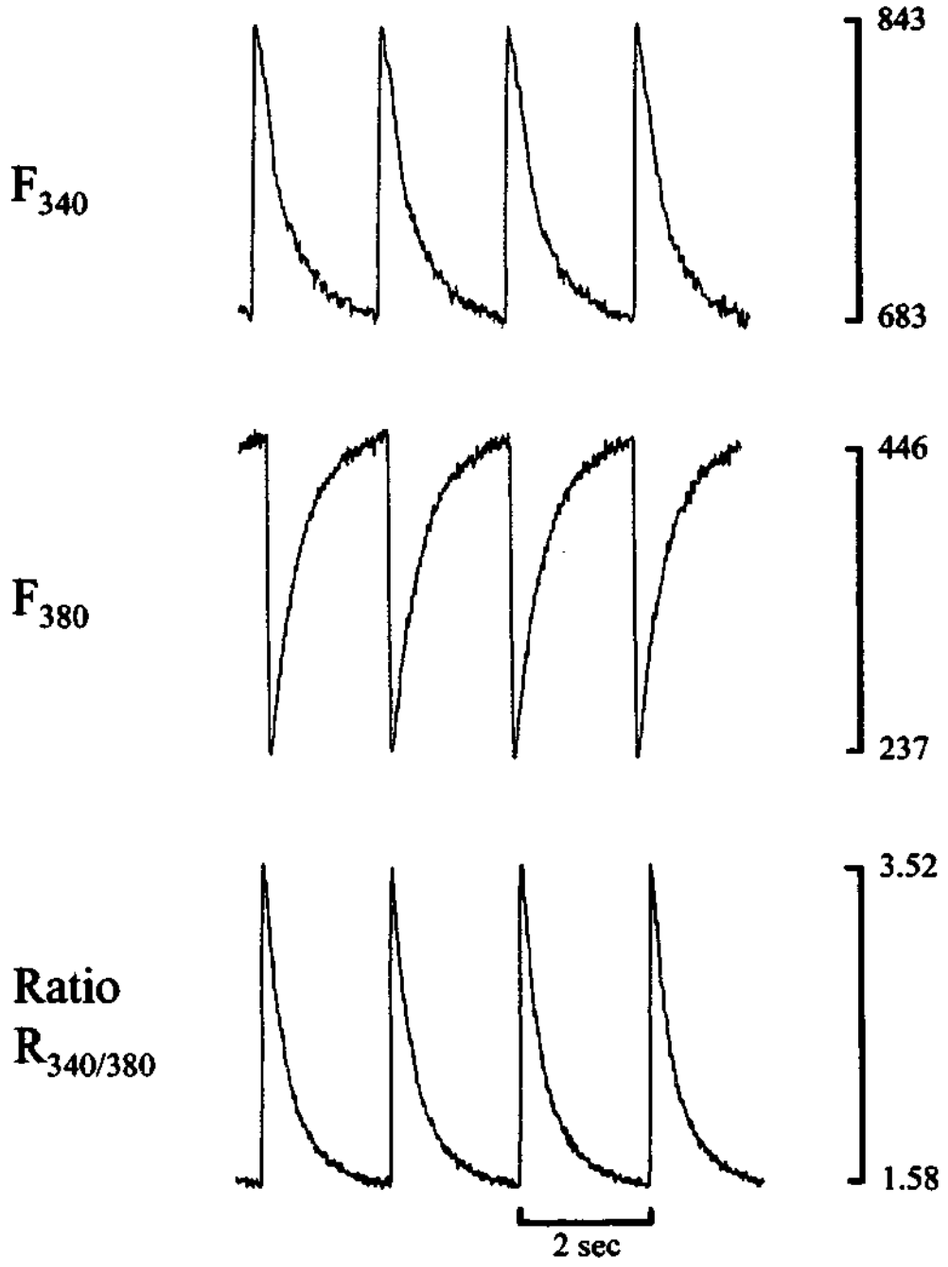


Figure 2.4: Original recordings of fluorescence changes from a cardiac myocyte stimulated to contract at 0.5 Hz. The upper trace illustrates the fluorescence recorded at an excitation wavelength of 340 nm, whilst the middle trace shows the fluorescence recorded at an excitation wavelength of 380 nm. Fluorescence signals are shown in arbitrary units. When fura-2 binds Ca^{2+} the 340 nm wavelength signal increases and the 380 nm wavelength signal decreases. The bottom trace represents the ratio of the fluorescence signal at 340 nm excitation to that at 380 nm excitation ($R_{340/380}$) which is an indication of the intracellular free Ca^{2+} concentration.

2.3.2 Loading of Cells with Fura-2

The myocytes were loaded with the acetoxymethyl ester form of fura-2 (Molecular Probes, Eugene, OR., USA). The fluorescent form of fura-2 is the free acid form which is membrane impermeant. The acetoxymethyl ester (AM) form, however, is lipid soluble and was therefore used to load the cells. The AM groups mask the negative charge on the carboxyl groups present in the indicator molecule (Kao, 1994). This form of the indicator is thus uncharged and hydrophobic, and therefore can cross the lipid membrane and gain entry into the cell. Because the carboxyl groups are essential for the indicator molecule to bind Ca^{2+}, the AM groups must be removed once the molecule is inside the cell. This is achieved by naturally occurring esterases which cleave off the AM group leaving the free acid form of fura-2. As previously mentioned, this free acid form is membrane impermeant and can theoretically, therefore, not pass out of the cell, leaving the fura-2 trapped inside the cell (Sipido and Callewaert, 1995).

Fura-2/AM was prepared as a stock solution to a concentration of 1 mM in dimethyl sulfoxide (DMSO). Appropriate small volumes of this fura-2/AM stock solution were added to aliquots of the cell suspension to yield a final fura-2/AM concentration of 5 μM. The cells were transferred to the experimental chamber on the stage of an inverted fluorescence microscope (Nikon Diaphot 300) and left to incubate at room temperature for 10 minutes in this solution. This was found to be an optimal time for loading the cells with fura-2 as it allowed sufficient time to load cells with enough fura-2 to emit fluorescence at readily detectable levels, whilst brief enough to greatly reduce the risk of overloading the cells, and thus avoiding compartmentalisation of the dye into intracellular organelles.

2.4 Measurement of Cell Fluorescence

Fura-2 loaded cardiac myocytes were placed in an experimental chamber (volume 600 μl) and allowed to settle and adhere to the coverslip which formed its base. The coverslip was attached with high vacuum silicone grease and was discarded and replaced with a new coverslip at the conclusion of recordings with each cell. The chamber was mounted on the stage of a Nikon Diaphot 300 inverted fluorescence microscope. Solution which was maintained at 25°C was then perfused through the experimental chamber at 0.7-1.0 ml/min by a Masterflex peristaltic pump (ColePalmer Instrument Co., Chicago, IL, USA). The outflow tubing had a wider diameter than the

inflow tubing to ensure a constant level of solution was maintained within the bath, and also to avoid overflow. The experimental temperature of 25°C was chosen because it prolongs the viability of the cells and reduces the rate of leakage of fura-2 from the cells. The leakage of fura-2 from the cells increases substantially with increasing temperature (Kao, 1994) placing a limitation on experiments conducted at higher temperatures. The leakage of fura-2 from the cell occurs through an extrusion mechanism for organic anions and can be blocked by inhibitors of uric acid transport. A reduction in temperature is a much preferred method for preventing fura-2 leakage as inhibitors have been shown to induce signs of cellular stress (Kao, 1994).

Fura-2 fluorescence was measured using a Cairn Spectrophotometer System (Cairn Research Limited, Kent, UK) coupled to the microscope. Light originated from a 75 watt xenon lamp and passed through a rotating filter wheel containing four 340 nm and two 380 nm filters to allow alternating excitation of the fura-2 loaded myocytes at these two wavelengths. The transmission of light through the optical path is poorer at 340 nm than at 380 nm, therefore when combining the signals for one revolution of the filter wheel, the sum of the intensity from the four 340 nm filters was approximately equal to the sum of the intensity from the two 380 nm filters. Once the light passed through the filter wheel it was reflected by a 410 nm dichroic mirror and focussed with a × 40 Nikon oil immersion objective (Fluor 40/1.30 Oil, Nikon, Japan) onto the cell under study. An adjustable rectangular diaphragm allowed one cell in the field of view to be masked off from the surrounding background. This ensured that only the fluorescence from this single cell was recorded. The typical recording field with a single cell masked off by the diaphragm can be seen in the top panel of Figure 2.1. The fluorescence emitted by the cell passed back through the objective and dichroic mirror, and then through a 510 nm emission filter to a photomultiplier tube. The output of the photomultiplier tube passes to the Cairn Spectrophotometer, which correlated the fluorescence signal with the particular excitation filter in the light path (F_{340nm} and F_{380nm}). The output current of the photomultiplier was converted to a voltage and these signals were then digitised for subsequent storage and analysis by personal computer. Single values for F_{340nm} and F_{380nm} were obtained from each rotation of the filter wheel by addition of the signals from the respective individual filters at each filter position. These values were then used to derive the F_{340} to F_{380} fluorescence ratio ($R_{340/380}$) which provides the measure of the intracellular free [Ca^{2+}]. In the majority of experiments the

filter wheel was rotated at 100 revolutions per second giving a sampling frequency for the recordings of 100 Hz.

To allow observation and measurement of the contractile behaviour of the cells throughout the experiment video images of the cells were displayed and captured on tape. To achieve this the cells were simultaneously illuminated with long wavelength red light (710 nm) from a halogen lamp mounted above the stage of the microscope. This transmitted light passed through essentially the same light path as the fluorescent light until the final stage. Here a second dichroic mirror (600 nm long pass) reflected the 510 nm fluorescent light to the photomultiplier while allowing the longer wavelength light (video image) to pass to the video camera. This experimental arrangement is schematically represented in Figure 2.5.

Throughout the course of the experiments completed for this thesis two different chambers were used for the cells. A deep conventional bath with a coverslip as its base was initially used. It had both an inlet and an outlet tube attached. A large amount of turbulent flow was apparent in this bath, however, which regularly detached cells from the base of the chamber. In response to this, a second chamber (90 mm × 40 mm × 13 mm) was constructed in a similar fashion to the first, however a small well (10 mm in diameter) was incorporated into the bottom of the chamber in an attempt to minimise the turbulent flow problem. In addition, larger outflow and smaller inflow tubing was attached to allow for more control over the rate at which solution was entering the chamber. This second chamber, as illustrated in Figure 2.6, proved to be much more successful than the first, with the indenting of the well allowing solution to wash over the cells without actually dislodging them.

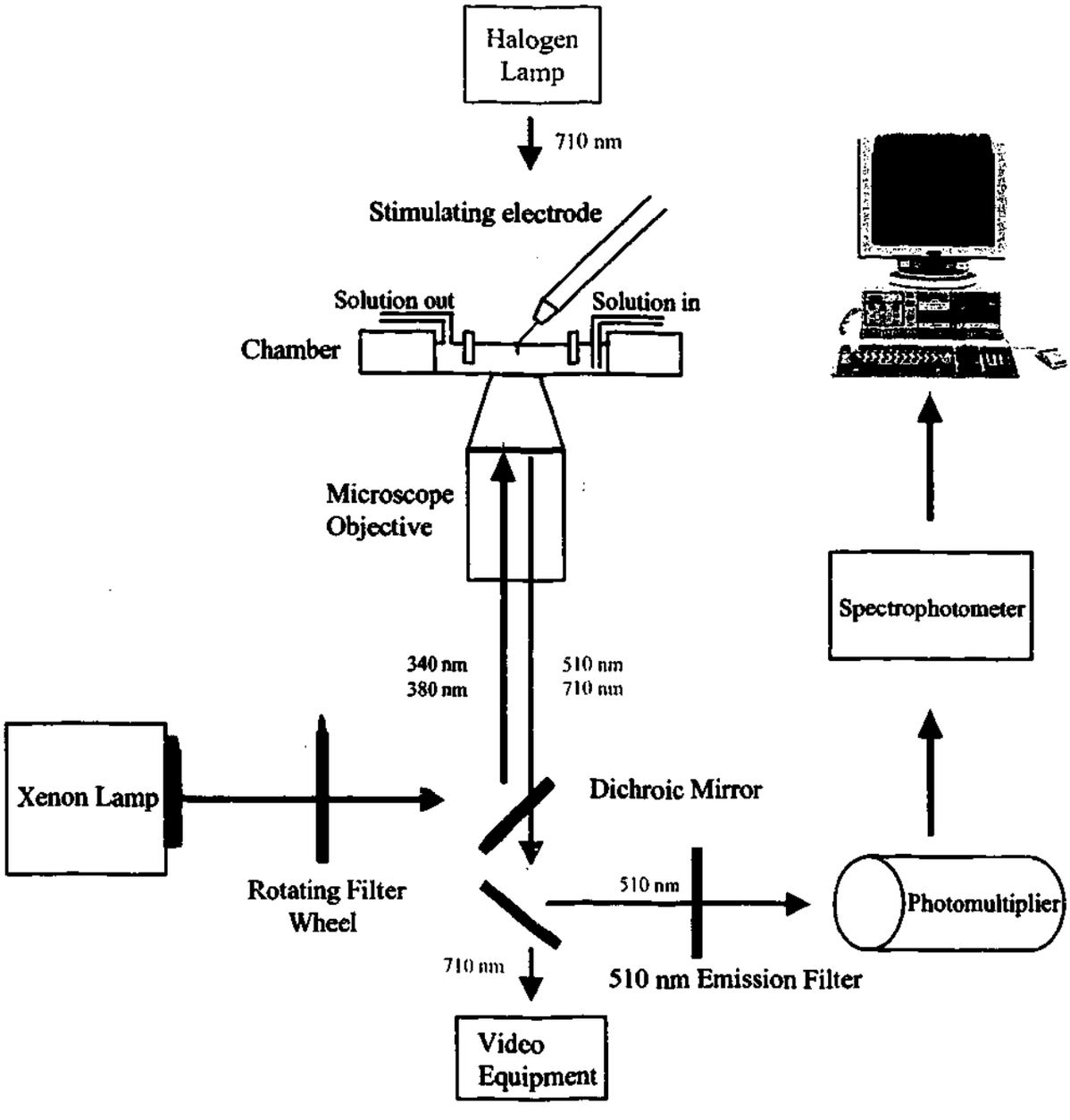


Figure 2.5: Schematic diagram of the experimental apparatus – light originating from a xenon lamp passed through 340 nm and 380 nm filters mounted in a rotating wheel. The filtered light was reflected by a 410 nm dichroic mirror though the microscope objective and focussed onto the cell under investigation. The emitted fluorescent light from the cell together with a long-wavelength (710 nm) image passed back through the objective where they were separated by a second dichroic mirror (600 nm). The fluorescence emitted by the cell passed through a 510 nm emission filter and was detected by a photomultiplier. The output from the photomultiplier passed to the spectrophotometer which digitised the information and stored it on a personal computer.

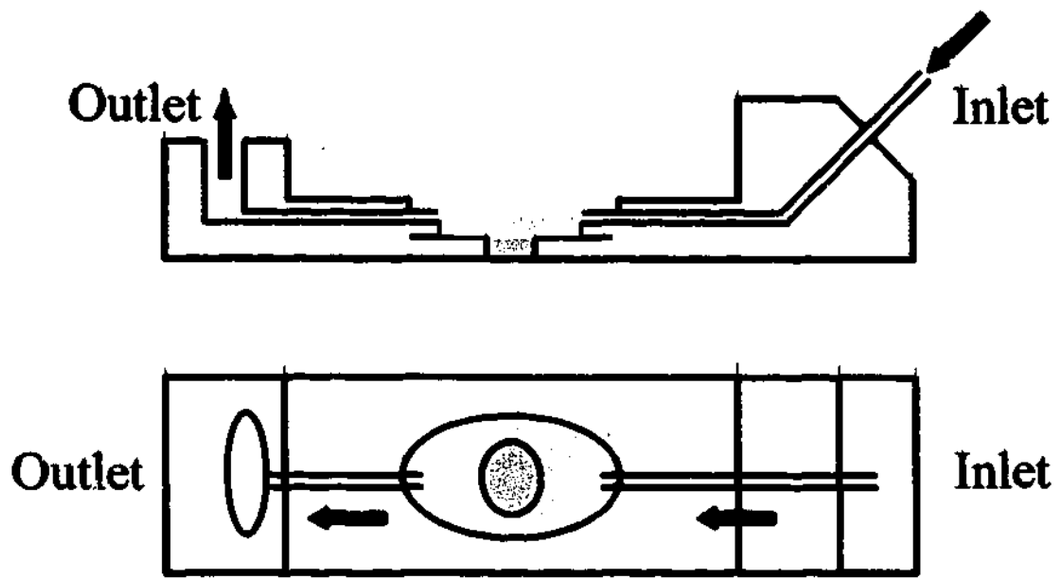


Figure 2.6: Schematic representation of the chamber that was used during fura-2 measurements from cells. The top panel shows a lateral view of the chamber. The lower panel shows a top view of the chamber. Improvements from the first chamber included an indented well for cells to sit in, and a smaller inlet tube to allow better control of solution flow into the chamber.

2.4.1 Calibration of Fura-2 Fluorescence Signals

It is possible to calibrate the $R_{340/380}$ obtained from the measured fluorescence values to obtain actual values of $[Ca^{2+}]$ using the following equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}] = K_d \times B \times (R-R_{min})/(R_{max}-R)$$

where:

K_d = The dissociation constant for fura-2 and Ca^{2+}

B = The ratio of the fluorescence emission intensity at 380 nm excitation in the absence of Ca^{2+} to that in the presence of saturating Ca^{2+}

R = Ratio of the fluorescence intensity at 340nm excitation to that at 380 nm excitation for any given Ca^{2+} concentration ($R_{340/380}$)

$R_{min} = R_{340/380}$ when no Ca^{2+} is bound to the fura-2

$R_{max} = R_{340/380}$ when all the fura-2 is in the Ca^{2+}-bound form (i.e. saturated)

The actual Ca^{2+} concentration represented by any given value of R can be determined if B, R_{max}, R_{min} and K_d are known. During the course of this thesis B, R_{min} and R_{max} were determined experimentally for each cell at the conclusion of the experiment. K_d was assumed to be 224 nM (Grynkiewicz *et al.*, 1985). The value for K_d is somewhat uncertain as it is dependent on experimental conditions, such as temperature and pH. Different values have been reported for the K_d of fura-2 in cardiac myocytes varying from around 200 nM (Martin *et al.*, 1998) to 371 nM (Haworth and Redon, 1998). A value of 224 nM was chosen for the K_d for experiments contained within this thesis because it has been widely used in other studies in cardiac myocytes (Yu *et al.*, 1995; Russ *et al.*, 1996; Zhang *et al.*, 2000). The K_d is essentially a constant value in the calibration equation, and therefore, inherent differences that are apparent within a data set should not be affected by a change in this number. This is illustrated in Figure 2.7 which shows mean data from male and female cardiac myocytes stimulated to contract at 0.5 Hz steady-state at various extracellular Ca^{2+} concentrations. The three different panels represent firstly the ratio values, secondly the Ca^{2+} concentration calculated with a K_d of 224 nM and thirdly the Ca^{2+} concentration calculated with a K_d of 371 nM recently cited in the literature (Haworth and Redon, 1998). It is evident from this figure that the difference in $[Ca^{2+}]_i$ between male and female cells is still apparent with the different K_d, in fact the increase in K_d has somewhat amplified the difference between the two. Some researchers have suggested that assuming a K_d of 224 nM leads to an underestimate of the true Ca^{2+} concentration in cells (Koshy *et al.*, 1997), therefore, the differences reported in this thesis may actually be greater than what is represented here.

It is also important to mention that many different studies performed previously do not include calibrated $[Ca^{2+}]_i$ values, but rather express all changes in ratio terms (Calaghan *et al.*, 1998; Hongo *et al.*, 1998; Palmer *et al.*, 1998; Ren *et al.*, 1999). It is interesting to notice from Figure 2.7 that the conversion from ratio to calibrated $[Ca^{2+}]_i$ values appears to have made no difference to the apparent shape of the two plots. In fact they are almost super-imposable suggesting that the calibrations undertaken were not distorting the trends in the data that has been collected.

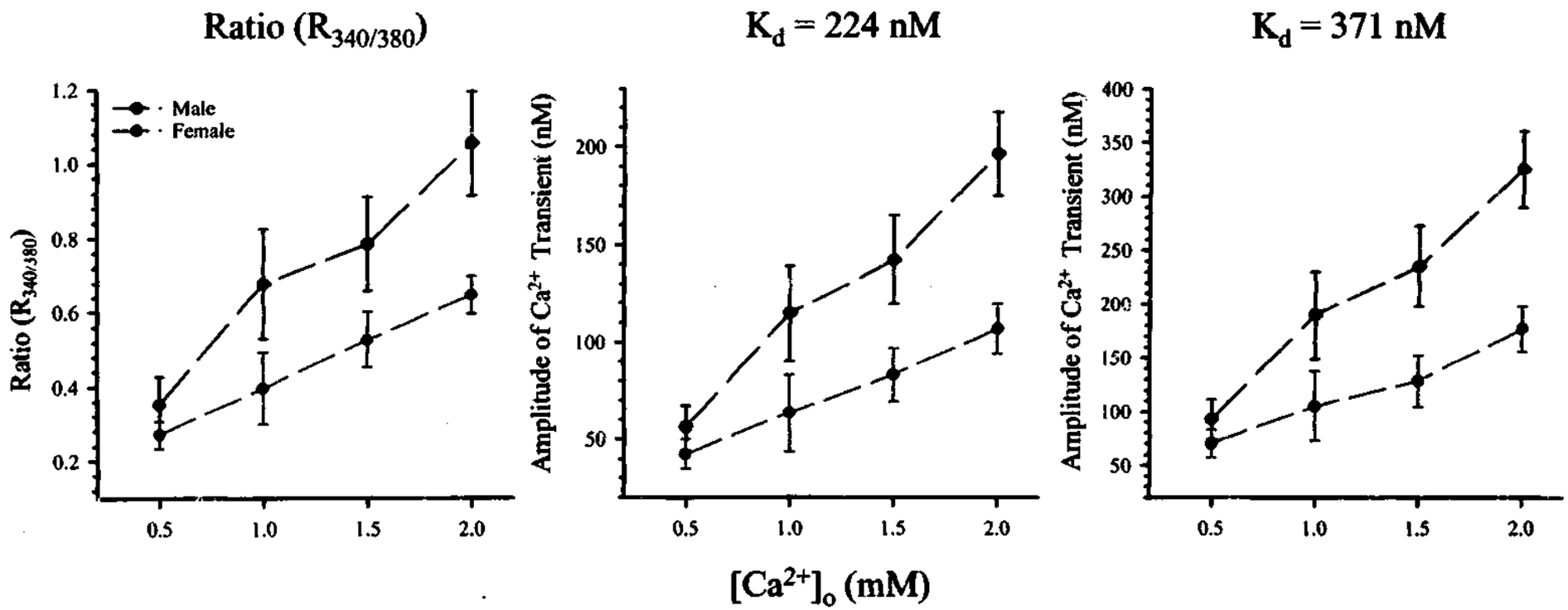


Figure 2.7: Comparison between the ratio, and the calibrated $[Ca^{2+}]_i$ with two different values of K_d used. The left hand panel illustrates ratio differences of the amplitude of the Ca^{2+} transient between male and female cardiac myocytes stimulated to contract at 0.5 Hz steady-state at different extracellular $[Ca^{2+}]$. The middle panel shows these differences with calibrated $[Ca^{2+}]_i$ values and a K_d of 224 nM. The right hand panel shows these differences with calibrated $[Ca^{2+}]_i$ values and a K_d of 371 nM.

R_{max} was determined by exposure of the cells to the Ca^{2+} ionophore ionomycin (7.5 μM) in the presence of solution containing 1.0 mM Ca^{2+}. Ionomycin renders the plasma membrane permeable to Ca^{2+} and allows equilibration of the extracellular and intracellular [Ca^{2+}] thus achieving saturation of the fura-2 (maximum fluorescence ratio) (Kao, 1994). Following determination of R_{max} an excess concentration (3 mM) of the Ca^{2+} chelator EGTA was added which effectively reduced the intracellular Ca^{2+}concentration to sub-nanomolar levels thus giving the minimum fluorescence value (R_{min}). A value for the constant B is the ratio of the maximum to the minimum fluorescence intensity levels recorded at 380 nm excitation during this procedure. An example of the recordings made during this calibration procedure is shown in Figure 2.8.

During the course of this study it was of some interest to determine if there was any difference in the calibration parameters between the two genders. Mean values of calibration parameters for male and female cardiac myocytes are presented in Table 2.3. There was no significant difference in any of the three calibration parameters between male and female cardiac myocytes.

Table 2.3: Mean values (± SEM) of calibration parameters obtained in male and female cardiac myocytes.

Calibration Parameter	Male ($n = 20$)	Female ($n = 21$)
R_{max}	8.57 ± 0.89	8.88 ± 0.56
R_{min}	0.76 ± 0.03	0.72 ± 0.02
B	6.28 ± 0.52	6.43 ± 0.37

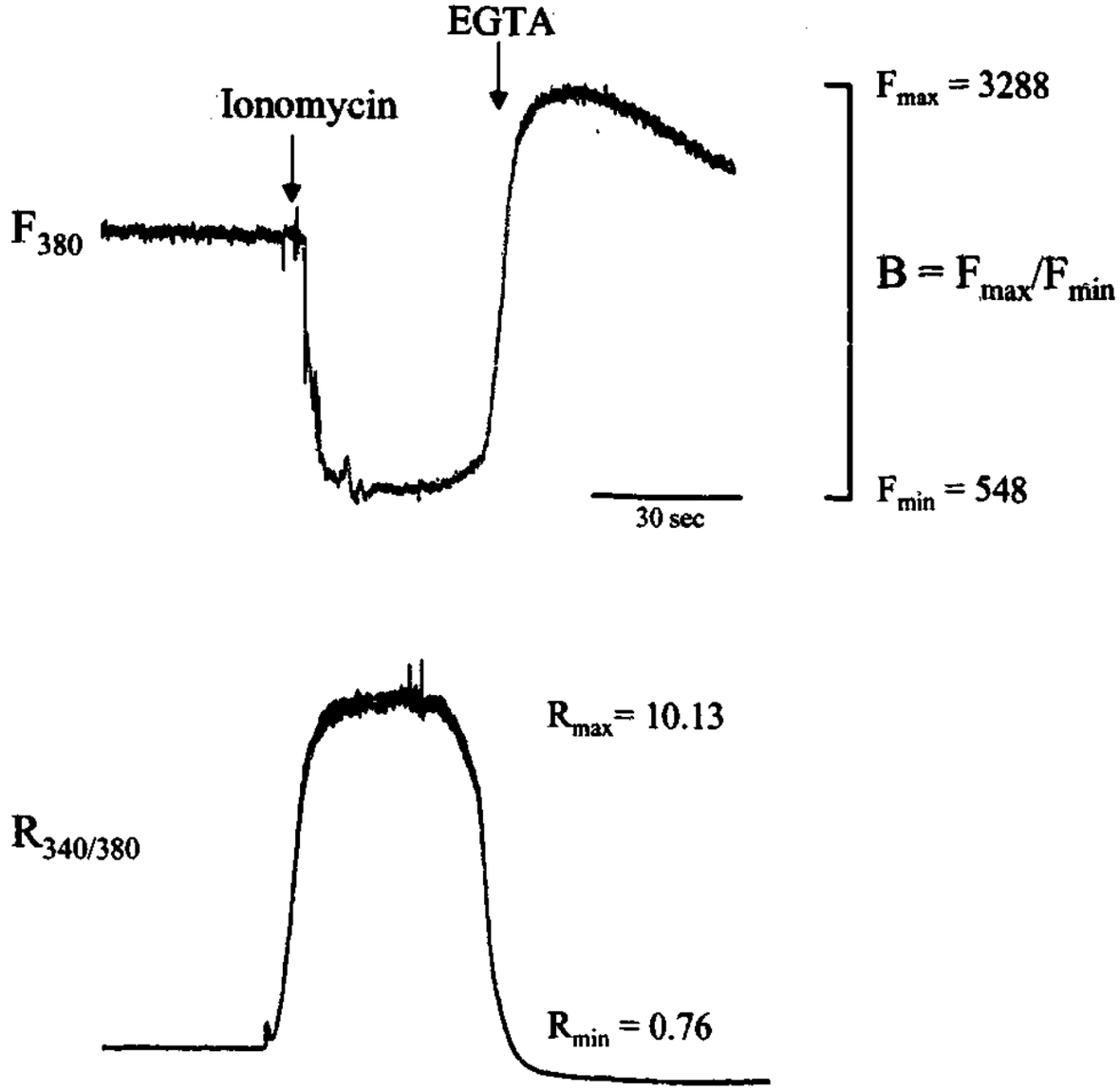


Figure 2.8: Original recordings of fluorescence changes during calibration of fura-2 fluorescence. The upper trace (F_{380}) shows the fluorescence recorded at an excitation wavelength of 380 nm, while the lower trace shows changes in the $R_{340/380}$ ratio. The fluorescence recorded at 340 nm excitation is not shown, but the changes are opposite in direction to those of F_{380}. The Ca^{2+} ionophore ionomycin was added to the bath to equilibrate intracellular and extracellular [Ca^{2+}]. Under these conditions all the fura-2 is bound to Ca^{2+}, giving the minimum F_{380} fluorescence, the maximum F_{340} fluorescence and hence the maximum value of $R_{340/380}$ (R_{max}). An excess of EGTA was then added to the bath to complex Ca^{2+} and reduce the [Ca^{2+}] to subnanomolar levels. Under these conditions all the fura-2 is unbound giving the maximum F_{380} fluorescence and the minimum values for F_{340} and $R_{340/380}$ (R_{min}). The constant B is the ratio of the fluorescence in the absence of Ca^{2+} (F_{max}) to that in the presence of Ca^{2+} (F_{min}).

Figure 2.9 demonstrates the relationship between increasing Ca^{2+} concentration and the ratio of the fura-2 fluorescence signal ($R_{340/380}$) as predicted from the mean values of the calibration parameters presented in Table 2.3. The relation between Ca^{2+} concentration and the ratio is non-linear, particularly at the higher ratio levels, approaching R_{max}. It is interesting to note, however, that the fluorescence recorded from all experiments reported in this thesis never exceeded 50% of R_{max} as determined by individual calibrations and in most cases were under 40% of R_{max}. This range corresponds to the lower portion of the graph in Figure 2.9 where the relationship between $R_{340/380}$ and $[Ca^{2+}]_i$ is reasonably close to linear.

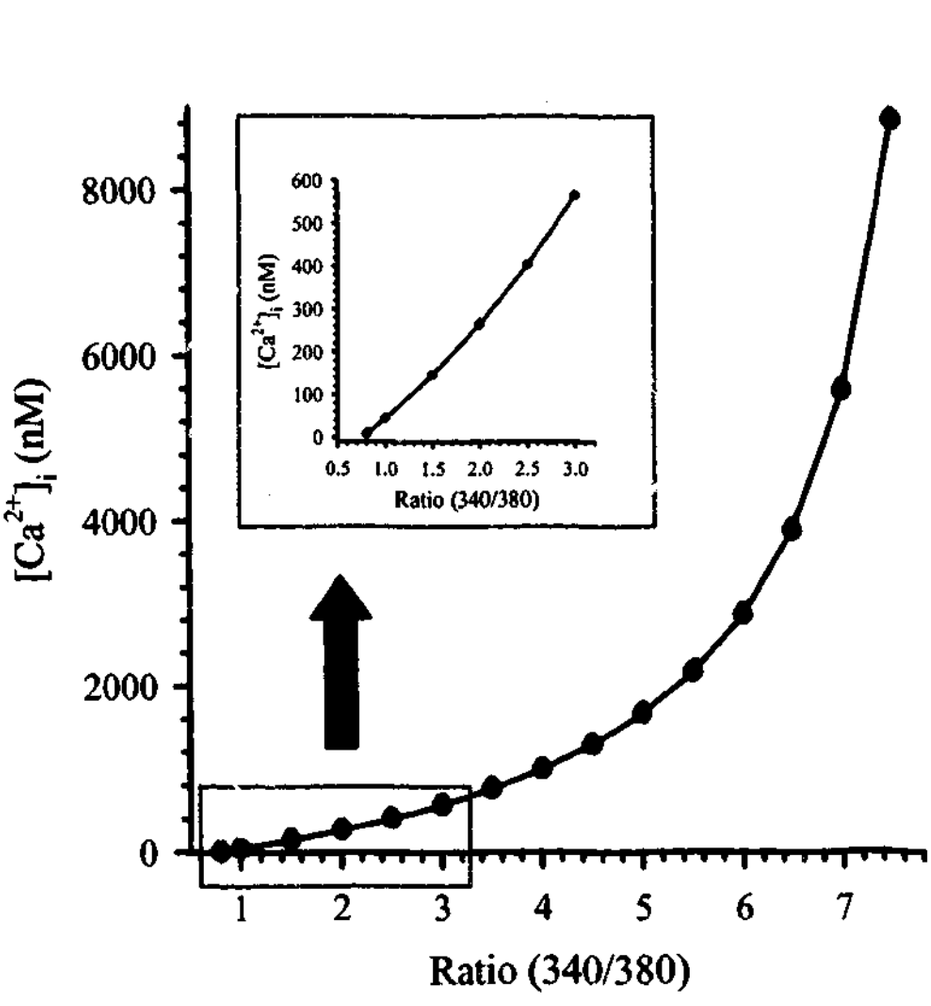


Figure 2.9: The relationship between increasing Ca^{2+} concentration and the ratio of the fura-2 fluorescence signal ($R_{340/380}$) as predicted from mean values of the calibration parameters shown in Table 2.3. At ratio values below 50% R_{max} the relationship is approximately linear (inset). As the ratio approaches R_{max} the relation between Ca^{2+} concentration and the ratio becomes increasingly non-linear.

2.4.2 Background Fluorescence

It is important to mention the background fluorescence. When using fura-2 in other tissues and preparations a background fluorescence recording is commonly made prior to loading with fura-2. This background fluorescence is then subtracted from the subsequent experimentally recorded values. The presence of the diaphragm in this setup assists in minimising any non-cellular background fluorescence as it masks off all other material around the cell and allows fluorescence to be recorded only from the cell in question. Even though this is the case, background fluorescence recordings were taken from cells that had not been loaded with fura-2 to make sure that there was no interfering background fluorescence of cellular origin which might influence the results. It was found that the background fluorescence that could be recorded from a single cell in this setup was so low as to be negligible in the overall recorded fluorescence level in cells loaded with fura-2.

2.4.3 Measurement of Cell Shortening

The extent of myocyte shortening was measured with a video edge detection system (Crescent Electronics, Utah, USA). Markers were placed on both ends of the cell and the trigger threshold adjusted to track the movement of the cell during shortening. In essence, in successive frames, a single raster line of the video image was analysed to track left and right cell-end motion (Delbridge and Roos, 1997). This voltage signal, once offset by the edge detector, was passed through the Cairn system and displayed, simultaneously with the fluorescence signal on a personal computer. The cell length was calibrated with the use of a graticule. The values obtained from the edge detector were then expressed as percentage change of resting cell length.

2.5 Experimental Protocol

For each experimental protocol in Chapters 3, 5 and 6, recordings were made only from cells that displayed normal morphology, had regular sarcomere spacing and were quiescent in the absence of electrical stimulation. A pair of platinum electrodes delivered square pulses of 10-20 V amplitude and 4 ms duration to electrically stimulate cells.

After the 10 minute loading time with fura-2, 1.0 mM Ca^{2+} HEPES-buffered Krebs-Henseleit solution was perfused through the bath to wash out the fura-2. The cells were

then left to equilibrate in this solution for approximately 15 minutes, during which time a suitable cell was identified. Once this equilibration period was completed cells were stimulated to contract at 0.5 Hz steady-state and were left at this frequency for 10 minutes before any recordings were made. Various protocols, as detailed in the relevant chapters, were then followed.

Three parameters were determined in the quantitative analysis of the changes in $[Ca^{2+}]_i$ at each frequency and $[Ca^{2+}]_o$. These were: (1) resting (diastolic) $[Ca^{2+}]_i$; (2) peak (systolic) $[Ca^{2+}]_i$; and (3) amplitude of the Ca^{2+} transient (peak-resting). For each recording, three contractions were measured and an average of these was taken. For the recordings taken at 0.5 Hz and 1.5 mM $[Ca^{2+}]_o$ the time course of decay of the Ca^{2+} transient was also analysed. This was done by fitting a single exponential function to the declining phase of the transient from 30-40 ms after the peak to 50 ms before the next stimulus. From this exponential fit a time constant for the decay of the Ca^{2+} transient was obtained for each cell. This is illustrated in Figure 2.10.

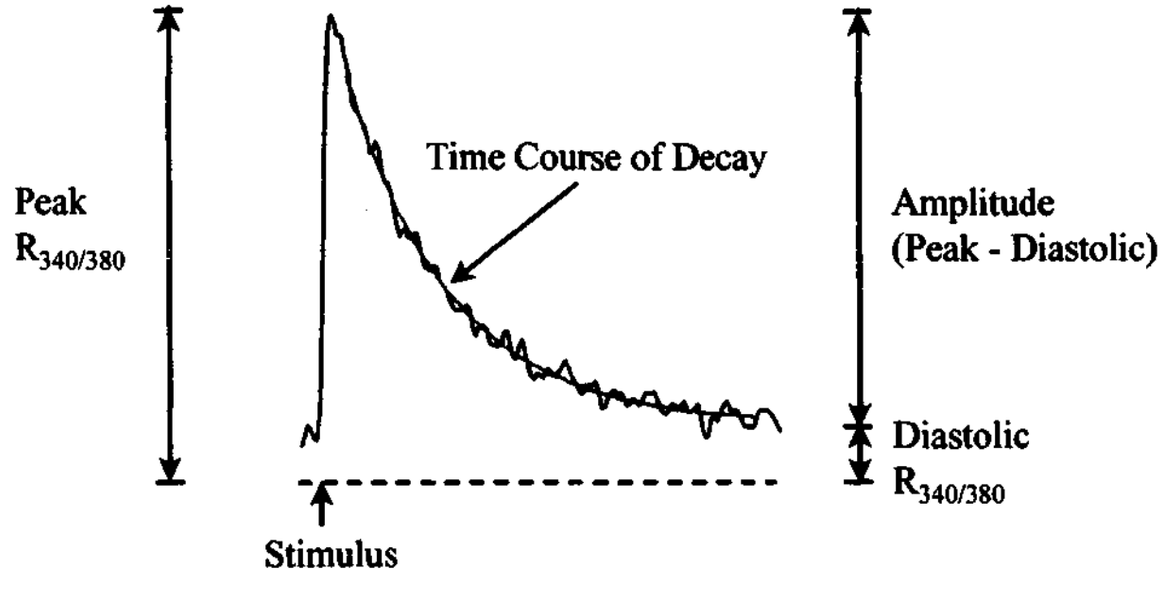


Figure 2.10: Example of the measurements made on Ca^{2+} transients. At the point indicated by the lower arrow the cell was electrically stimulated giving rise to a rapid increase in intracellular Ca^{2+} which then declines back to its resting level. Transients were analysed in terms of their diastolic or baseline level, peak or systolic level, as well as their amplitude (peak – diastolic). A single exponential function was fitted to the declining phase of the transient to give an indication of the time-constant of decay. This measurement provides an indication of the rate of relaxation of the cardiac myocyte.

2.6 Statistics

All data is represented as mean ± standard error of the mean (SEM). In each chapter the number of cells and animals are quoted with n = number of cells and N = number of animals. Statistical analysis for unpaired group comparisons were undertaken using analyses of variance (ANOVA) appropriate for the given experimental design. P-values of less than 0.05 ($p<0.05$) were considered significant. A more detailed description of the statistical tests employed for each study is presented in the relevant section in the appropriate chapter.

Chapter 3

Chapter 3

Effects of Gender on Intracellular Calcium

Over recent years it has become recognised that estrogen may exert significant cardiovascular protective actions. This stems originally from epidemiological evidence for gender-based differences in the incidence of cardiovascular disease, which was found to be significantly lower in pre-menopausal women than in age-matched men, (Barrett-Connor and Bush, 1991; Stampfer *et al.*, 1991; Grady *et al.*, 1992; Chow, 1995; Gardin *et al.*, 1995). After menopause, however, the risk of developing cardiovascular disease increases in women to be at levels similar to that in men of comparable age, unless the women receive estrogen replacement therapy in which case they remain at a significantly lower risk (Stampfer *et al.*, 1991; Grady *et al.*, 1992; Collins *et al.*, 1993; Chow, 1995).

Estrogen is known to have actions in the systemic and vascular systems, but the discovery of estrogen receptors within the heart itself has led to speculation that estrogen possesses the potential to exert direct actions on the heart (Stumpf *et al.*, 1977; Grohe *et al.*, 1997). The Ca^{2+} antagonistic actions of estrogen are one example of these direct effects on the heart with both genomic and non-genomic pathways implicated. Johnson *et al.* (1997) found an increase in Ca^{2+} channel current in estrogen receptor

knockout mice (ERKO) mice which they showed was a result of increased expression of the cardiac L-type Ca^{2+} channel. More rapid effects of estrogen have also been shown with acute application of 17β-estradiol resulting in a negative inotropic effect (Raddino *et al.*, 1986; Sitzler *et al.*, 1996) and inhibition of L-type Ca^{2+} current (Jiang *et al.*, 1992; Grohe *et al.*, 1996; Meyer *et al.*, 1998) in isolated cardiac muscle preparations and myocytes.

Previous studies that have attempted to determine gender-specific differences in the contractile function of cardiac muscle have resulted in contradictory outcomes. Capasso *et al.* (1983) reported that isolated papillary muscles from female rats had greater contractile performance than those from male rats. This finding was not supported by Schaible and Scheuer (1984) who used the isolated working rat heart to demonstrate that intrinsic cardiac function is moderately greater in male rats than in female rats. Similarly, Leblanc *et al.* (1998) demonstrated that papillary muscles from female rats older than 6 months displayed smaller isometric and isotonic contractions than age-matched males. Brown *et al.* (1996) however, reported no difference in intrinsic contractile performance between male and female rat hearts.

Given the crucial role of Ca^{2+} in regulating the contractile function of cardiac muscle, the experiments in this chapter were undertaken to seek direct evidence for gender-specific differences in Ca^{2+} handling and contractility by ventricular myocytes isolated from the hearts of male and female rats.

3.1 Methodology

Intracellular $[Ca^{2+}]$ measurements were made in isolated male and female rat cardiac myocytes. A description of the protocol used for the cell isolation has been previously detailed in Section 2.1.1, whilst details of the apparatus employed to measure $[Ca^{2+}]_i$ and cell shortening are explained in Section 2.4 and 2.4.3 respectively. The number of cells for each group and for each experiment have been included in the results section within the text where appropriate. Body weights were significantly greater ($p<0.05$) in male rats (429±10 g; N=18) when compared with female rats (263±4 g; N=15), whilst no significant difference in age was apparent between male (92±11 days) and female (92±17 days) rats.

3.1.1 Vaginal Smearing

The stage of the estrous cycle at the time of post mortem for each female rat was determined using a vaginal smearing technique. A small amount of saline was drawn up into a blunt pipette, which was inserted into the vagina and aspirated several times. The cells that are obtained by this method are indicative of the condition of the reproductive tract and therefore the ovaries, thus giving an indication of the stage of the estrous cycle. A small amount of the aspirated saline was placed on a slide and allowed to dry. Once dry, the cells were stained with methyl blue. The different appearance of the cells will give an indication of the time of the estrous cycle. The results of the smearing tests showed a random distribution of the four stages of the estrous cycle, demonstrating that the female rats used in this study were utilised at indiscriminate times during their estrous cycle. Approximately 14% of rats were in the pro-estrous phase, 43% were in estrous, 14% were in metestrous and 29% were in diestrous.

3.1.2 Varying Extracellular $[Ca^{2+}]$

For each cell, changes in $[Ca^{2+}]_i$ were recorded during steady-state stimulation at 0.33, 0.5 and 1.0 Hz with the cell superfused with the standard K-H solution containing varying levels of $[Ca^{2+}]_o$ (0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM). With each change in stimulus frequency cells were allowed to equilibrate to the new rate of pacing for 3 minutes before any recordings were made. When $[Ca^{2+}]_o$ was changed, a 15 minute equilibration period was allowed. Only results from cells from which a complete set of recordings over the whole range of $[Ca^{2+}]_o$ were included in any statistical analyses.

3.1.3 Caffeine-Induced Calcium Release

Caffeine-induced calcium release was utilised in an attempt to assess the Ca^{2+} content of the SR. Caffeine has been shown to release the calcium store from the SR and to also inhibit uptake of calcium back into the SR along with sensitising the contractile machinery to $[Ca^{2+}]_i$ (Wendt and Stephenson, 1983; Konishi *et al.*, 1984; Bassani *et al.*, 1992). In accordance with this cells were stimulated to contract at 0.5 Hz in the presence of 1.5 mM $[Ca^{2+}]_o$, given a 10 second rest, and then rapidly exposed to 20 mM caffeine. The amplitude of the caffeine-induced calcium release was then compared between the two genders.

3.1.4 Effects of the β-adrenergic Agonist Isoprenaline

The effect of the β-adrenergic agonist isoprenaline was studied in male and female cardiac myocytes. Isoprenaline has been shown to have strong positive inotropic actions that are mediated through actions on both the L–type Ca^{2+} current and SR calcium uptake and release (Tada and Katz, 1982; Skeberdis *et al.*, 1997). In this study, cells were stimulated to contract at 0.5 Hz in the presence of 1.5 mM $[Ca^{2+}]_o$, and varying concentrations of isoprenaline (10^{-12} to 10^{-7} M). The cells were left to equilibrate in each concentration of isoprenaline for 10 min before recordings were taken.

3.1.5 Statistics

All data are represented as mean ± standard error of the mean (SEM). The effects of varying $[Ca^{2+}]_o$ on male and female cells were analysed using a three-factor ANOVA with repeated measures. All other protocols used during the course of experiments undertaken in this chapter were analysed using a one-way ANOVA with two layers of nesting.

3.2 Results

3.2.1 Effects of Stimulation Frequency on Extracellular $[Ca^{2+}]$

Figure 3.1 shows an example of Ca^{2+} transient recordings from a male and a female cell in 1.5 mM Ca^{2+} stimulated to contract at three different frequencies. It is apparent from this figure that an increase in stimulation frequency results in an increase in the baseline $[Ca^{2+}]_i$, peak $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} transient in both male and female cardiac myocytes. In addition, it is also evident that the increase in the Ca^{2+} transient parameters were smaller in each case in the female cells when compared with the male cells.

Figures 3.2 to 3.5 illustrate the mean effects of increasing stimulation frequency on baseline $[Ca^{2+}]_i$, peak $[Ca^{2+}]_i$, and the amplitude of the Ca^{2+} transient at each of the four different extracellular Ca^{2+} concentrations tested. At all extracellular Ca^{2+} concentrations the increase in stimulation frequency resulted in a significant increase ($p<0.001$) in each of the $[Ca^{2+}]_i$ parameters in both male (n=7 cells, N=7 animals) and female (n=7, N=7) cardiac myocytes. In addition, statistical analysis reveals a clear gender based difference with significantly higher ($p=0.01$) peak $[Ca^{2+}]_i$ and amplitude of the Ca^{2+} transient ($p=0.015$) in male cardiac myocytes when compared with female cardiac myocytes.

3.2.2 Effects of Frequency on the Shortening Profile

Figure 3.6 shows the effects of increasing stimulus frequency on original recordings of cell shortening from both a male and a female cell in 1.5 mM Ca^{2+}. The shortening profiles follow a similar pattern to the Ca^{2+} transient recordings in that the extent of shortening increases with increasing stimulus frequency in both male and female cardiac myocytes. In addition, this increase appears to be more pronounced in male cells when compared with female cells. A decrease in the length of each cell is also noticeable as the stimulation frequency is increased. This finding is also consistent with the $[Ca^{2+}]_i$ data, as the reduction in length of the cell corresponds to the increase in diastolic $[Ca^{2+}]_i$.

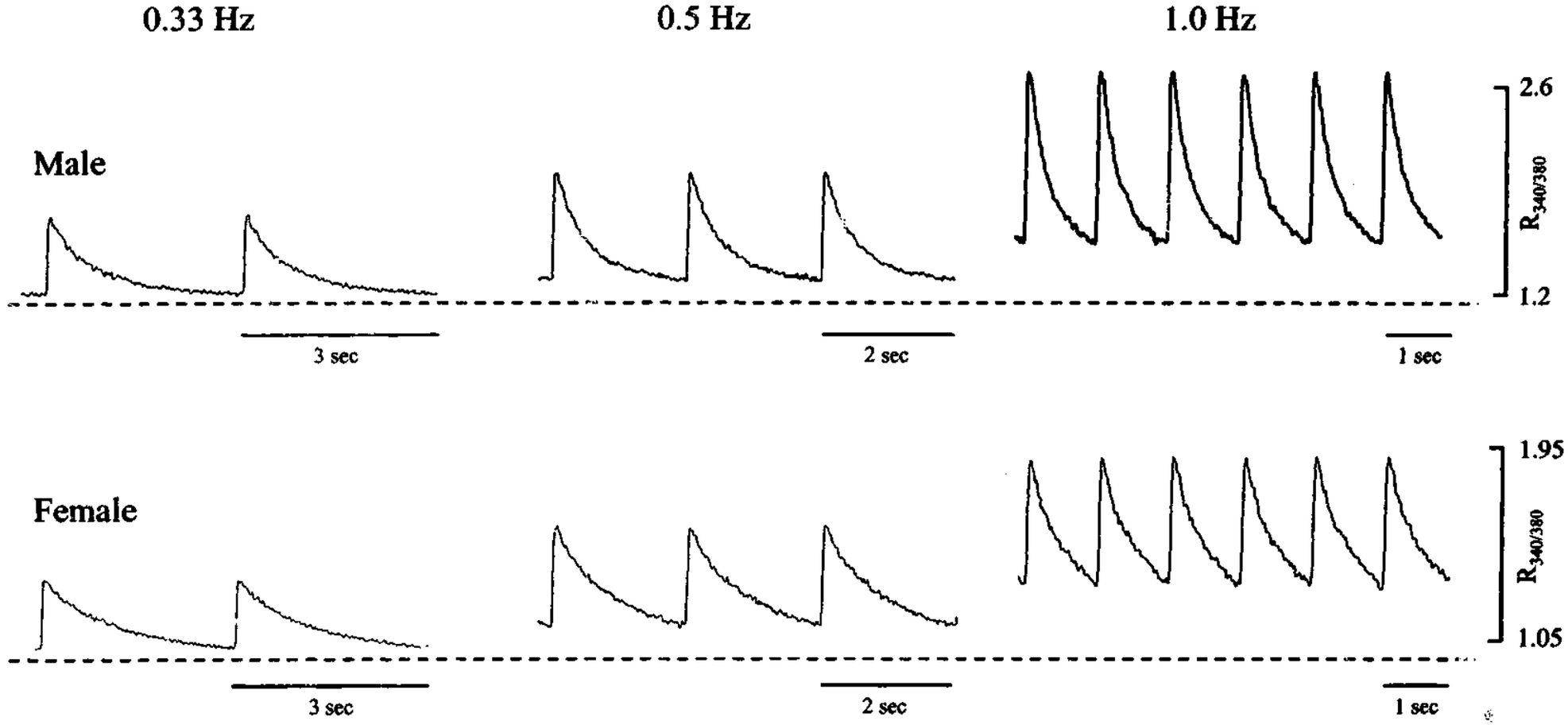


Figure 3.1: Example of Ca^{2+} transients recorded from a male and a female cardiac myocyte at three different stimulus frequencies in 1.5 mM $[Ca^{2+}]_o$. The top panel shows Ca^{2+} transients recorded during steady-state conditions at 0.33, 0.5 and 1.0 Hz in a male cell, whilst the bottom panel shows Ca^{2+} transients from a female cell under the same conditions.

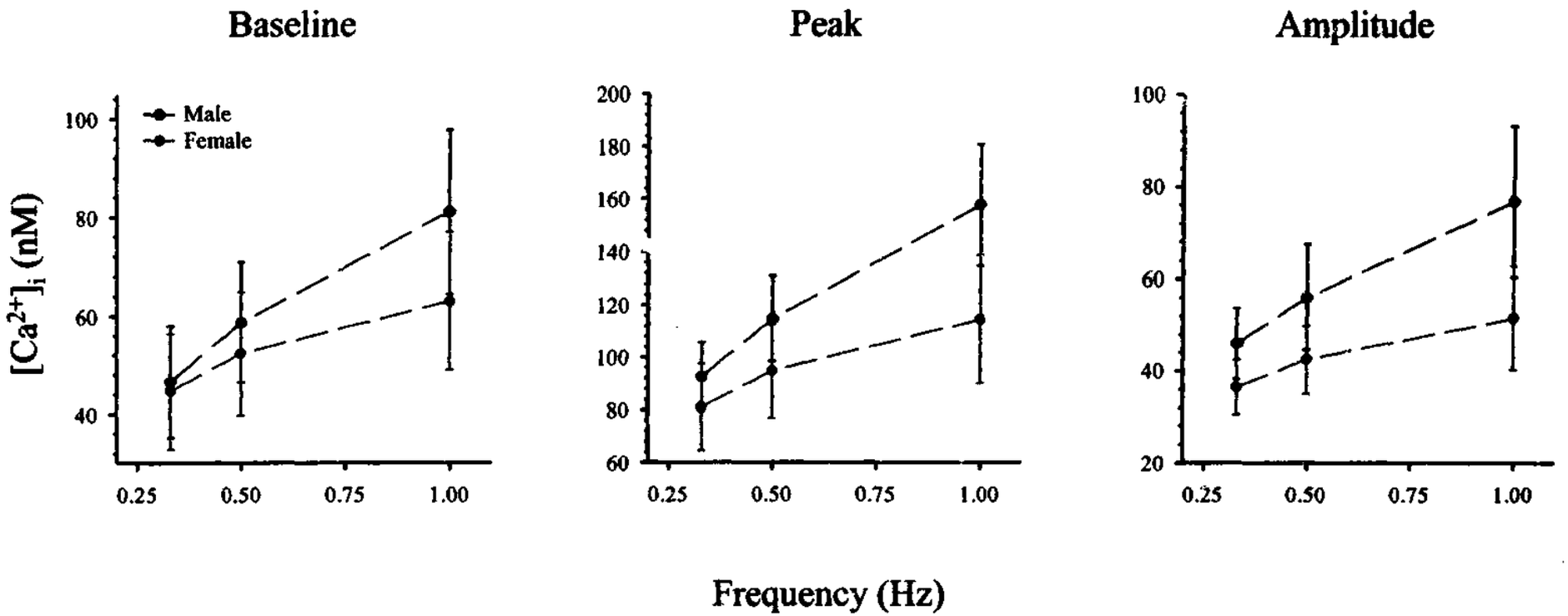


Figure 3.2: Effects of varying stimulus frequency on baseline $[Ca^{2+}]_i$, peak $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} transient in male (n=7, N=7) and female (n=7, N=7) cardiac myocytes in 0.5 mM $[Ca^{2+}]_o$. Values are mean ± SEM.

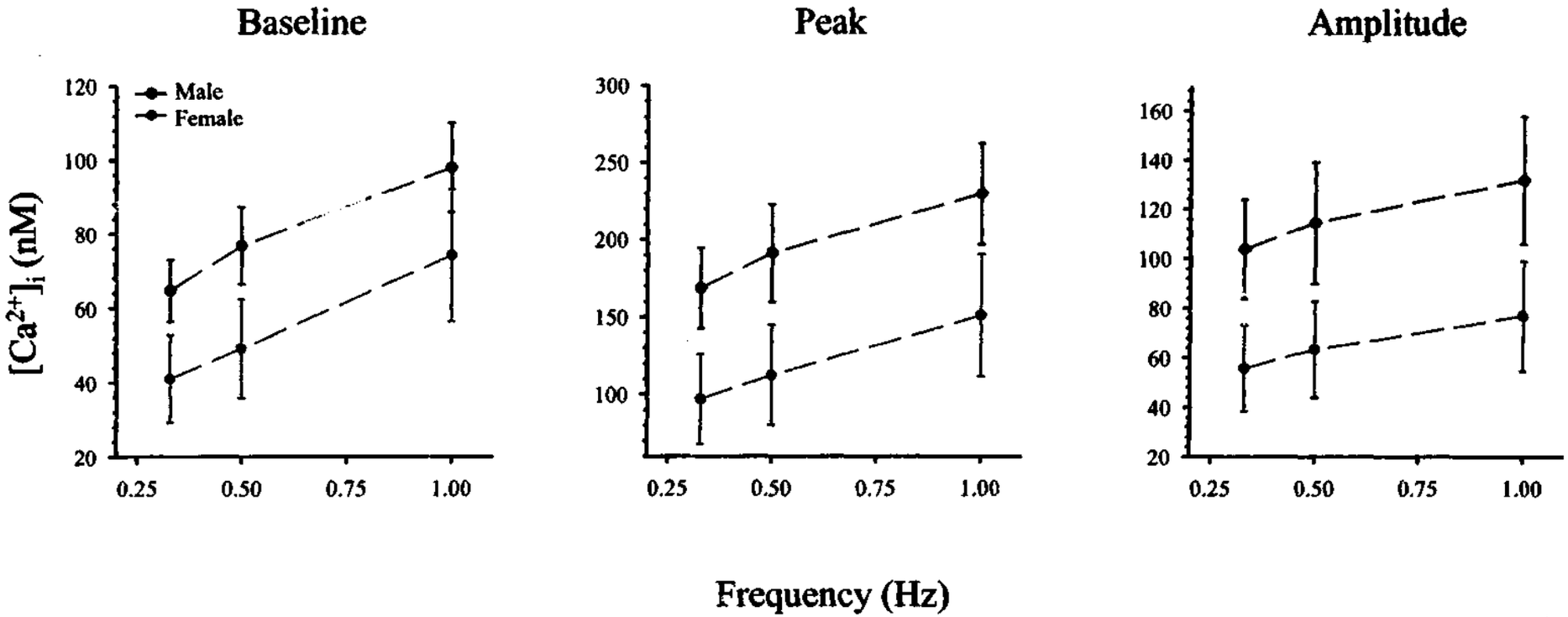


Figure 3.3: **Effects of varying stimulus frequency on baseline $[Ca^{2+}]_i$, peak $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} transient in male (n=7, N=7) and female (n=7, N=7) cardiac myocytes in 1.0 mM $[Ca^{2+}]_o$. Values are mean ± SEM.**

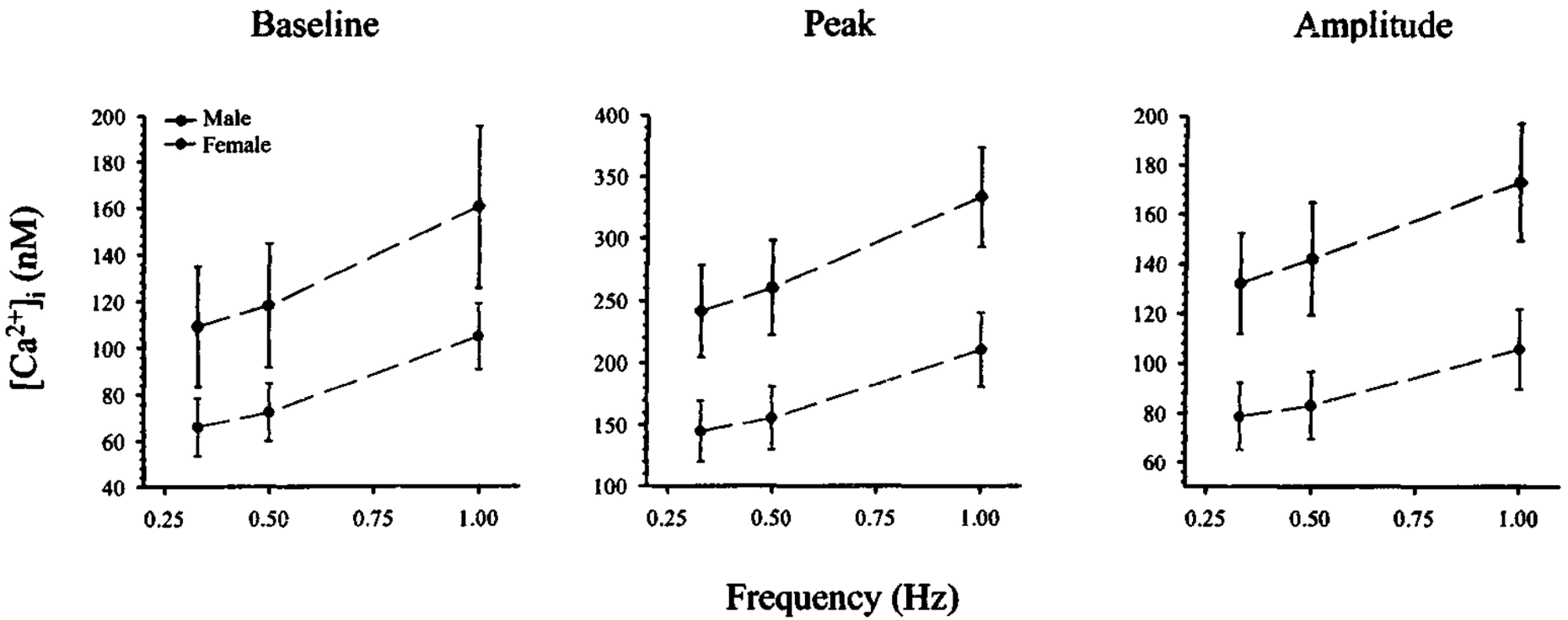


Figure 3.4: Effects of varying stimulus frequency on baseline $[Ca^{2+}]_i$, peak $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} transient in male (n=7, N=7) and female (n=7, N=7) cardiac myocytes in 1.5 mM $[Ca^{2+}]_o$. Values are mean ± SEM.

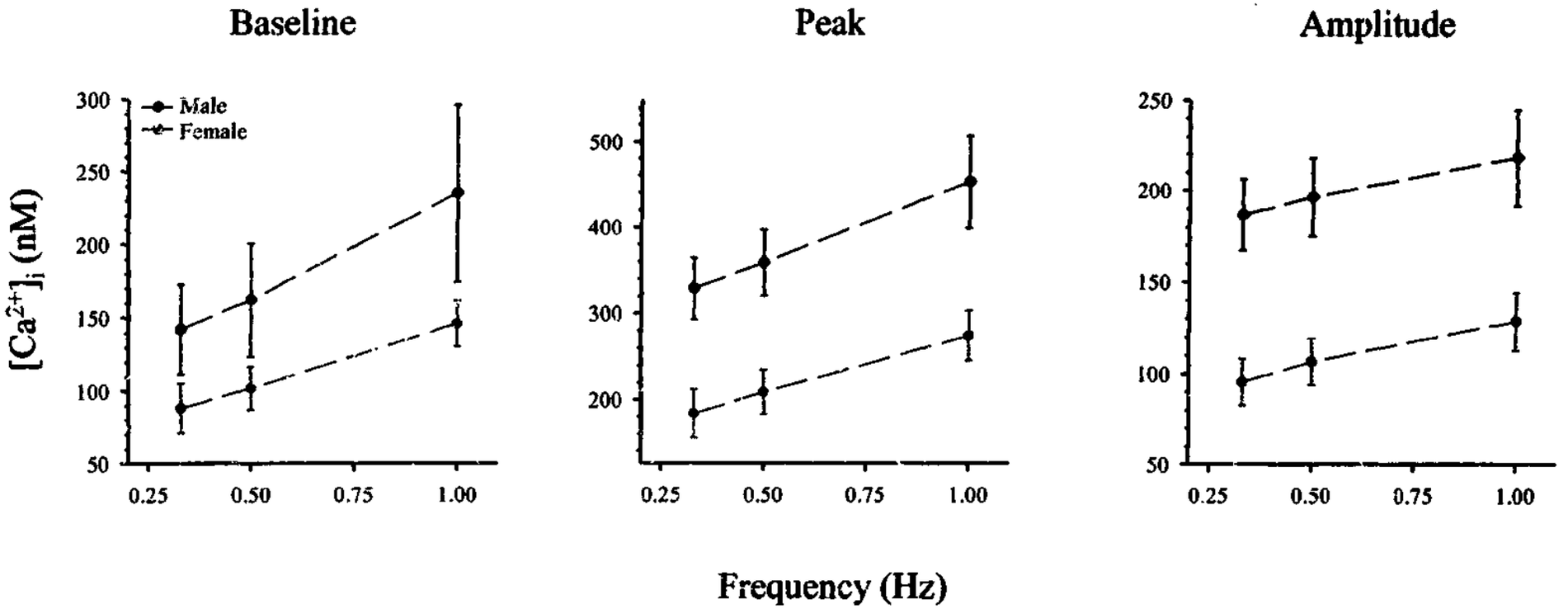


Figure 3.5: Effects of varying stimulus frequency on baseline $[Ca^{2+}]_i$, peak $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} transient in male (n=7, N=7) and female (n=7, N=7) cardiac myocytes in 2.0 mM $[Ca^{2+}]_o$. Values are mean ± SEM.

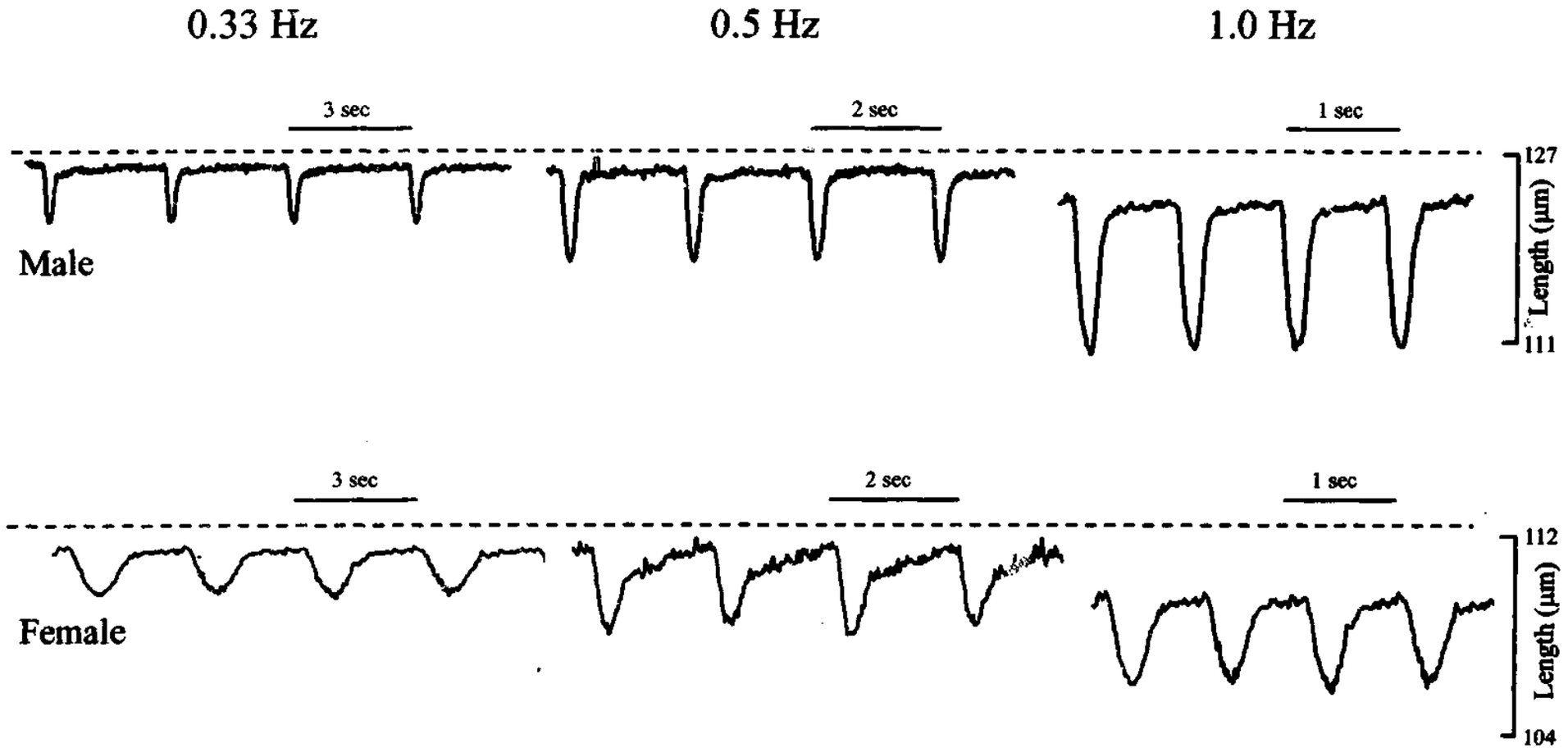


Figure 3.6: Example of shortening traces from a male and a female cardiac myocyte in 1.5 mM $[Ca^{2+}]_o$ and varying stimulus frequencies. The top panel shows shortening recorded from a male cell stimulated to contract at 0.33, 0.5 and 1.0 Hz steady-state, whilst the bottom panel shows a female cell under the same conditions.

A summary of the effects of increasing stimulus frequency on the extent of shortening is shown in Figure 3.7. There is a clear tendency for cell shortening to increase with increasing stimulus frequency which is evident in both the male and female cardiac myocytes. In addition, the extent of shortening of the cardiac myocyte appears to be greater in male cardiac myocytes when compared with female cardiac myocytes ($n \geq 15$ for each gender). This difference was not, however, statistically significant due to considerable variability within the data set.

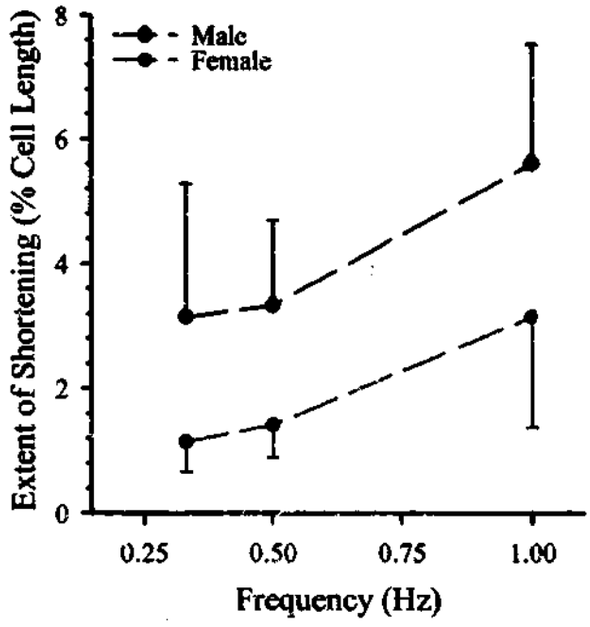


Figure 3.7: The average extent of shortening in male and female cardiac myocytes ($n \geq 15$ for each gender) in response to different stimulus frequencies at 1.5 mM $[Ca^{2+}]_o$.

3.2.3 Effect of Varying Extracellular $[Ca^{2+}]$ on Intracellular $[Ca^{2+}]$

Ca^{2+} transient recordings from both a male and a female cardiac myocyte stimulated to contract at 0.5 Hz in four different $[Ca^{2+}]_o$ concentrations are shown in Figure 3.8. As the extracellular Ca^{2+} concentration was increased, it is evident that the baseline $[Ca^{2+}]_i$, peak $[Ca^{2+}]_i$ and amplitude of the Ca^{2+} transient increased in both male and female cardiac myocytes. From these traces it appears, however, that this increase is greater in the male cells when compared with the female cells.

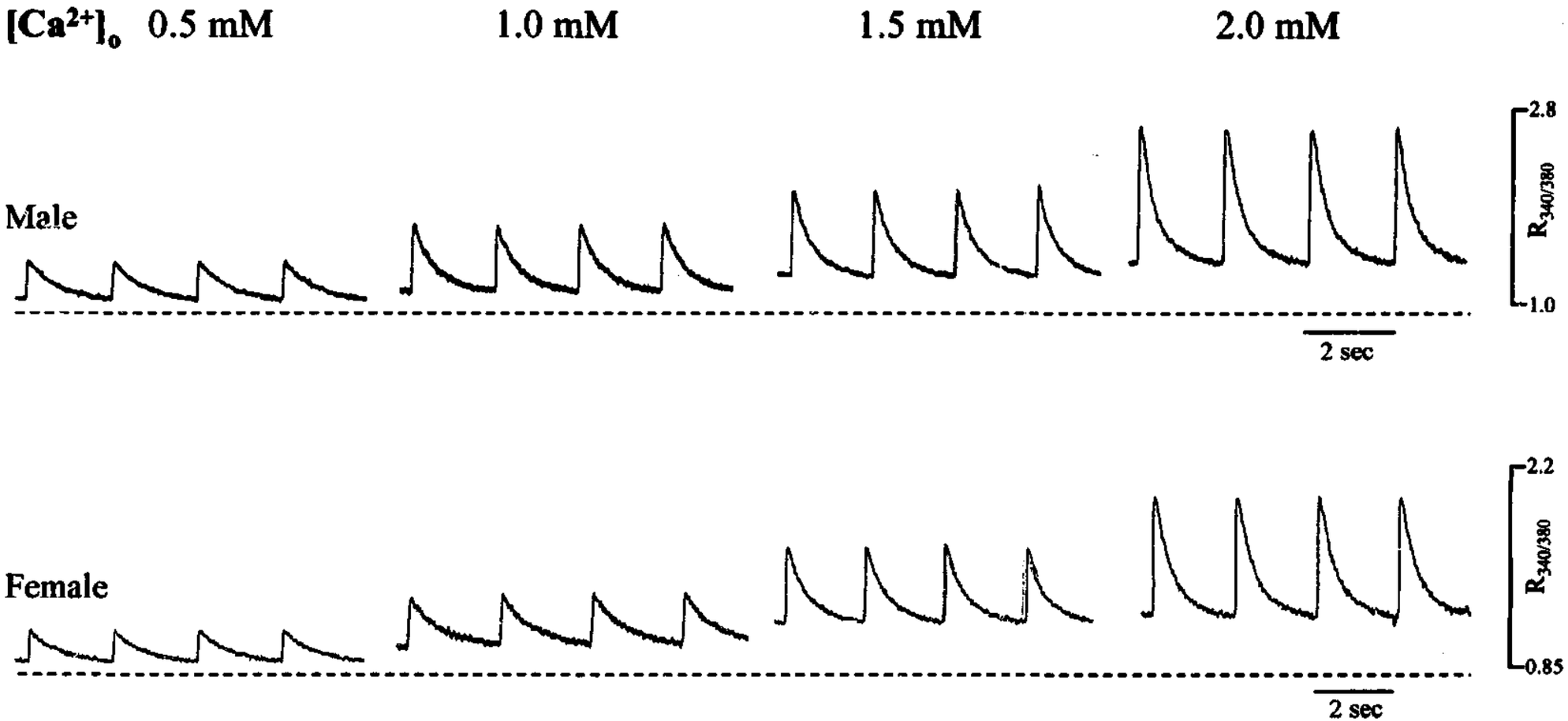


Figure 3.8: **Example of Ca^{2+} transients recorded from a male and a female cardiac myocyte at 0.5 Hz steady-state stimulation and different extracellular $[Ca^{2+}]$. The top panel shows Ca^{2+} transients recorded from a male cell at 0.5, 1.0, 1.5 and 2.0 mM $[Ca^{2+}]_o$, whilst the bottom panel shows a female cell under the same conditions.**

Figure 3.9 illustrates mean baseline $[Ca^{2+}]_i$ values in male and female cardiac myocytes at three different stimulus frequencies and varying $[Ca^{2+}]_o$. As both $[Ca^{2+}]_o$ and frequency were increased, baseline $[Ca^{2+}]_i$ significantly increased in both male (n=7, N=7) and female (n=7, N=7) cardiac myocytes ($p<0.001$ for each). Although a trend is apparent which shows that the male cells seem to have higher levels of baseline $[Ca^{2+}]_i$ than female cells, a statistically significant gender difference was not detected.

The mean peak $[Ca^{2+}]_i$ values in male (n=7, N=7) and female (n=7, N=7) cardiac myocytes in varying $[Ca^{2+}]_o$ and at different stimulus frequencies is shown in Figure 3.10. Similar to the baseline $[Ca^{2+}]_i$ data, peak $[Ca^{2+}]_i$ significantly increased ($p<0.001$) at each stimulus frequency when $[Ca^{2+}]_o$ was increased, in both male and female cardiac myocytes. In addition, at each stimulus frequency, peak $[Ca^{2+}]_i$ was, on average, significantly higher ($p<0.02$) in male cardiac myocytes than in female cardiac myocytes.

The relationship between the amplitude of the Ca^{2+} transient and $[Ca^{2+}]_o$ in male (n=7, N=7) and female (n=7, N=7) cardiac myocytes at different stimulus frequencies is shown in Figure 3.11. As was the case with peak $[Ca^{2+}]_i$, the amplitude of the Ca^{2+} transient increased significantly with increasing $[Ca^{2+}]_o$ and at all stimulus frequencies, in both male and female cardiomyocytes ($p<0.001$ for each). Similarly, significant differences ($p<0.03$) in the relationship between the amplitude of the Ca^{2+} transient and $[Ca^{2+}]_o$ were evident between male and female cardiac myocytes at each stimulus frequency.

3.2.4 Effect of Varying Extracellular $[Ca^{2+}]$ on Cell Shortening

Figure 3.12 shows an example of shortening trace recordings from a male and a female cell at 0.5 Hz steady-state stimulation in varying extracellular Ca^{2+} concentrations. It is evident from these traces that as the extracellular $[Ca^{2+}]$ is increased, the extent of shortening is also increased in both male and female cardiac myocytes. It appears that this increase is greater in the male cells when compared with the female cells.

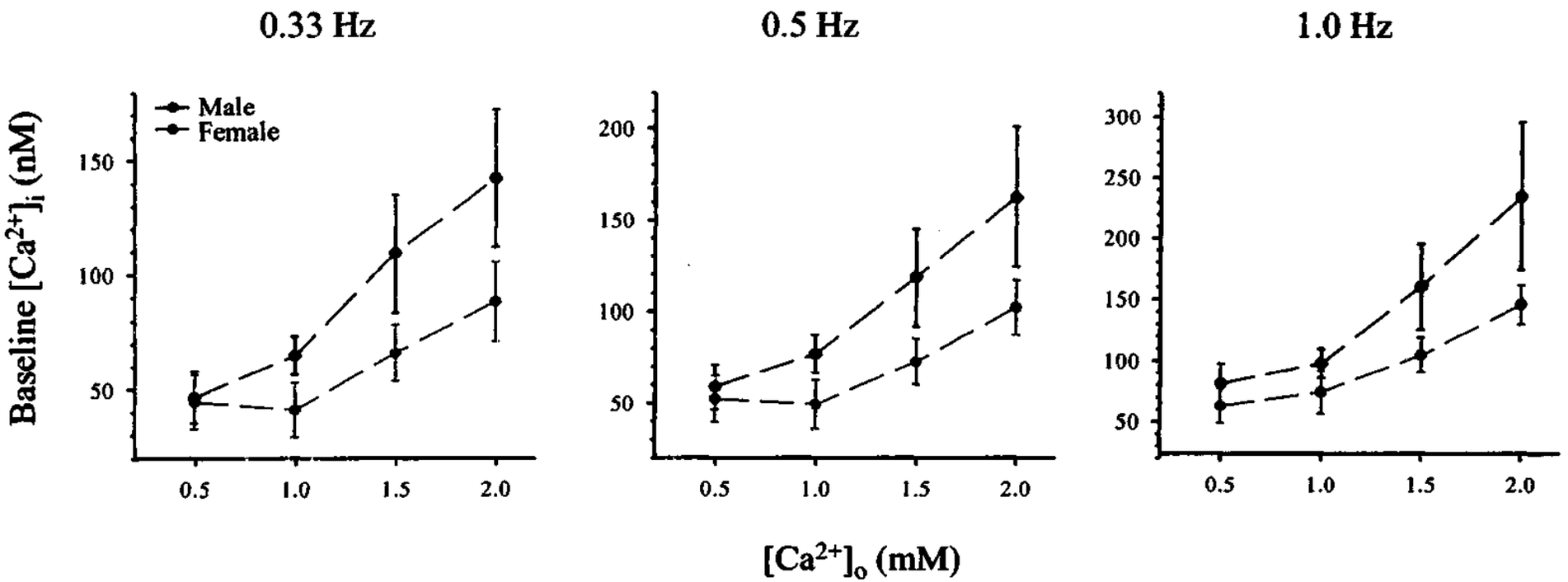


Figure 3.9: **The effects of varying extracellular $[Ca^{2+}]$ and stimulus frequency on baseline $[Ca^{2+}]_i$ in male (n=7, N=7) and female (n=7, N=7) cardiac myocytes. Values are mean ± SEM.**

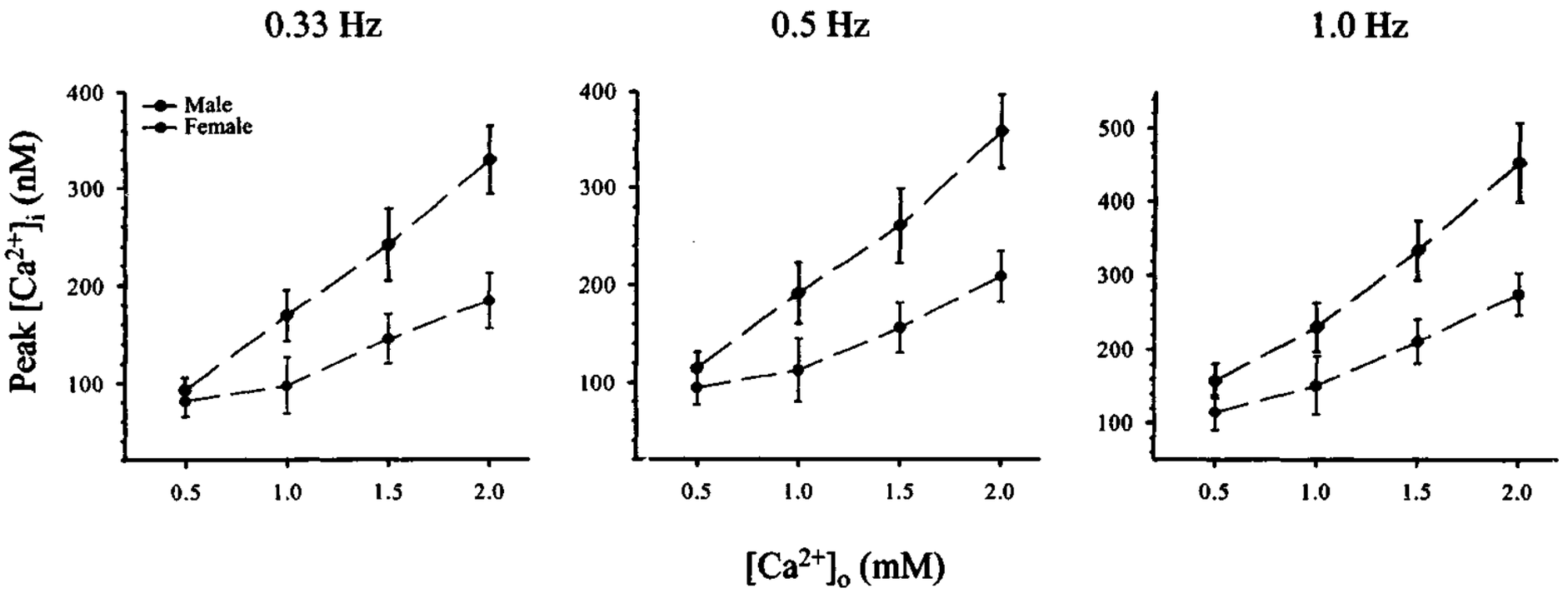


Figure 3.10: **The effects of varying extracellular $[Ca^{2+}]$ and stimulus frequency on peak $[Ca^{2+}]_i$ in male (n=7, N=7) and female (n=7, N=7) cardiac myocytes. Values are mean ± SEM.**

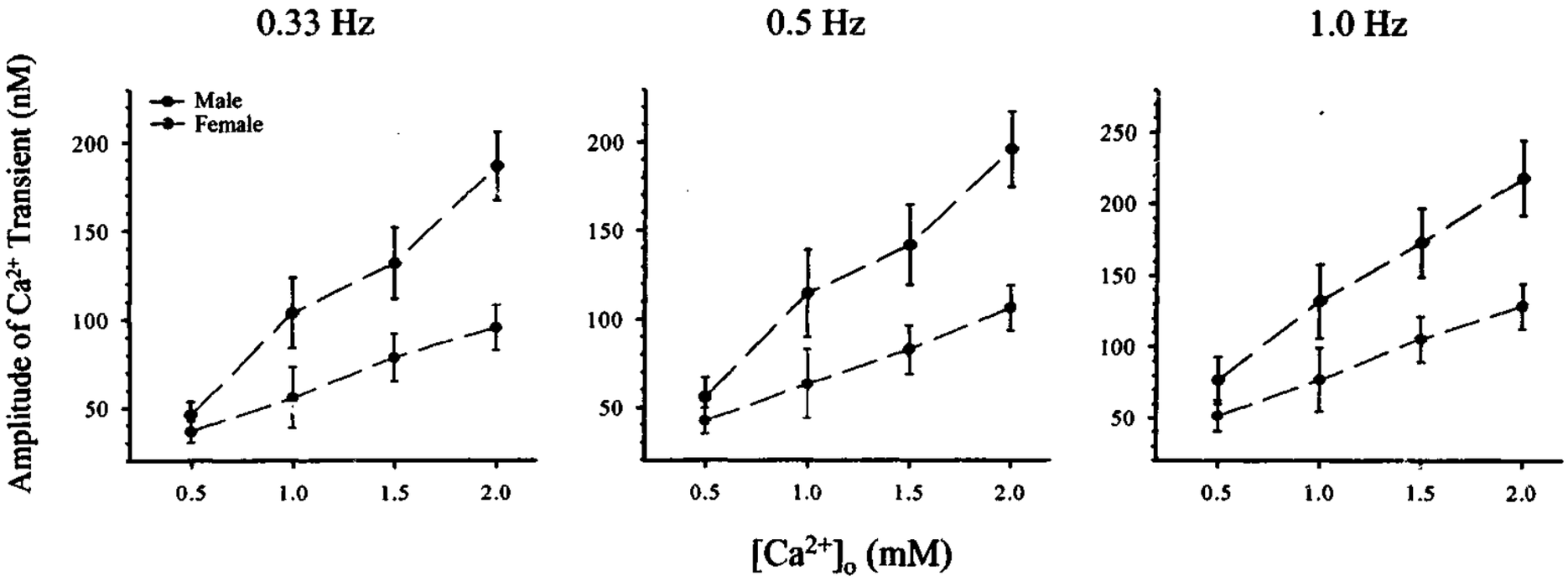


Figure 3.11: The effects of varying extracellular $[Ca^{2+}]$ and stimulus frequency on the amplitude of the Ca^{2+} transient in male (n=7, N=7) and female (n=7, N=7) cardiac myocytes. Values are mean ± SEM.

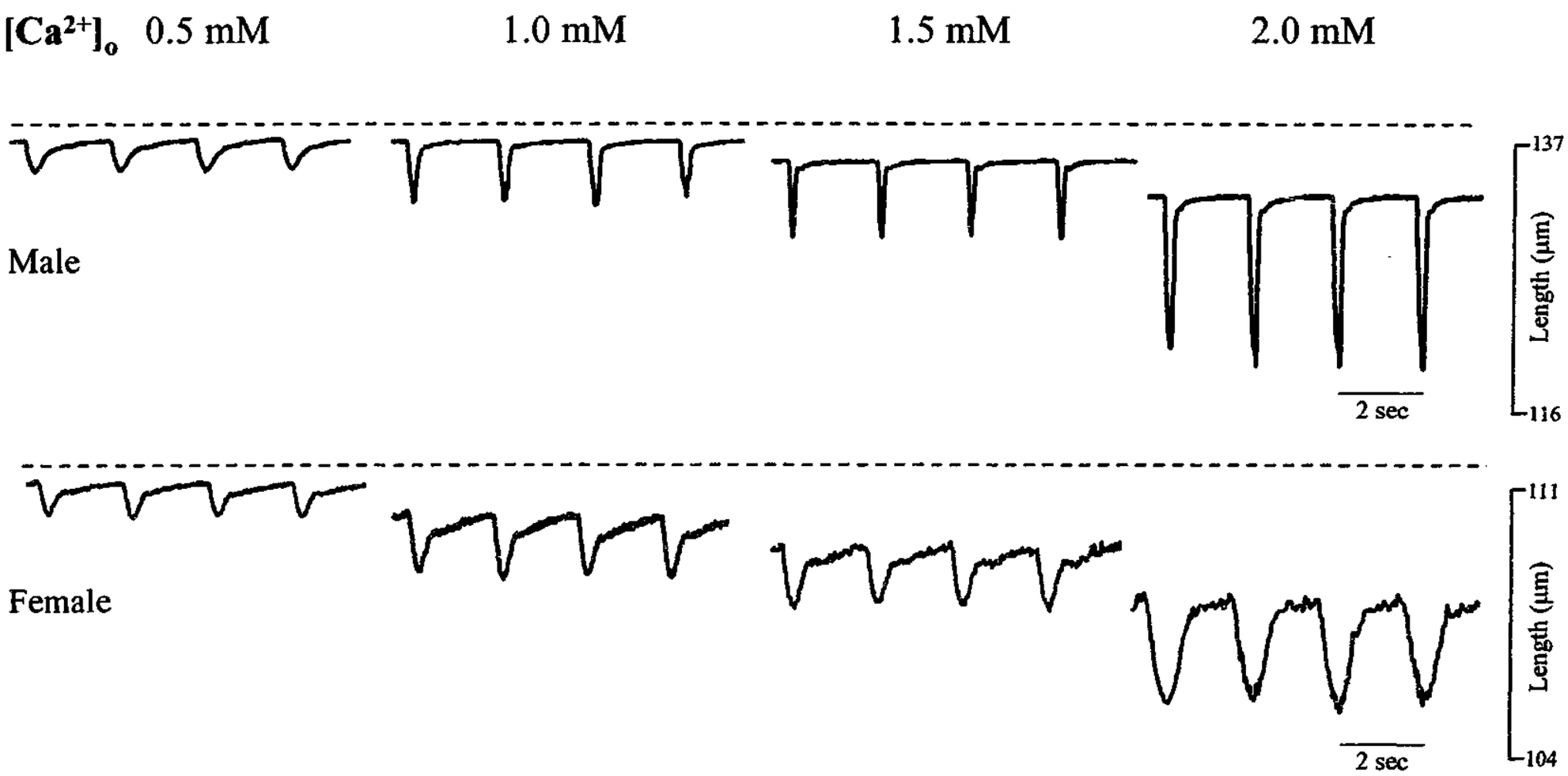


Figure 3.12: Example of shortening traces from a male and a female cardiac myocyte stimulated to contract at 0.5 Hz steady-state in varying extracellular $[Ca^{2+}]$. The top panel shows shortening traces from a male cell which has been exposed to 0.5, 1.0, 1.5 and 2.0 mM $[Ca^{2+}]_o$, whilst the bottom panel shows a female cell under the same conditions.

A summary of the effects of varying $[Ca^{2+}]_o$ on the extent of shortening is shown in Figure 3.13. These average values confirm the increase in the extent of shortening in response to increased $[Ca^{2+}]_o$ in both male and female cardiac myocytes ($n \geq 6$ for both genders). They also highlight a possible gender based difference in the extent of shortening when the extracellular Ca^{2+} concentration is increased.

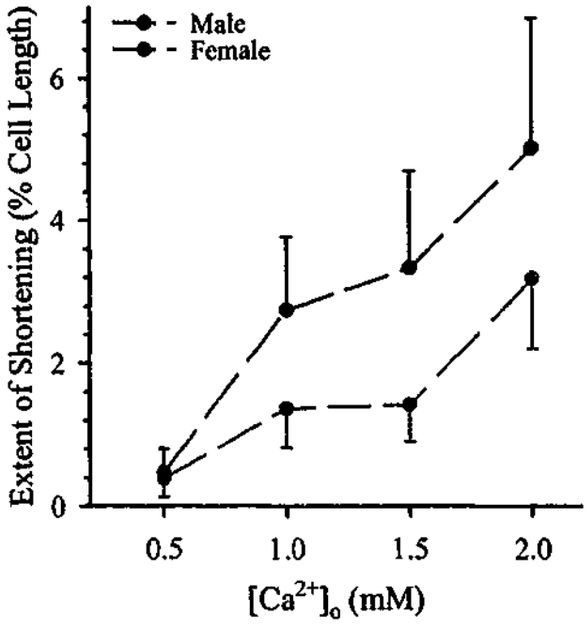


Figure 3.13: The average extent of shortening values in male and female cardiac myocytes ($n \geq 6$ for both genders) in response to varying extracellular $[Ca^{2+}]$ at 0.5 Hz steady-state stimulation.

The average extent of shortening values seen in the graph above include only cells that underwent the full varying extracellular $[Ca^{2+}]$ protocol. During subsequent experiments involving caffeine and isoprenaline cells were stimulated to contract at 0.5 Hz in 1.5 mM Ca^{2+} solution. When this single point was studied and all of the data was combined ($n \geq 15$ for each gender), a significant gender difference ($p<0.02$) in the extent of shortening of the cardiac myocyte was apparent. This is illustrated in Figure 3.14.

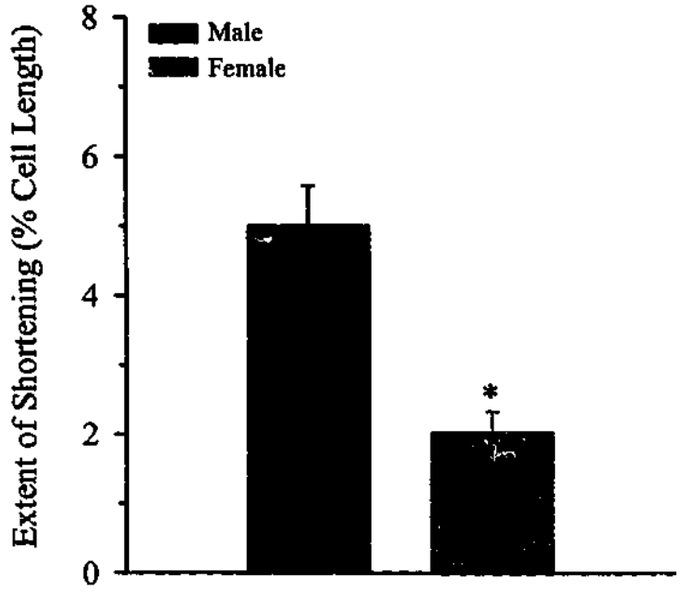


Figure 3.14: The extent of shortening in male and female cardiac myocytes stimulated to contract at 0.5 Hz steady-state in 1.5 mM Ca^{2+} solution. Values are mean ± SEM. * denotes that the value from the female cardiac myocyte is significantly different ($p<0.02$) from the corresponding value for the male cardiac myocyte.

3.2.5 Effects of Isoprenaline on Intracellular $[Ca^{2+}]$

The effects of the β-adrenergic agonist isoprenaline were studied in both male (n=10, N=6) and female (n=9, N=5) cardiac myocytes in order to further explore any gender-based calcium handling differences at the level of the sarcolemma and SR. Figure 3.15 shows Ca^{2+} transient recordings from a male and a female cell exposed to different concentrations of isoprenaline whilst being stimulated to contract at 0.5 Hz steady-state in 1.5 mM $[Ca^{2+}]_o$ solution. Recordings were taken after 10 min following exposure to 0, 10^{-12}, 10^{-9} and 10^{-7} M isoprenaline. It is evident from these traces that an increase in the concentration of isoprenaline results in increased Ca^{2+} transients in both male and female cardiac myocytes. In addition, this increase seems to be greater in the male cells when compared with the female cells. At the highest concentration of isoprenaline tested (10^{-7} M) the majority of the male cells (seven out of ten) became spontaneous and showed signs of Ca^{2+} overload. This is evident in Figure 3.15. The female cells, on the

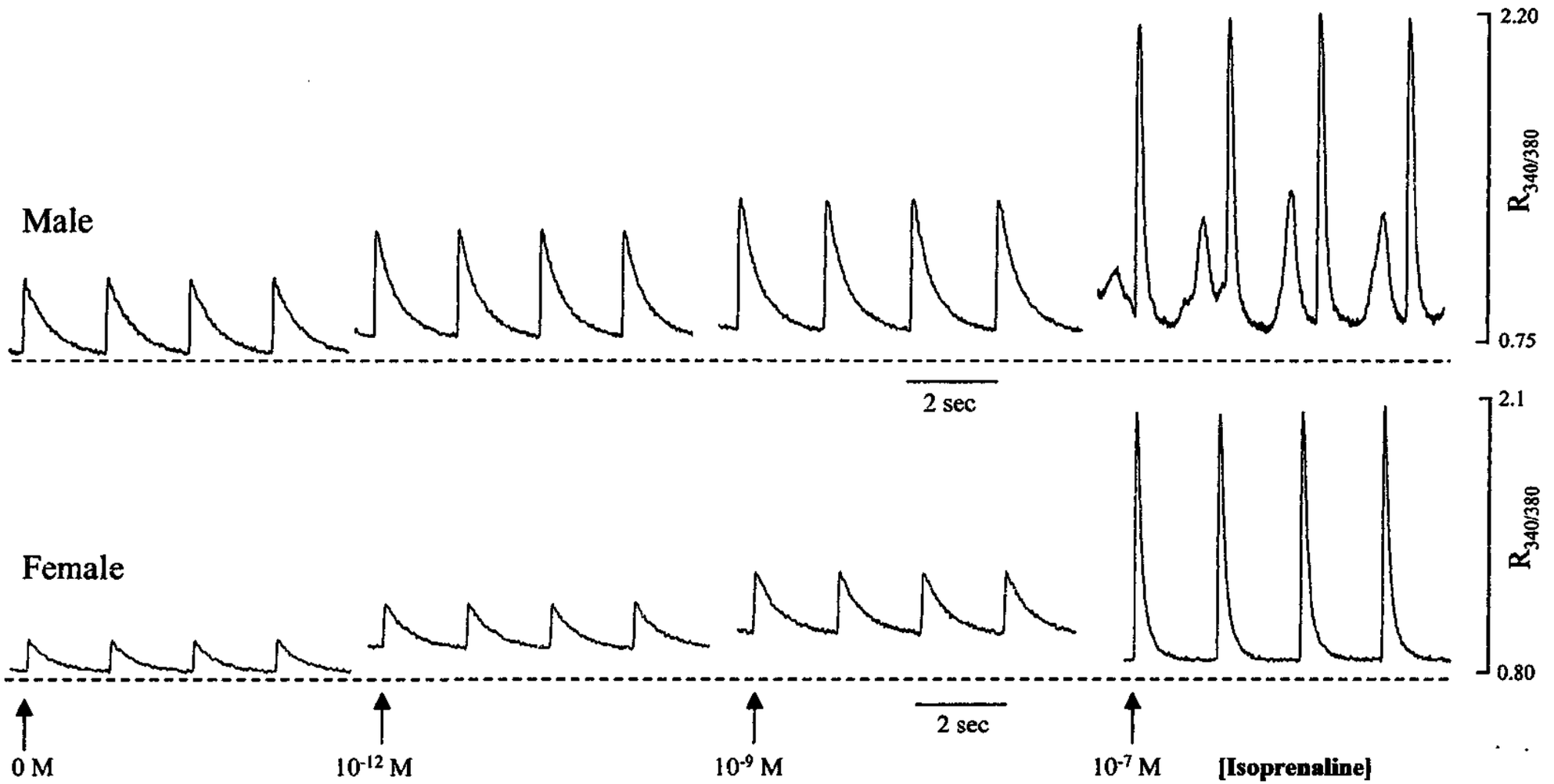


Figure 3.15: Example of Ca^{2+} transient recordings from a male and a female cardiac myocyte at 0.5 Hz steady-state stimulation, 1.5 mM $[Ca^{2+}]_o$ and different concentrations of isoprenaline. The top panel shows Ca^{2+} transients from a male cell which has been exposed to 0, 10^{-12}, 10^{-9} and 10^{-7} M isoprenaline, whilst the bottom panel shows Ca^{2+} transients from a female cell under the same conditions.

other hand, tolerated this concentration of isoprenaline well with none of the nine cells studied exhibiting any sign of developing spontaneous activity or Ca^{2+} overload.

The average data for the effects of isoprenaline on the baseline $[Ca^{2+}]_i$, peak $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} transient are shown in Figure 3.16. Values are expressed as a percentage change from steady state levels in the absence of isoprenaline. At each of the isoprenaline concentrations used the increases in the baseline, peak and amplitude of the Ca^{2+} transient, when expressed relative to steady-state values, were significantly smaller ($p<0.03$) in the female cardiac myocytes when compared with the male cardiac myocytes. Data taken at 10^{-7} M isoprenaline were omitted due to the high incidence of spontaneous activity seen in the male cells at this concentration.

3.2.6 Effects of Isoprenaline on Cell Shortening

The effects of the addition of isoprenaline on the shortening of male (n=10, N=6) and female (n=8, N=5) cardiac myocytes is shown in Figure 3.17. Values are expressed as a percentage increase from shortening at 0 M isoprenaline. It is apparent from this figure that the addition of isoprenaline resulted in increased shortening in both male and female cardiac myocytes, with the higher isoprenaline concentration resulting in larger increases in myocyte shortening for both genders. The increase in shortening appears to be greater in the male cardiac myocytes when compared with the female cardiac myocytes, however due to the large variability in the data set, this difference was not significant.

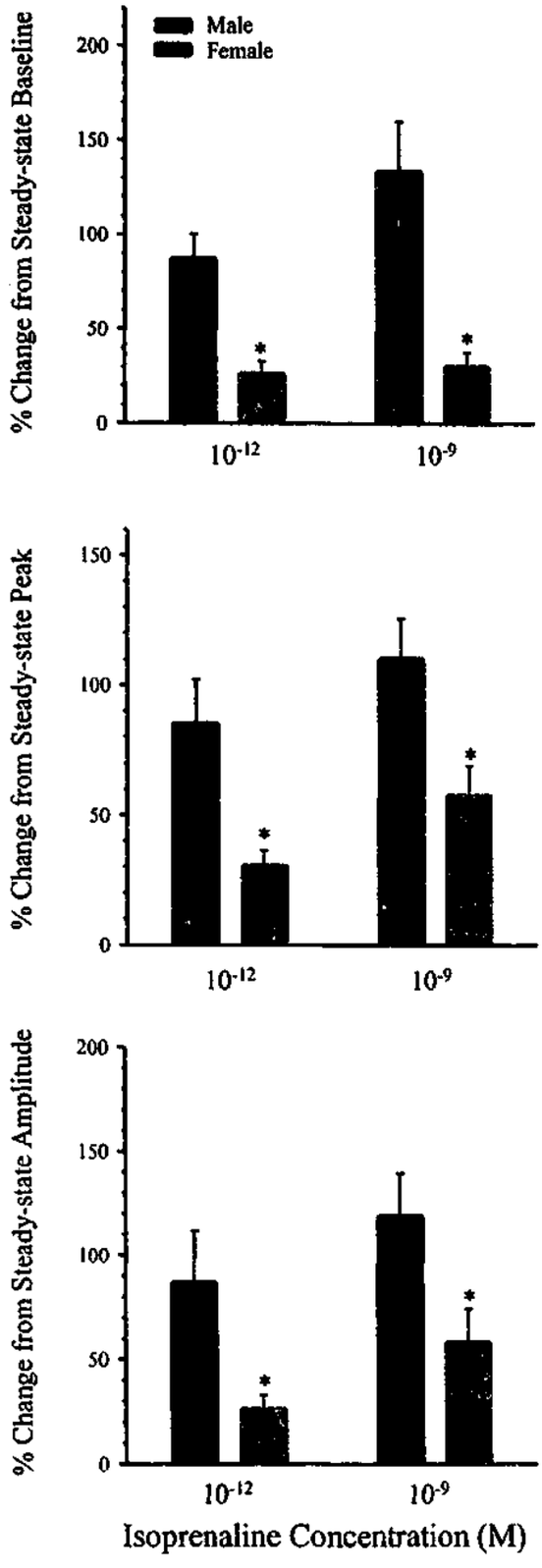


Figure 3.16: Effects of different concentrations of isoprenaline in male (n=10, N=6) and female (n=9, N=5) cardiac myocytes. Isoprenaline-induced changes in baseline $[Ca^{2+}]_i$, peak $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} transient values are presented as a percentage change from steady-state levels in the absence of isoprenaline. Values are mean ± SEM. * denotes that the value from the female cardiac myocyte is significantly different ($p<0.05$) from the corresponding value for the male cardiac myocyte.

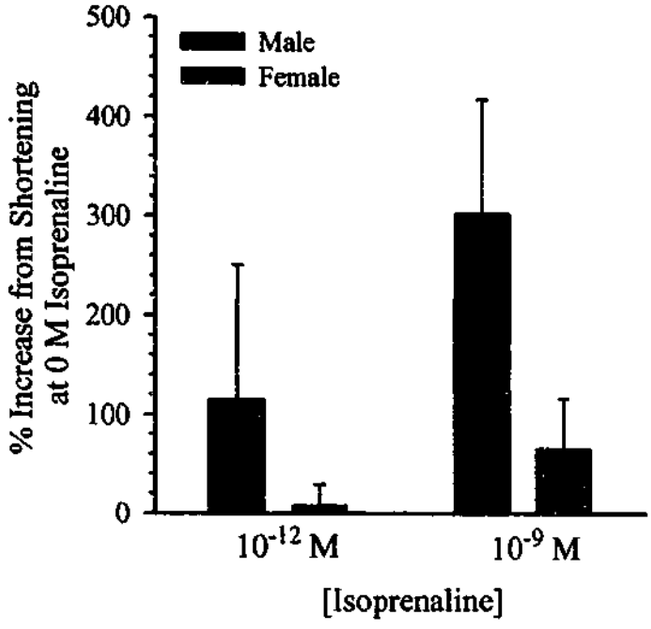


Figure 3.17: Effects of isoprenaline on the shortening of male (n=10, N=6) and female (n=8, N=5) cardiac myocytes stimulated to contract at 0.5 Hz in 1.5 mM $[Ca^{2+}]_o$. Values are expressed as a percentage increase from shortening at 0 M isoprenaline and are mean ± SEM.

3.2.7 Time Course of the Ca^{2+} Transient Decay

Figure 3.18 shows traces from male and female cardiac myocytes stimulated to contract at 0.5 Hz steady-state in 1.5 mM Ca^{2+}. The top trace in this figure shows single Ca^{2+} transients recorded from a male and a female cell adjusted to the same baseline value to facilitate comparison. These traces show firstly, that the peak and amplitude of the Ca^{2+} transient are larger in the male cardiac myocyte, when compared with the female cardiac myocyte. In addition the time course of decay of the Ca^{2+} transients appears faster in the male cells when compared with the female cells. This is confirmed in the bottom traces which again show the same transients from male and female cardiac myocytes recorded at 0.5 Hz stimulation frequency in 1.5 mM $[Ca^{2+}]_o$. However, in the lower panel of Figure 3.18 the transients have been normalised to the same peak amplitude and superimposed to facilitate comparison of their time course. It is clear from these records that the rate of decay of the Ca^{2+} transient is faster in the male cell. Transients recorded from each cell under these conditions were fitted with a single exponential function from which a time constant was obtained.

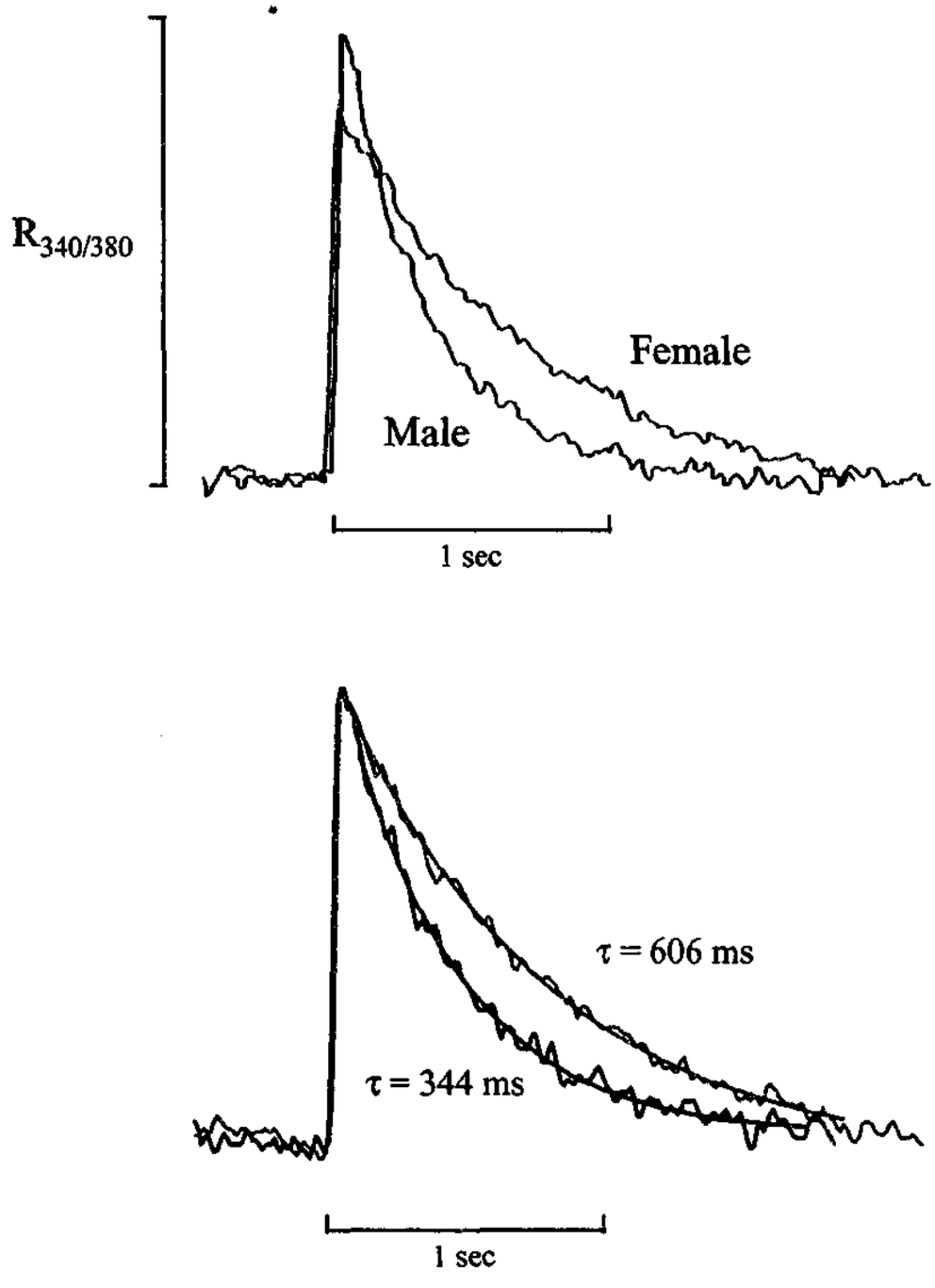


Figure 3.18: Example of single Ca^{2+} transients stimulated to contract at 0.5 Hz steady-state in 1.5 mM $[Ca^{2+}]_o$ from a male and a female cardiac myocyte. The top panel shows a comparison of raw data traces. The transients in the bottom panel have been scaled to the same amplitude to facilitate comparison. Both of the transients in the bottom panel have been fitted with a single exponential function from which a time constant was derived. The solid line shows the fitted exponential function. Time constant values shown here are representative of each individual trace.

Figure 3.19 shows the average time constants of the rate of decay of the Ca^{2+} transient for male (n=31, N=13) and female (n=32, N=14) cardiac myocytes. It is evident that the rate of decay of the calcium transient was significantly slower ($p<0.001$) in female cardiac myocytes than male cardiac myocytes.

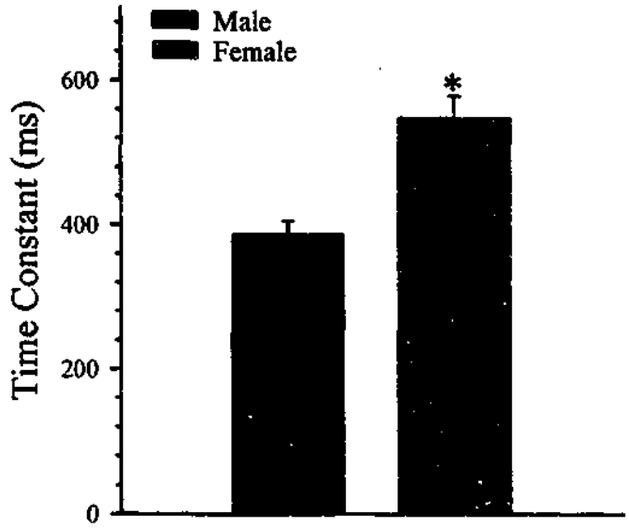


Figure 3.19: Average time constant values for the rate of decay of the Ca^{2+} transient in male (n=31, N=13) and female (n=32, N=14) cardiac myocytes. Values are mean ± SEM. * denotes that the value for the female cardiac myocytes is significantly different ($p<0.001$) from the corresponding value for the male cardiac myocytes.

Figure 3.20 shows the effects of isoprenaline on the time course of decay. The top panel of this figure illustrates the increase in the rate of decay of the Ca^{2+} transient with the addition of isoprenaline. This increase was expressed as a percentage of 0 M isoprenaline, and the mean values for this are shown in the bottom panel of Figure 3.20. Although there appears to be a much greater increase in the rate of decay of the Ca^{2+} transient in the male (n=8, N=6) cardiac myocytes when compared with the female (n=8, N=5) cardiac myocytes, this difference was not statistically significant. This is most likely due to the variability apparent within the data set.

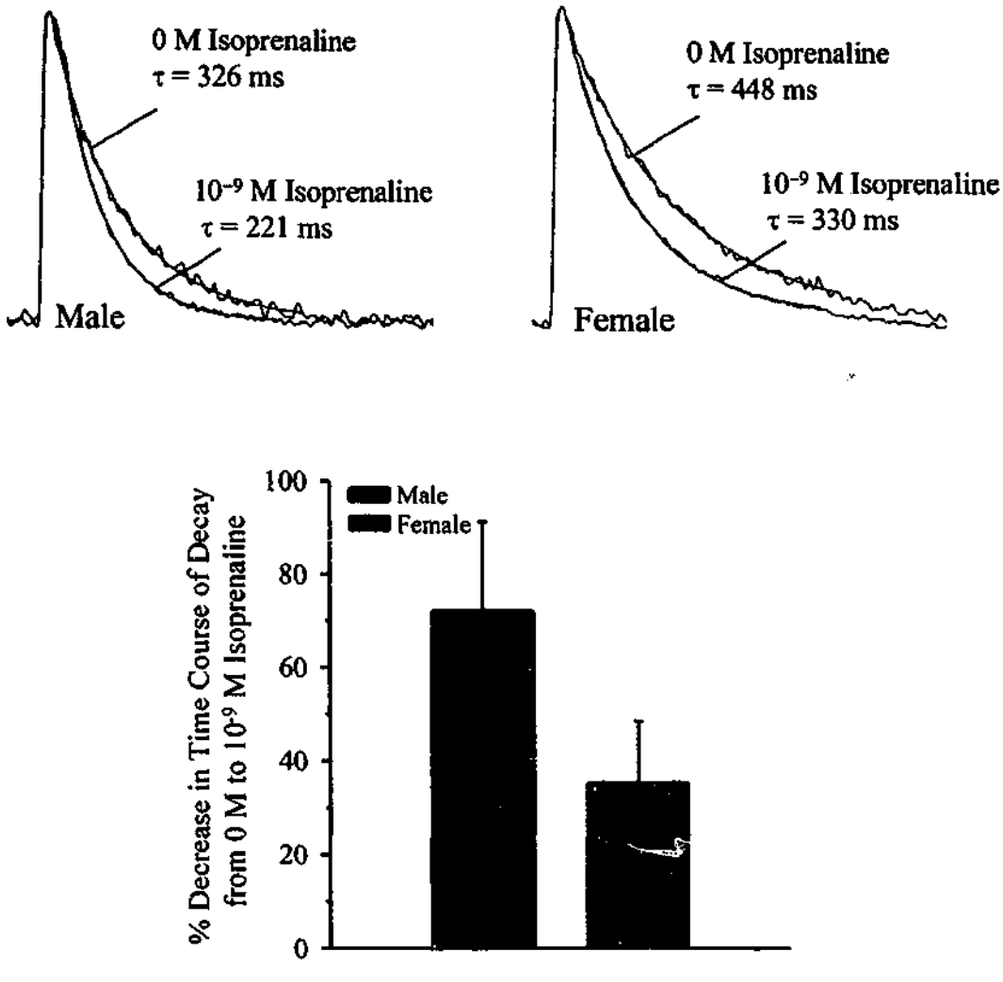


Figure 3.20: Effects of isoprenaline on the time course of decay of the Ca^{2+} transient. The top panel shows male (n=8, N=6) and female (n=8, N=5) Ca^{2+} transients exposed to 0 M and 10^{-9} M isoprenaline. The transients have been scaled to the same amplitude to facilitate comparison, and have been fitted with a single exponential function from which a time constant was derived. The bottom panel shows the percentage decrease in the time constant from 0 M to 10^{-9} M isoprenaline in male and female cardiac myocytes.

3.2.8 Time Course of Myocyte Shortening and Re-lengthening

The differences in amplitude and time course of the Ca^{2+} transient were accompanied by differences in the cells contractile responses recorded in terms of cell length changes. Data on the amplitude of cell shortening has already been presented in Section 3.2.4. In this section aspects of the time course of the changes in cell length will be considered. Cell shortening and re-lengthening was analysed for cells stimulated at 0.5 Hz in 1.5 mM $[Ca^{2+}]_o$. Figure 3.21 shows length traces from a male and a female cell that have been normalised to the same peak amplitude and superimposed to facilitate comparison of the time to peak shortening and time to 50% relaxation. Analysis of the results from

a number of cells (n>15 for each gender) revealed that, in the female cardiac myocytes, the time to peak shortening and the time to 50% relaxation were both significantly longer ($p<0.02$) than in male cardiac myocytes.

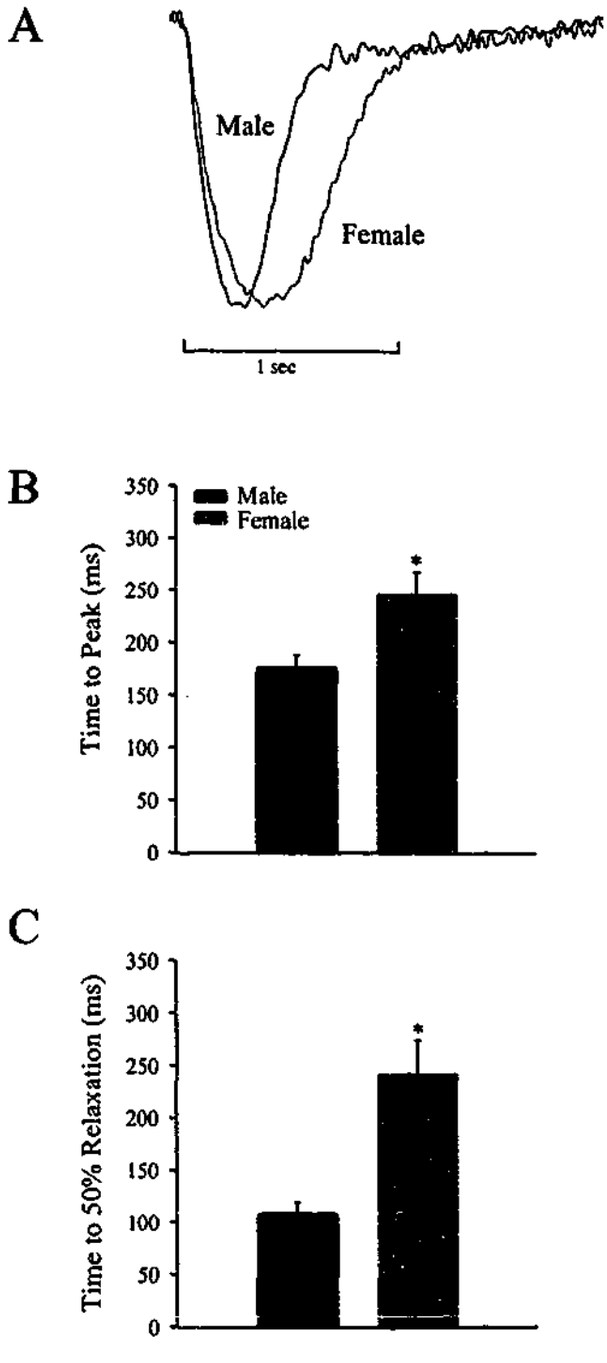


Figure 3.21: Time course of cell length changes at 0.5 Hz steady-state stimulation in 1.5 mM $[Ca^{2+}]_o$. **A.** Original recordings of changes in cell length from a male and a female cardiac myocyte. The traces have been normalised to the same amplitude and superimposed to facilitate comparison. **B** and **C.** Mean values for the time to peak shortening and time to 50% relaxation respectively in male (n=17) and female (n=16) myocytes. Values are mean ± SEM. * denotes that the value for the female cardiac myocyte is significantly different ($p<0.05$) from the corresponding value for the male cardiac myocyte.

3.2.9 Determination of SR Ca^{2+} Content in Male and Female Cardiac Myocytes

Figure 3.22 illustrates a typical recording from the protocol employed to investigate the SR Ca^{2+} content using caffeine-induced Ca^{2+} release in male and female cardiac myocytes. Cells were stimulated to contract at 0.5 Hz, given a 10 second rest, followed by a rapid application of 20 mM caffeine which resulted in a large calcium release. The mean amplitude of this caffeine-induced calcium release in male (n=13, N=3) and female (n=14, N=5) cardiac myocytes is presented in the bottom panels of Figure 3.22. When comparing the amplitude of the caffeine-induced Ca^{2+} release alone, it appears that the male cardiac myocytes had a larger release of Ca^{2+} from the SR than the female cardiac myocytes. This difference was not, however, statistically significant. If, however, the amplitude of the caffeine-induced Ca^{2+} release is divided by the pre-caffeine steady-state amplitude in each cell, it would appear that the female cardiac myocytes in fact had a greater caffeine-induced Ca^{2+} release when expressed relative to steady-state levels. This difference, however, was not statistically significant.

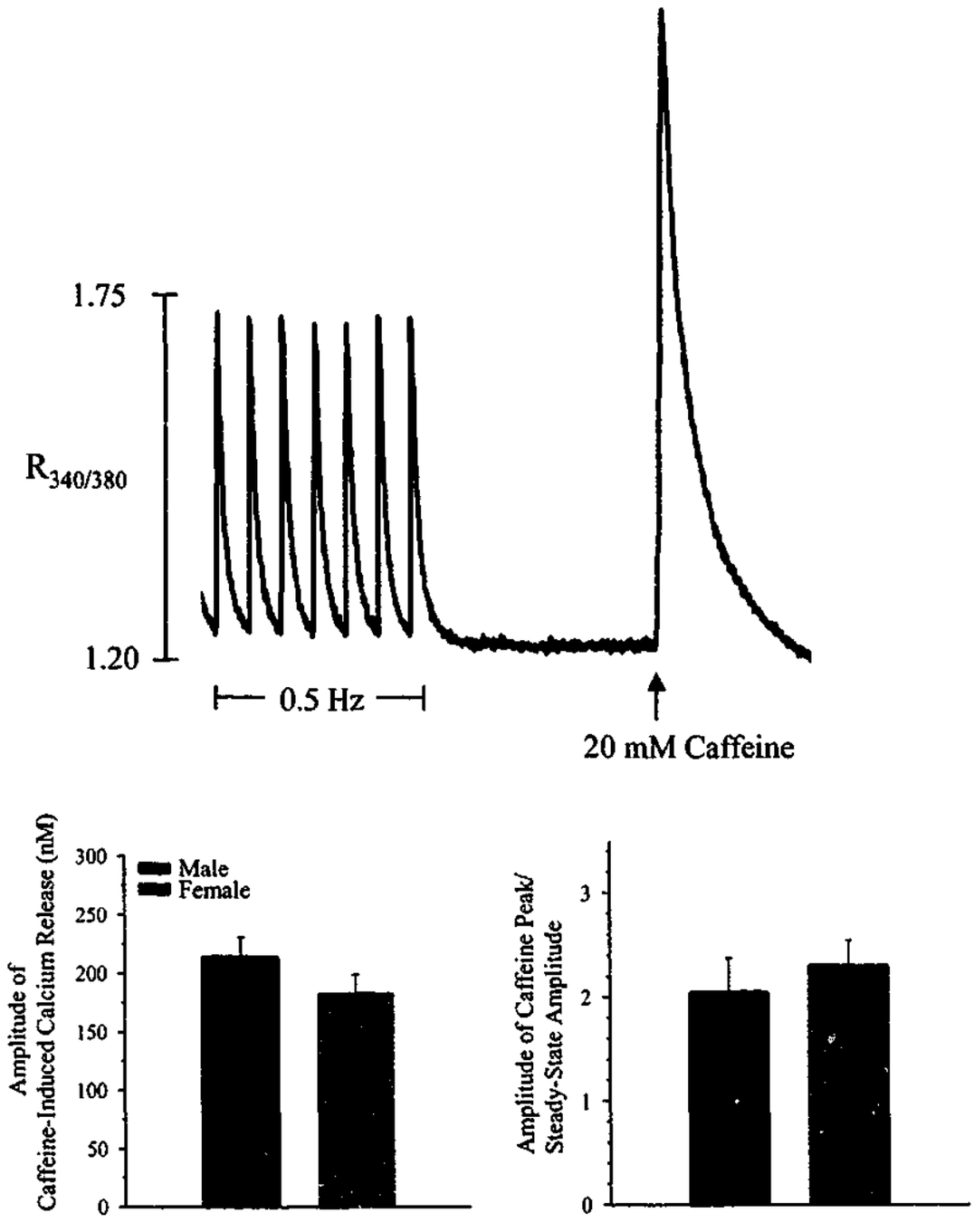


Figure 3.22: Effect of rapid application of 20 mM caffeine on $[Ca^{2+}]_i$ in a male cardiac myocyte. Male and female cells were stimulated to contract at 0.5 Hz steady-state, given a 10-s rest followed by addition of 20 mM caffeine. The mean amplitude of the caffeine-induced calcium release in both male (n=13, N=3) and female (n=14, N=5) cardiac myocytes is shown in the bottom panel, along with the mean values for the amplitude of the caffeine peak divided by the steady-state amplitude. Values are mean ± SEM.

3.3 Discussion

The risk of developing cardiovascular disease has a gender-based element, with pre-menopausal women having a significantly lower risk than age-matched men (Barrett-Connor and Bush, 1991; Stampfer *et al.*, 1991; Grady *et al.*, 1992; Chow, 1995; Gardin *et al.*, 1995). This reduction in risk has been linked to the sex steroid estrogen and its actions on the vascular system (Barrett-Connor and Bush, 1991; Gerhard and Ganz, 1995; Farhat *et al.*, 1996; Pelzer *et al.*, 1997) and more recently to effects on the heart itself (Grohe *et al.*, 1997; Meyer *et al.*, 1998). The precise nature of possible direct actions of estrogen on the heart are still unclear, however, it is possible that estrogen may produce its beneficial effects through changes in Ca^{2+} movements within the heart (Jiang *et al.*, 1992; Collins *et al.*, 1993; Leblanc *et al.*, 1998; Meyer *et al.*, 1998). The present study has shown that male cardiac myocytes consistently have a higher $[Ca^{2+}]_i$ than female cardiac myocytes under a variety of conditions.

Increases in $[Ca^{2+}]_o$ resulted in increases in resting $[Ca^{2+}]_i$, peak $[Ca^{2+}]_i$, and the amplitude of the Ca^{2+} transient in both male and female cardiac myocytes. Increasing $[Ca^{2+}]_o$ increases the concentration gradient between the extracellular and intracellular compartments and thus the driving force for Ca^{2+} entry. As $[Ca^{2+}]_o$ increases, Ca^{2+} influx upon excitation will increase, resulting in larger amounts of trigger calcium available for calcium-induced calcium release. Since it is now well recognised that the amount of calcium that enters the cell through the L-type calcium channel determines the amount of calcium released from the SR (Bassani *et al.*, 1995), the increased amounts of trigger calcium available would be expected to result in a larger release of the calcium from the SR, greater calcium activation of the contractile machinery and hence increased cardiac contraction.

Increases in stimulation frequency also resulted in increases in resting $[Ca^{2+}]_i$, peak $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} transient in both male and female cardiac myocytes. This positive staircase or force-frequency phenomenon was first described by Bowditch (1871), who demonstrated that an increase in stimulation frequency resulted in enhanced myocardial contractility. This increase has been attributed to an increased entry of Ca^{2+} into cardiac muscle (increased Ca^{2+} current) leading to higher diastolic $[Ca^{2+}]_i$ as a result of more action potentials per unit time and also less time for Ca^{2+} to be extruded from the cell during contractions. As a result of the increased diastolic

$[Ca^{2+}]_i$ there will also be an increase in SR Ca^{2+} available for release (Bers, 1991). In addition, the increase in frequency would result in increased Na^+ entry due to more action potentials per unit time, which results in greater $[Na^+]_i$ which would favour increased Ca^{2+} entry via the Na^+/Ca^{2+} exchanger (Borzak *et al.*, 1991). Overall this results in enhanced Ca^{2+} entry, greater release of Ca^{2+} from the SR and therefore greater amounts of Ca^{2+} available to activate the contractile machinery.

Interestingly, studies of isolated rat cardiac muscle generally report a negative force-frequency relationship with increases in stimulation frequency resulting in reduced amounts of force produced (Capogrossi *et al.*, 1986; Bouchard and Bose, 1989). There is disagreement about whether the rate staircase of the rat is strictly negative (Borzak *et al.*, 1991) with a study by Frampton *et al.* (1991) even showing both positive and negative force-frequency relationships within the one study. The rat cardiac muscle force-frequency relationship has, however, been shown to be exceptionally sensitive to experimental conditions such as $[Ca^{2+}]_o$, frequency range examined and temperature (Layland and Kentish, 1999), perhaps indicating that the varied results seen in many different studies undertaken can be related to experimental conditions.

At all $[Ca^{2+}]_o$, the peak $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} transient were, on average, significantly higher in male cells when compared with female cells and this was observed across a range of stimulus frequencies. It is interesting to note that as the $[Ca^{2+}]_o$ is increased, the differences between the male and female peak $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} transient become greater. This suggests that the more $[Ca^{2+}]_o$ is increased, the greater the gender-based difference. The smaller Ca^{2+} transient in the female cells may be attributed to a smaller trigger flux of Ca^{2+} into the cell through the L-type Ca^{2+} channels.

Several studies have reported acute inhibitory effects of 17β-estradiol on the L-type Ca^{2+} current in both cardiac (Jiang *et al.*, 1992; Sitzler *et al.*, 1996; Meyer *et al.*, 1998) and smooth muscle (Han *et al.*, 1995; Nakajima *et al.*, 1995). This appears to occur through a non-genomic pathway (Meyer *et al.*, 1998) but at present has only been demonstrated with exogenous 17β-estradiol applied at concentrations two to three orders of magnitude higher than physiological plasma concentrations. Perhaps of more relevance to the present experiments is the possible genomic regulation of cardiac L-type Ca^{2+} channel expression by estrogen. Johnson *et al.* (1997) have reported increased expression of the cardiac L-type Ca^{2+} channel in mice whose estrogen receptor has been

disrupted (ERKO). If estrogen serves to downregulate L-type Ca^{2+} channel expression, then one predicted consequence would be a reduced Ca^{2+} influx in cardiac myocytes from females as compared to males. This would most likely lead to a lower peak $[Ca^{2+}]_i$ and smaller amplitude Ca^{2+} transient in female cardiac myocytes, as was observed in the present study.

The change in cell shortening of both male and female cardiac myocytes was also measured in an attempt to see if the differences in the Ca^{2+} transient were reflected in contractile performance. The extent of shortening was significantly smaller in the female cells when compared with the male cells, along with a longer time to peak and time to 50% relaxation. These results, which are consistent with the Ca^{2+} data, are also suggestive of less Ca^{2+} entry and intracellular Ca^{2+} mobilisation in the female cell.

Another possible basis for the reduced amplitude of the Ca^{2+} transient and peak $[Ca^{2+}]_i$ in the female cardiac myocytes could be a reduced SR Ca^{2+} content and a reduced Ca^{2+} release as a consequence of this. Several investigators have suggested the possibility of gender-based differences in SR function (Penpargkul *et al.*, 1981; Capasso *et al.*, 1983; Leblanc *et al.*, 1998). In rat ventricular myocytes the SR is believed to be responsible for approximately 90% of the cytosolic Ca^{2+} removal during relaxation (Bassani *et al.*, 1994). Therefore, measurement of the rate of relaxation or decay of the Ca^{2+} transient would give a good indication of the rate of SR Ca^{2+} uptake. The rate of decay of the Ca^{2+} transient was found to be significantly slower in the female myocytes when compared with the male myocytes. This corresponds well with the slower time to 50% relaxation observed in the female myocyte. Leblanc *et al.* (1998) also reported that Ca^{2+} transients in 10-month-old female rats were significantly reduced and had a diminished rate of decay when compared with those of age-matched male rats. The most obvious reason for this outcome would be a reduction in SR Ca^{2+}-ATPase activity, as suggested by Penpargkul *et al.* (1981), who found that SR isolated from the hearts of female rats tended to have lower enzymatic activities than those from male rats. Although differences in SR Ca^{2+}-ATPase activity provides a plausible explanation for the differences in the rate of decay of the Ca^{2+} transient, it is important to recognise that differences in other Ca^{2+} extrusion mechanisms such as the Na^+/Ca^{2+} exchanger could also play a role.

The slower time course of decay of the Ca^{2+} transient in the female cells suggests a reduction in the rate of SR Ca^{2+} uptake, and this might be expected to lead to a

reduction in SR Ca^{2+} content. Caffeine-induced Ca^{2+} release responses were used to compare the Ca^{2+} content of the SR (under steady-state conditions) in male and female cardiac myocytes. However, although the caffeine-induced Ca^{2+} release from the SR tended to be smaller in the female cells when compared with the male cells, the difference was not found to be statistically significant. This suggests that the reduced amplitude of the Ca^{2+} transient, under conditions of steady-state stimulation, in the female cells is more likely to be the result of a reduced trigger Ca^{2+} influx with a consequent reduction in SR Ca^{2+} release. In addition to the above findings, the amplitude of the caffeine-induced Ca^{2+} release normalised to pre-caffeine steady-state Ca^{2+} transient levels was slightly larger in the female cardiac myocytes when compared with male cardiac myocytes. This difference was not however, statistically significant. If the SR Ca^{2+} content is the same in male and female cardiac myocytes, then the release of the Ca^{2+} store with caffeine would be expected to result in similar amounts of Ca^{2+} in the cytoplasm in both genders. However, when the amplitude of the caffeine-induced Ca^{2+} release is expressed relative to the pre-caffeine steady-state Ca^{2+} transient amplitude, this ratio would be expected to be larger in the female because of the reduced amplitude of the steady-state Ca^{2+} transient in female cardiac myocytes when compared with the male cardiac myocytes. Therefore this finding supports the idea that differences in trigger influx of Ca^{2+} from the extracellular space are responsible for the difference in the amplitude of the Ca^{2+} transient between male and female cardiac myocytes, rather than a change in the Ca^{2+} content of the SR.

β-adrenergic agonists, such as isoprenaline, are known stimulators of Ca^{2+} flux through the L-type Ca^{2+} channels and the SR (Tada and Katz, 1982; Kurihara and Konishi, 1987). Isoprenaline increased baseline $[Ca^{2+}]_i$, peak $[Ca^{2+}]_i$, and the amplitude of the Ca^{2+} transient in both male and female cardiac myocytes, and these effects were concentration dependent. Proportionately, as a change from steady-state levels in the absence of isoprenaline, the increases in each parameter of $[Ca^{2+}]_i$ induced by isoprenaline were greater in the male cells when compared with the female cells. These data are again consistent with a greater Ca^{2+} influx in the male cardiac myocytes and a greater enhancement of Ca^{2+} influx by isoprenaline in these cells as compared to the female myocytes. Whilst the differences between male and female cardiac myocytes in their responses to isoprenaline are consistent with a greater Ca^{2+} influx in male cells, it is important to recognise that gender-related differences in β-adrenergic receptor

responsiveness could also contribute to this. There are some early observations that are suggestive of a downregulation of β-adrenergic responsiveness in estrogen treated rats (Fregly and Thrasher, 1977). Isoprenaline also increased the rate of decay of the Ca^{2+} transient in both male and female myocytes and although this effect appeared to be more pronounced in the male cells, there was considerable variability, with differences in the isoprenaline-induced increase in the time constant of decay of the Ca^{2+} transient not significantly different between male and female myocytes. This suggests that the ability of isoprenaline to stimulate SR Ca^{2+} uptake is similar in cardiac myocytes from male and female rats.

Interestingly, the male cells appeared to be unable to tolerate the highest concentration of isoprenaline employed (10^{-7} M) without exhibiting spontaneous activity. In contrast, all female cells studied were able to contract continuously and without spontaneity at this concentration of isoprenaline. The spontaneous activity observed at 10^{-7} M isoprenaline in male cells is indicative of Ca^{2+} overload and may have arisen because of very high levels of Ca^{2+} influx into these cells under these conditions. If the female cardiac myocytes do indeed have a reduced expression of the L-type Ca^{2+} channel they may be afforded some level of protection against Ca^{2+} overload under these conditions.

The use of vaginal smearing confirmed that the female rats utilised for this study were at random stages of their estrous cycle. Concern regarding results obtained at different times during the female estrous cycle have been raised, and were addressed by analysing data according to the different stage of the estrous cycle of each rat. No significant rei.tionship between the time of the estrous cycle and the $[Ca^{2+}]_i$ levels measured was detected. In addition, even without these measurements, the large difference that is apparent between the male and female groups in the amplitude of the Ca^{2+} transient suggests that the estrous cycle is not a factor. If certain female animals had small amplitude Ca^{2+} at one point in the estrous cycle, whilst other animals had large amplitude Ca^{2+} at another point in the estrous cycle, then these values would be averaged to bring about a modest decrease, or no change in the amplitude of the Ca^{2+} transient. The size of the difference in amplitude Ca^{2+} between male and female cardiac myocytes supports the idea that the stage of the estrous cycle has little bearing on the $[Ca^{2+}]_i$ measured.

The experiments undertaken in this chapter have shown clear differences in intracellular Ca^{2+} levels and cell shortening between cardiac myocytes from male and female rats.

Given the results obtained from these experiments and the evidence that estrogen may regulate expression of the cardiac L-type Ca^{2+} channel (Johnson *et al.*, 1997), the possibility exists that estrogen may serve to limit Ca^{2+} entry in the female heart as a way of protecting against Ca^{2+} overload within the female cardiac myocyte.

Chapter 4

Chapter 4

Effects of Gender on Papillary Muscle Contractile Force

The previous chapter highlighted differences in intracellular Ca^{2+} handling and cell shortening between male and female cardiac myocytes. The experiments in this chapter were undertaken to compliment the previous findings by trying to elucidate differences in contractile force between intact, multicellular cardiac muscle preparations from male and female rats.

The papillary muscle is found in the heart attached at one end to the ventricular wall and at the other end to the chordae tendineae. The main purpose of these muscles is to ensure that the atrioventricular (AV) valve is not forced back into the atrium when ventricular contraction occurs (Moffett *et al.*, 1993). Papillary muscles can be isolated from the heart with minimal dissection, thereby limiting injury to cardiac tissue during dissection. This, together with their favourable geometry, makes them one of the most suitable preparations with which to study myocardial contraction *in vitro* (Kiriazis and Gibbs, 1995).

Previous studies investigating gender differences in male and female papillary muscles have led to contradictory outcomes. Leblanc *et al.* (1998) studied papillary muscles from 2 to 14 month old male and female rat hearts under both isotonic and isometric

conditions. They showed that papillary muscles from female rats that were 6 months or over displayed smaller isometric and isotonic contractions, as well as slower maximal rates of tension development and decay, and slower shortening velocities than age-matched males. In accordance with this finding, Patterson *et al.* (1998) showed that estrogen replacement in ovariectomised rabbits decreased isometric force produced by papillary muscles when exposed to increased extracellular [Ca^{2+}]. In contrast, Wang *et al.* (1998) found a significant difference in the Ca^{2+} concentration-response curves between male and female papillary muscles, with females producing higher levels of developed tension at equivalent extracellular [Ca^{2+}]. Capasso *et al.* (1983) found no difference in peak isometric tension between male and female papillary muscles, however they did find that male rats showed significantly greater isometric time-to-peak tension and time to half relaxation with a depression of both the maximum rate of tension development and maximum rate of decay.

Given the contradictory outcomes of previous studies involving gender and papillary muscle contractile characteristics, and the findings of the previous chapter, it was the aim of the current set of experiments to elucidate if the gender differences in Ca^{2+} handling that were apparent in freshly isolated cardiac myocytes are also reflected in the force of contraction of intact male and female papillary muscles.

4.1 Methodology

4.1.1 Papillary Muscle Dissection Procedure

Rats were killed by decapitation under deep chloroform anaesthesia. The heart was rapidly excised and washed in bicarbonate-buffered Krebs-Henseleit solution (composition given in Section 4.1.2) containing 1.6 mM Ca^{2+}. Following removal of the pericardium and other adhering tissue, the heart was transferred into a beaker containing the same solution as above. The heart was allowed to beat in this beaker for approximately 1 minute and was then transferred to a second beaker containing the same solution. This process allowed residual blood to be removed from the heart. The heart was then removed from the beaker and attached, via an aortic cannula, to a syringe and perfused with 20 ml of bicarbonate-buffered Krebs-Henseleit solution containing 1.6 mM Ca^{2+} and 30 mM BDM to arrest mechanical activity. The heart was then placed in a dissection dish with a Sylgard (Dow Corning Corporation, Midland, MI., USA) base containing bicarbonate-buffered Krebs-Henseleit solution with 1.6 mM Ca^{2+} and 30 mM BDM bubbled with carbogen (95% O_2/5% CO_2). The heart was pinned to the base of the dish by the aorta and at the apex. The free wall of the right ventricle was located and reflected back and away from the heart by cutting along the septal border. An incision was then made through the midline of the interventricular septum exposing the papillary muscles in the left ventricle. Once the left ventricle was opened the heart was left to sit in the BDM solution for 20 minutes. BDM is known to arrest mechanical activity and protect against tissue cutting injury, and it has been shown that papillary muscle experiments which utilise BDM to protect against cutting injury during the dissection procedure show no difference in energetic and mechanical properties when compared with muscles that have not been exposed to BDM (Kiriazis and Gibbs, 1995). Following this 20 minute incubation time, the papillary muscles were separated from the heart wall and, if their diameter was considered too large, were split longitudinally before being tied at either end with 4/0 non-capillary silk thread. The muscle was then placed under tension using a stainless steel spring clamp attached to the silk threads before being removed from the heart and transferred to the force recording apparatus.

At the conclusion of each experiment, the muscle length was recorded whilst the muscle was still mounted on the force recording apparatus and, therefore, still at the experimental length. The silk ties were then removed and the blotted wet weight of the

muscle was determined. The papillary muscles from male animals (n=26) used in the study had a length of 5.6 ± 0.2 mm with a weight of 5.2 ± 0.3 mg whilst the muscles from female animals (n=25) had a length of 4.7 ± 0.2 mm and a weight of 4.3 ± 0.4 mg.

The cross-sectional area of each muscle was calculated from the length and mass of the muscle, assuming a uniform cross-sectional area and a muscle density of 1.06 g/cm^3. All forces were subsequently expressed normalised for cross-sectional area (i.e. as mN/mm^2).

4.1.2 Solutions

The physiological saline solution used in the experiments described in this chapter was a bicarbonate-buffered Krebs-Henseleit solution. It had the following composition (in mM): 118.0 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 24.8 $NaHCO_3$, 1.6 $CaCl_2$ and 10.0 glucose. For experiments involving varying extracellular [Ca^{2+}], the $CaCl_2$ was simply omitted from the original solution and was then added back in appropriate amounts to achieve the desired concentrations.

Nifedipine (Sigma, St. Louis, MO, USA) was made up as a 10^{-2} M stock solution in dimethyl sulfoxide (DMSO) on the day of each experiment. Appropriate aliquots of this stock solution were added to the standard bicarbonate-buffered Krebs-Henseleit solution containing 1.6 mM Ca^{2+} to obtain the required final concentrations of nifedipine. The maximum addition of DMSO was 3 parts in 1000 which, by itself, had no effect on the contractile response of papillary muscles from either male or female rats.

Bay K8644 (1,4 Dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-3-pyridine carboxylic acid methyl ester) (Sigma, St.Louis, MO, USA) was made up as a 10^{-3} M stock solution in DMSO on the day of each experiment. Aliquots of this were added to the standard bicarbonate-buffered Krebs-Henseleit solution containing 0.8 mM Ca^{2+} to achieve the desired final bath concentrations. In the experiments involving Bay K8644 the Ca^{2+} concentration in the solution was lowered from 1.6 mM to 0.8 mM. Initially when 1.6 mM $[Ca^{2+}]_o$ was used, only very small or non-existent increases in force were observed when Bay K8644 was added to the bath. The $[Ca^{2+}]_o$ was, therefore, lowered to 0.8 mM to reduce the contractility of the muscle and provide greater scope for possible increases in force induced by Bay K8644.

4.1.3 Force Measurements

Isometric force recordings were used to monitor the contractile state of the papillary muscles. The muscle was mounted vertically on a stainless steel holder with the lower tie fixed to the base of the holder and the upper tie attached, via a stainless steel rod, to a light-weight magnesium alloy lever. The lever had a set of Entran semi-conductor strain gauges (Model ESB-160-350, Entran Devices Inc., Fairfield, NJ, USA) bonded to its upper and lower surfaces to allow force measurements to be made. The lever also allowed adjustment of the muscle length. Once the papillary muscle was attached to the lever system, it was immersed in a temperature regulated tissue bath. The experiments were conducted at 32°C.

In all experiments, once the muscle was attached and in position, it was placed in bicarbonate-buffered Krebs-Henseleit solution containing 1.6 mM Ca^{2+} and was stimulated at 0.2 Hz with 10 V, 2 msec square pulses delivered via platinum electrodes positioned on either side of the muscle at its upper and lower ends. The muscle was left to contract isotonically under a 1 g load for 1 hour before any experimental manipulations commenced. At the conclusion of this equilibration period the muscle length was fixed such that all further contractions were isometric. Once the isometric contractions had stabilised the muscle length was adjusted, if necessary, to be optimal for force development. The output of the force transducer was directed to a MacLab data recording and acquisition system (ADInstruments, NSW, Australia) where it was displayed and stored using Chart 3.3 software. Continuous recordings were made throughout the duration of the experiment. During equilibration times, however, the sampling rate was reduced to 40 samples/sec to minimise the size of the file. Once a steady-state was attained the sampling rate was increased to 200 samples/sec.

4.1.4 Protocols

4.1.4.1 Varying Extracellular [Ca^{2+}]

Following equilibration of the muscle in the normal 1.6 mM Ca^{2+} containing Krebs-Henseleit solution this was replaced with a solution containing 0.2 mM Ca^{2+}. The muscles were allowed to contract in this solution until a steady-state level of developed force was attained. Recordings were then taken at this Ca^{2+} concentration before further Ca^{2+} was cumulatively added to reach the next concentration desired. The force output of the muscle strips was recorded in response to the following extracellular Ca^{2+}

concentrations: 0.2, 0.4, 0.8, 1.2, 1.6, 2.0, 3.0 and 5.0 mM. At each concentration recordings were not made until developed force had stabilised at the new steady-state.

4.1.4.2 Nifedipine

Once the varying extracellular [Ca^{2+}] protocol was completed, the solution was replaced with the normal bicarbonate-buffered Krebs-Henseleit solution containing 1.6 mM [Ca^{2+}]. After the muscle had equilibrated to this concentration of $[Ca^{2+}]_o$, nifedipine was added cumulatively to the tissue bath to give final concentrations of: 10^{-7}, 3×10^{-7}, 10^{-6}, 3×10^{-6}, 10^{-5} and 3×10^{-5} M. After each addition of nifedipine, the muscle was left to contract until a new steady-state had been attained before the next addition of nifedipine.

4.1.4.3 Bay K8644

The effects of Bay K8644 were investigated in fresh preparations (not exposed to nifedipine) because of uncertainty as to whether nifedipine could be fully washed out following its addition. Therefore, a new muscle was always employed for the Bay K8644 protocol. Following the equilibration period, muscles were placed in a tissue bath containing bicarbonate-buffered Krebs-Henseleit solution with 0.8 mM [Ca^{2+}]. Once concentrations had reached a new steady-state recordings were made to establish a control, 0 M recording for Bay K8644. Bay K8644 was then added cumulatively to the tissue bath to give final concentrations of: 10^{-7}, 3×10^{-7}, 10^{-6} and 3×10^{-6} M. After each addition of Bay K8644, the muscle strip was allowed to attain a new steady-state before the concentration of Bay K8644 was increased.

4.1.5 Statistics

All data are presented as mean ± standard error of the mean (SEM). All protocols undertaken within this chapter were analysed using a one-way ANOVA with repeated measures.

4.2 Results

4.2.1 Effects of Gender and Varying Extracellular [Ca^{2+}] on Contractile Force

Figure 4.1 illustrates original force recordings from a male and a female papillary muscle exposed to different extracellular [Ca^{2+}]. The top panel shows recordings from a male papillary muscle whilst the bottom panel shows a female papillary muscle under the same conditions. In all instances the muscle was being stimulated at 0.2 Hz and the single contraction shown at each $[Ca^{2+}]_o$ represents the steady-state response under that condition. From these traces it is evident that, for both genders, as extracellular [Ca^{2+}] is increased, the contractile force produced by the papillary muscle also increases.

Figure 4.2 shows average active force values for both male (n=8) and female (n=9) papillary muscles at the different extracellular [Ca^{2+}]. This figure demonstrates the expected significant increase ($p<0.01$) in contractile force with increasing extracellular [Ca^{2+}] in both male and female papillary muscle strips. It is also interesting to note that in both genders force increases more rapidly at the lower Ca^{2+} concentrations and appears to begin to plateau once higher extracellular Ca^{2+} concentrations are reached, suggesting a point of saturation in the force response. This figure also shows that, on average, force is significantly greater ($p<0.04$) in the male papillary muscles when compared with female papillary muscles.

The data, when the force responses for each muscle were expressed as a percentage of the maximum developed by that muscle in the presence of 5.0 mM extracellular [Ca^{2+}], is shown in Figure 4.3. Similarly to Figure 4.2, an increase in extracellular [Ca^{2+}] results in a significant increase ($p<0.01$) in normalised force in both male and female muscles. In addition, at $[Ca^{2+}]_o$ below 5.0 mM the relative force was, on average, significantly greater ($p<0.04$) in male papillary muscles when compared with female papillary muscles.

The time to peak tension and time to 50% relaxation were also determined in both male (n=8) and female (n=9) papillary muscles when exposed to 1.6 mM $[Ca^{2+}]_o$. Figure 4.4 illustrates that no significant difference in either time to peak ($p=0.49$) or time to 50% relaxation ($p=0.71$) was apparent between male and female papillary muscles.

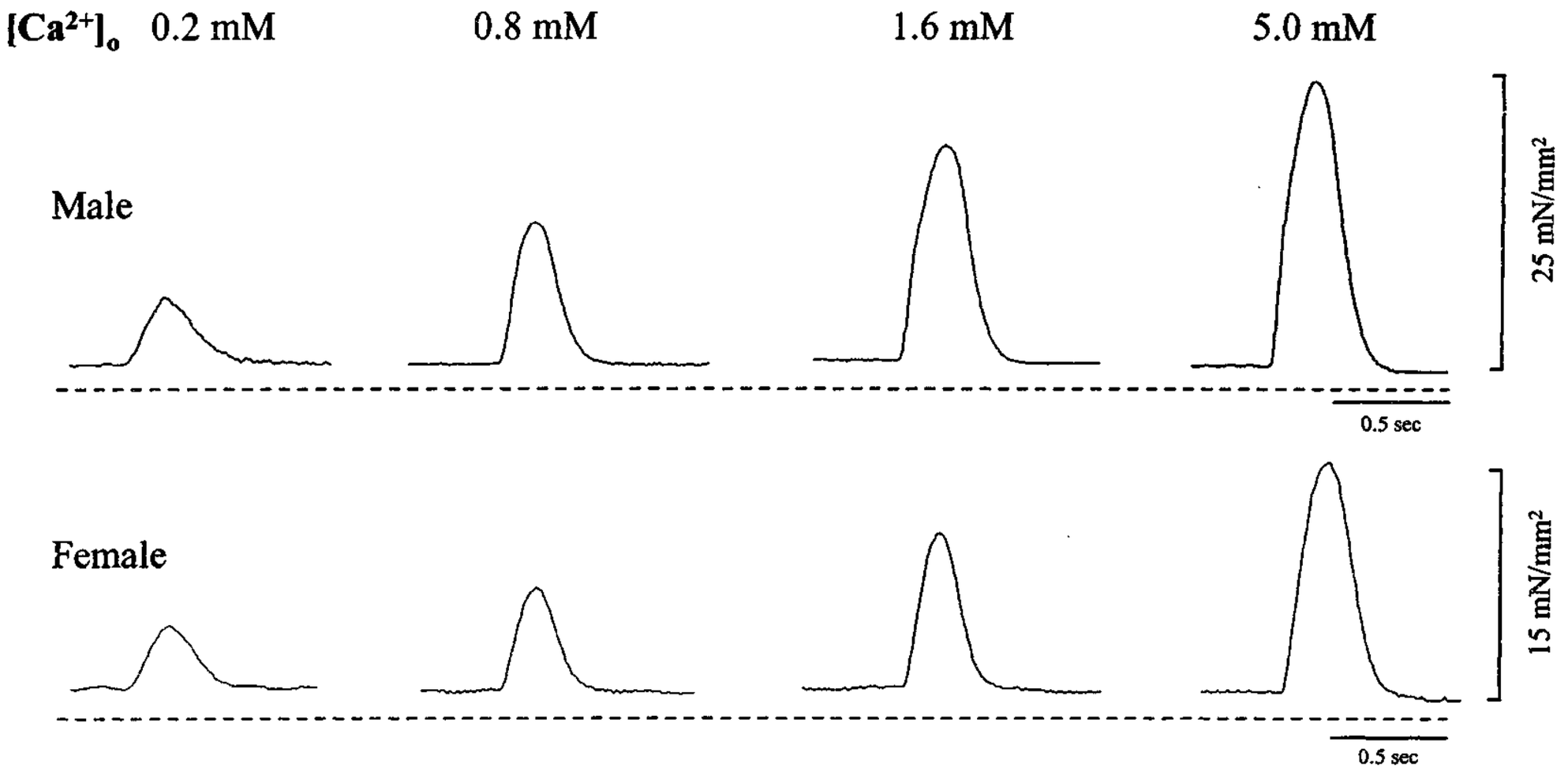


Figure 4.1: Example of force recordings from papillary muscles stimulated to contract at 0.2 Hz steady-state and exposed to different extracellular $[Ca^{2+}]$. The top panel shows force recordings from a male papillary muscle at 0.2, 0.8, 1.6 and 5.0 mM $[Ca^{2+}]_o$, whilst the bottom panel shows a female papillary muscle under the same conditions. The dashed line represents the point of zero force.

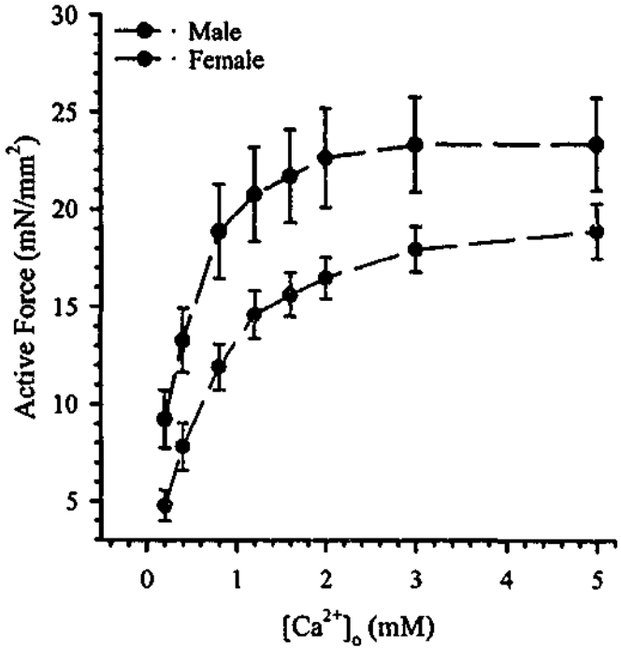


Figure 4.2: Average active force values in male (n=8) and female (n=9) papillary muscles stimulated to contract at 0.2 Hz steady-state in varying extracellular [Ca^{2+}]. Values are mean ± SEM.

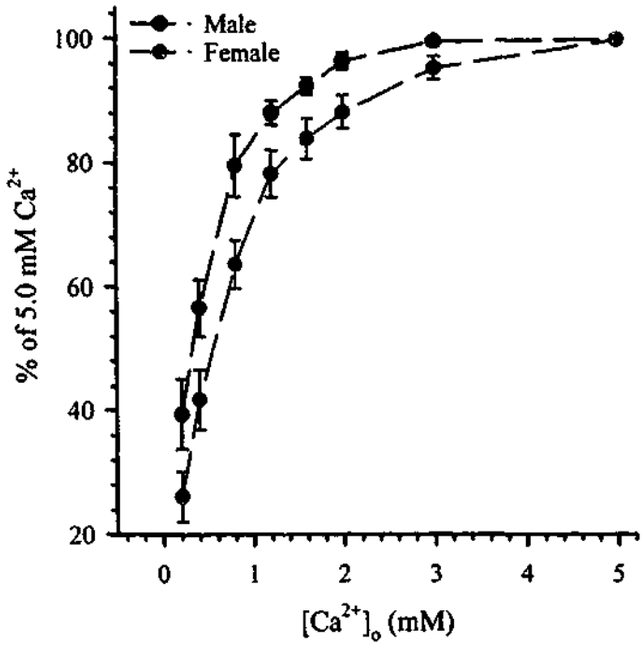


Figure 4.3: Steady-state active force of male (n=8) and female (n=9) rat papillary muscles stimulated at 0.2 Hz in the presence of varying extracellular [Ca^{2+}]. For each muscle, force has been expressed relative to that developed in the presence of 5.0 mM [Ca^{2+}]$_o$. Values are mean ± SEM.

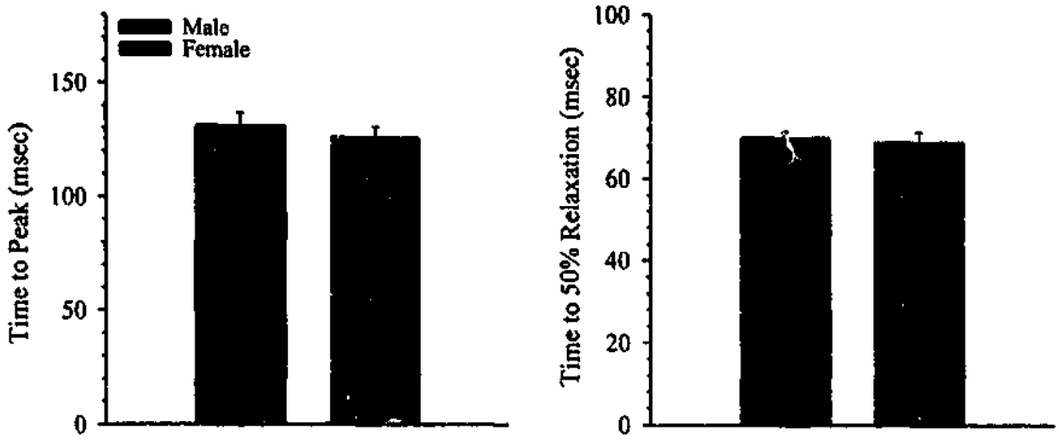


Figure 4.4: Average time to peak force (left panel) and time to 50% relaxation (right panel) in male (n=8) and female (n=9) papillary muscles in the presence of 1.6 mM $[Ca^{2+}]_o$. Values are mean ± SEM.

4.2.2 Gender and the Effects of the Calcium Channel Antagonist Nifedipine on Contractile Force

The effects of nifedipine on papillary muscle force generation are shown in Figure 4.5. This figure demonstrates individual traces from male and female papillary muscles at varying nifedipine concentrations. The top panel shows original force recordings from a male papillary muscle stimulated to contract at 0.2 Hz steady-state in 1.6 mM $[Ca^{2+}]_o$ and at varying nifedipine concentrations, whilst the bottom panel shows a female papillary muscle under the same conditions. A decrease in the contractile force is evident as the concentration of nifedipine is increased.

Figure 4.6 illustrates the average contractile force data for male (n=8) and female (n= 10) papillary muscles at the different concentrations of nifedipine. It is evident from this figure that nifedipine significantly ($p<0.001$) decreases developed force in both male and female papillary muscles in a concentration-dependent manner. It is also apparent that the male papillary muscle force always remained significantly greater ($p<0.02$) than the female papillary muscle force.

The data expressed as a percentage of the control (0 M nifedipine) steady-state force is shown in Figure 4.7. This further highlights that nifedipine significantly decreases ($p<0.001$) force in both male and female papillary muscles. In addition, it reveals that the concentration dependence of this decrease was significantly different between the

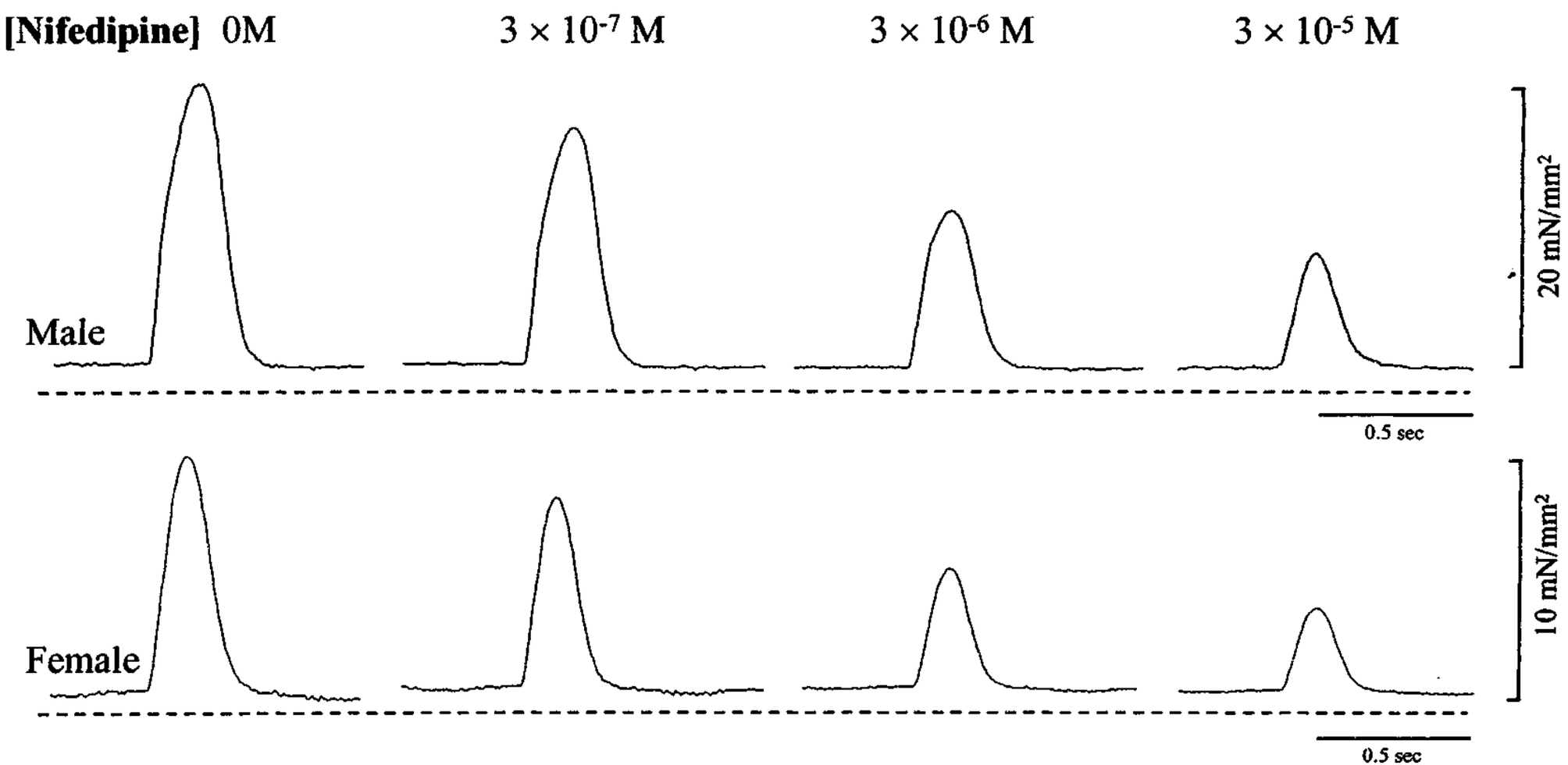


Figure 4.5: Example of force recordings from papillary muscles stimulated to contract at 0.2 Hz steady-state in 1.6 mM $[Ca^{2+}]_o$ and exposed to different concentrations of nifedipine. The top panel shows force recordings from a male papillary muscle at 0, 3×10^{-7}, 3×10^{-6} and 3×10^{-5} M nifedipine, whilst the bottom panel shows a female papillary muscle under the same conditions. The dashed line represents the point of zero force.

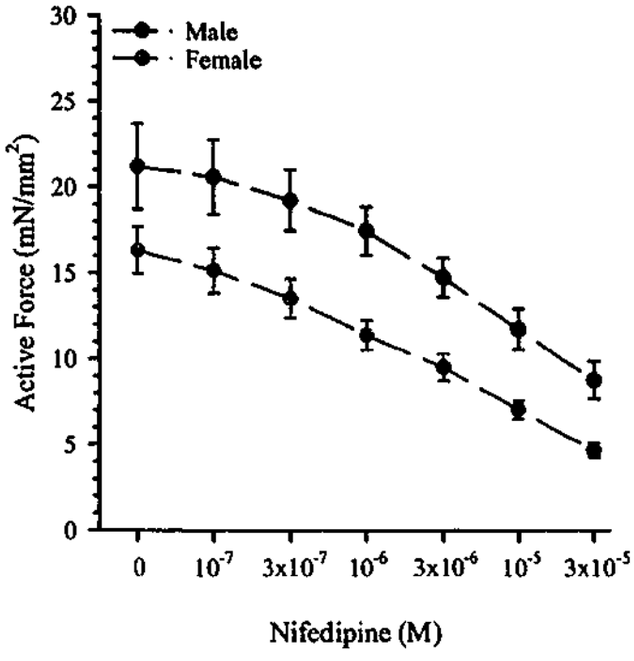


Figure 4.6: Average active force data for male (n=8) and female (n=10) papillary muscles stimulated to contract at 0.2 Hz steady-state in 1.6 mM $[Ca^{2+}]_o$ and at varying concentrations of nifedipine. Values are mean ± SEM.

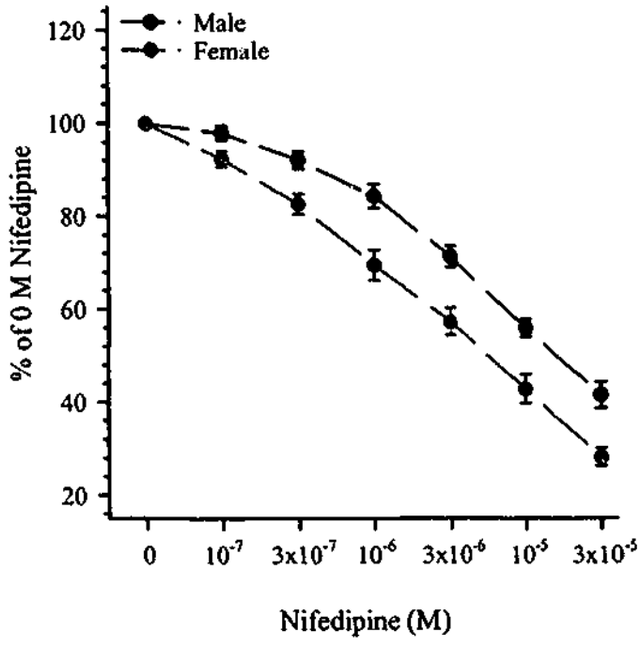


Figure 4.7: Effect of varying concentrations of nifedipine on male (n=8) and female (n=10) papillary muscle force expressed as a percentage of the control (0 M nifedipine) force response. All recordings were steady-state responses obtained from muscles stimulated to contract at 0.2 Hz. Values are mean ± SEM.

two genders ($p<0.002$), with the response of the female papillary muscles proportionately decreasing by a greater extent at each nifedipine concentration.

4.2.3 Gender and the Effects of the Calcium Channel Agonist Bay K8644 on Contractile Force

Figure 4.8 shows the effects of the addition of Bay K8644 on contractile force in male and female papillary muscles. The top panel shows original force recordings from a male papillary muscle stimulated to contract at 0.2 Hz steady-state in 0.8 mM $[Ca^{2+}]_o$ and at varying Bay K8644 concentrations, whilst the bottom panel shows a female papillary muscle under the same conditions. It is evident from this figure that Bay K8644 was able to modestly increase the contractile force of the male papillary muscle, however, it appears that it had a lesser effect on the contractile force of the female papillary muscle.

The average active tension values for male ($n=9$) and female ($n=11$) papillary muscles at the different Bay K8644 concentrations are shown in Figure 4.9. As the Bay K8644 concentration increased so too did the active tension ($p<0.001$) in male papillary muscles. A significant increase was, however, not detected in the female papillary muscles ($p=0.228$). In accordance with these findings the concentration-dependence of the increase in active force with the addition of Bay K8644 was significantly different ($p<0.003$) in female papillary muscles when compared with male papillary muscles. A significant difference ($p<0.03$) in the contractile force response between male and female papillary muscles at each concentration of Bay K8644 was also evident. Data are only shown for Bay K8644 concentrations up to 3×10^{-6} M since at concentrations above this the contractile force decreased in both male and female papillary muscles.

Figure 4.10 shows the increase in active force produced by Bay K8644 expressed as a percentage change from the steady-state force development in the absence of Bay K8644. Analysis of the data expressed in this manner also revealed that there was a significant concentration-dependent increase in force with Bay K8644 in male papillary muscles ($p<0.01$) but not in the female papillary muscles ($p=0.690$). Consequently statistical analysis revealed a significant difference in the concentration dependence of the effect of Bay K8644 on force development between male and female papillary muscles ($p<0.03$).

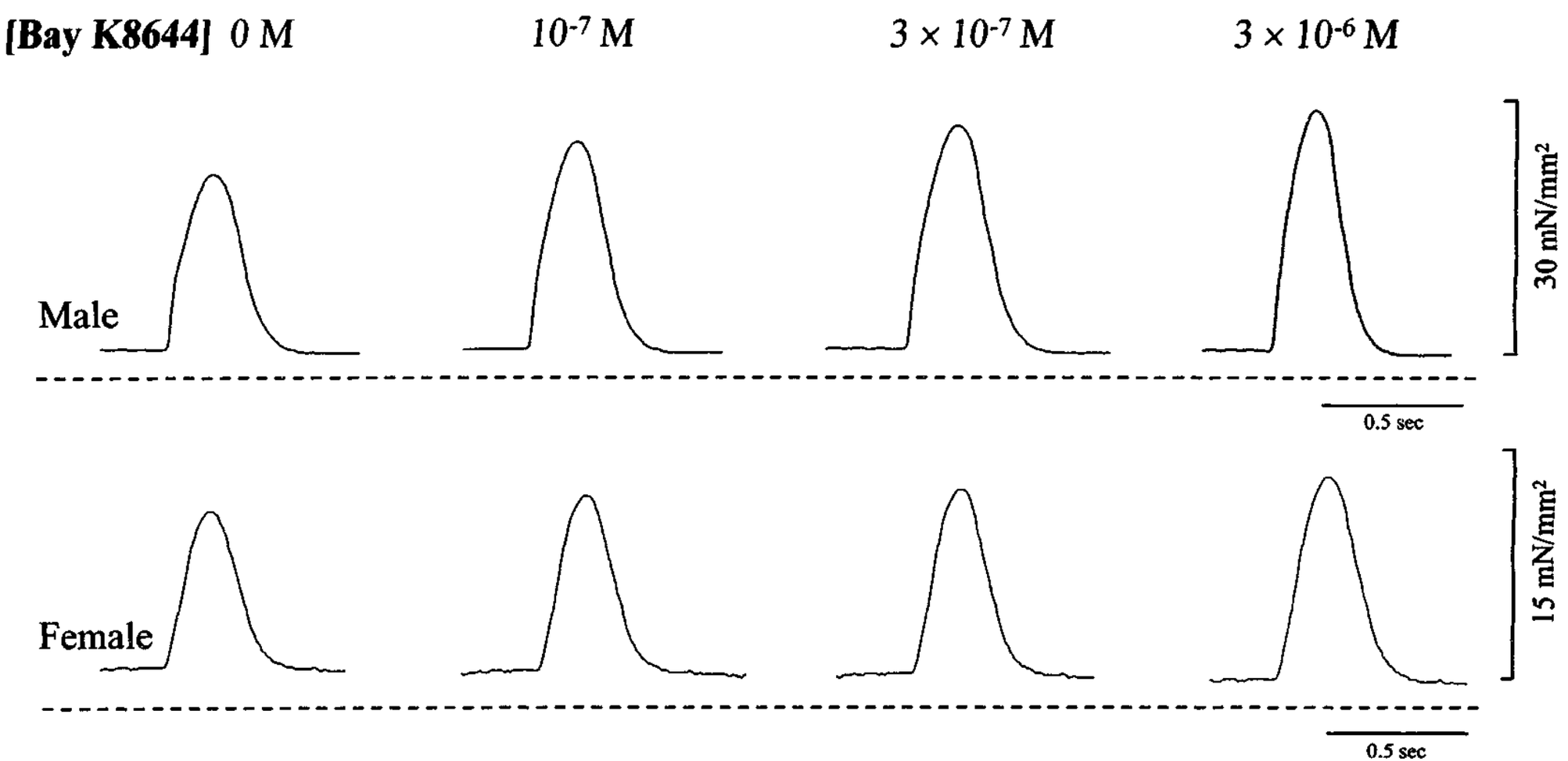


Figure 4.8: Example of force recordings from papillary muscles stimulated to contract at 0.2 Hz steady-state in 0.8 mM $[Ca^{2+}]_o$ and exposed to different concentrations of Bay K8644. The top panel shows force recordings from a male papillary muscle at 0, 10^{-7}, 3×10^{-7} and 3×10^{-6} M Bay K8644, whilst the bottom panel shows a female papillary muscle under the same conditions. The dashed line represents the point of zero force.

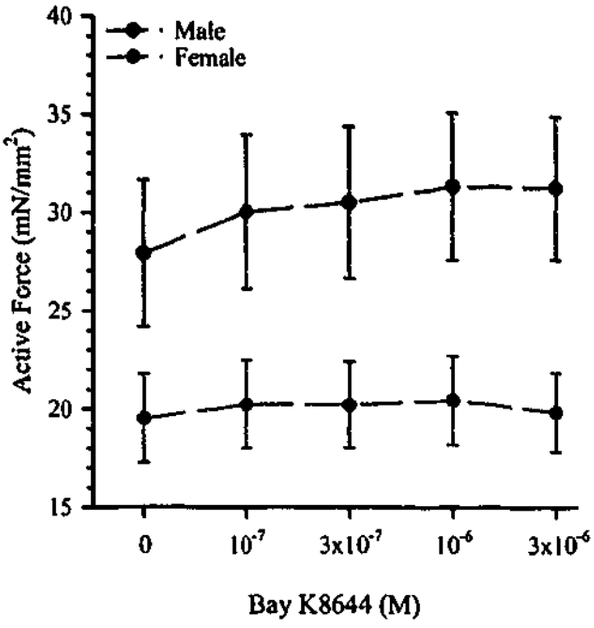


Figure 4.9: Average active force values in male (n=9) and female (n=11) papillary muscle stimulated to contract at 0.2 Hz steady-state in 0.8 mM $[Ca^{2+}]_o$ and at varying concentrations of Bay K8644. Values are mean ± SEM.

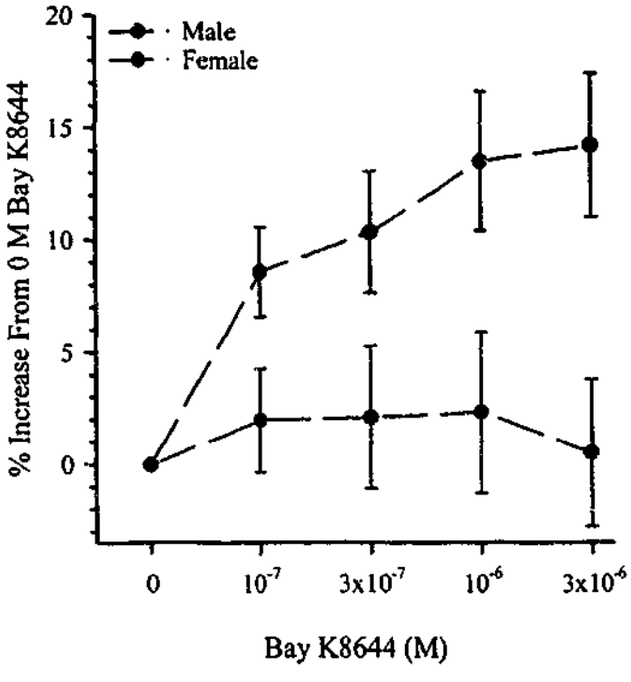


Figure 4.10: Effect of varying concentrations of Bay K8644 on male (n=9) and female (n=11) papillary muscle force expressed as a percentage increase of the control response in the absence of Bay K8644. Muscles were stimulated to contract at 0.2 Hz steady-state in the presence of 0.8 mM $[Ca^{2+}]_o$ and the appropriate concentration of Bay K8644. Values are mean ± SEM.

4.3 Discussion

Many factors have been linked to the gender based differences that are apparent in the incidence of cardiovascular disease. The sex steroid estrogen has been recognised as a key factor in these differences with beneficial effects on both the lipid profile and the vasculature (Barrett-Connor and Bush, 1991; Gerhard and Ganz, 1995). More recently, however, effects of estrogen on the heart itself have also been suggested to contribute to the reduced risk apparent in women. In particular a role for estrogen in regulating Ca^{2+} handling within the heart and, therefore, cardiac muscle contraction has been suggested (Jiang *et al.*, 1992; Collins *et al.*, 1993; Leblanc *et al.*, 1998; Meyer *et al.*, 1998). The aim of the current study was to elucidate gender differences in the contractile force of rat papillary muscle, and to provide further complimentary evidence to the gender differences in Ca^{2+} handling that were found in the previous chapter. The present study has shown that, under comparable experimental conditions, male papillary muscles consistently developed higher contractile force than female papillary muscles.

Increases in $[Ca^{2+}]_o$ resulted in increases in the contractile force in both male and female papillary muscles. Increases in contractile force with increases in $[Ca^{2+}]_o$ are widely reported for cardiac muscle and would be expected from what is known of excitation-contraction coupling in cardiac muscle. In the steady-state as $[Ca^{2+}]_o$ increased there would be both an increased Ca^{2+} entry and an increased SR Ca^{2+} content. These increases would be expected to result in a larger amount of Ca^{2+} available to instigate the calcium-induced calcium release mechanism which would lead to an increased amount of Ca^{2+} released from the SR and therefore greater calcium activation of the contractile machinery (Bassani *et al.*, 1995; Bers and Perez-Reyes, 1999). There are, however, species differences in the $[Ca^{2+}]_o$ dependence of force. In the rat force attains maximal levels at relatively lower $[Ca^{2+}]_o$ than in other commonly used species such as rabbits (Bers *et al.*, 1981). The profile of the changes in active force over the $[Ca^{2+}]_o$ range employed in the present study is similar to what has been previously reported for the rat (Zavecz *et al.*, 2000).

At all $[Ca^{2+}]_o$, the active force was, on average, significantly greater in male papillary muscles when compared with female papillary muscles. These results are in accordance with those reported by Leblanc *et al.* (1998) who found isometric tension to be less in female papillary muscles when compared with male papillary muscles. In addition a

study involving ovariectomy and estrogen replacement in rabbits undertaken by Patterson *et al.* (1998) demonstrated reduced isometric force in rabbits that had undergone ovariectomy and then estrogen replacement. This data, along with the current findings suggests a role for estrogen within the heart, influencing the contractile force of papillary muscle.

It is, however, also important to mention that others have reported results that differ from those reported here. Wang *et al.* (1998) found that atrial preparations from female rats were more sensitive to extracellular Ca^{2+} than those from male rats. The Ca^{2+} concentration-response curve for left ventricular papillary muscles from male rats also appeared to be shifted toward higher $[Ca^{2+}]_o$ compared to that for female papillary muscles, although there was no significant difference in the $[Ca^{2+}]$ EC_{50} values between the genders. Wang *et al.* (1998) also reported higher isometric force development in female compared to male papillary muscles although again the differences were not statistically significant. The reasons for the discrepancy between the results reported in this chapter and those of Wang *et al.* (1998) are not clear. There are some experimental differences worth noting. These include the fact that Wang *et al.* (1998) used a higher stimulation frequency (1.5 Hz c.f. 0.2 Hz) and included a β-adrenergic antagonist in their solution. They also expressed force relative to tissue dry weight rather than muscle cross-sectional area. Since it is likely that the male rats would have been considerably larger than the females it is possible that the male papillary muscles were also larger (i.e. larger cross-sectional area), unless specifically chosen, or dissected, to be of similar dimensions. An inverse relation between papillary muscle stress (i.e. force per cross-sectional area) and cross-sectional area has been reported (Delbridge and Loiselle, 1981). This could lead to apparently lower forces in male papillary muscles if these were in fact considerably larger than female muscles.

The gender differences in the contractile force with increasing $[Ca^{2+}]_o$ observed in these experiments are consistent with the findings of the previous chapter. The amplitude of the Ca^{2+} transient was significantly greater in male rat cardiac myocytes when compared with female cardiac myocytes. Both sets of results from myocytes and papillary muscles are consistent with an effect of estrogen on the expression of the L-type Ca^{2+} channel as suggested by Johnson *et al.* (1997). Their study demonstrated that estrogen affects the genomic regulation of the cardiac L-type Ca^{2+} channel, with increased expression in estrogen receptor knockout (ERKO) mice. In support of these findings are those by

Patterson *et al.* (1998) who showed that 17β-estradiol treatment decreased the number of nitrendipine binding sites in left ventricular membrane preparations from ovariectomised rabbits. It is, therefore, possible that estrogen is limiting the amount of Ca^{2+} that is able to enter the female heart via reduced expression of the L-type Ca^{2+} channel. This would lead to a reduced cellular Ca^{2+} content, reduced amount of Ca^{2+} available to initiate calcium-induced calcium release and would therefore reduce the overall contractile force produced by female papillary muscles.

The time to peak force and time to 50% relaxation were not significantly different between male and female papillary muscles. This is consistent with the findings of Leblanc *et al.* (1998) who showed no difference in time-to-peak tension and duration of contraction in papillary muscles from 2 and 4 month old male and female rats. Nevertheless these results are somewhat surprising considering that in the previous chapter a significant gender difference in time to peak and time to 50% relaxation were found in the shortening of cardiac myocytes. It is important to recognise that unloaded shortening of single isolated myocytes and isometric force development by multicellular papillary muscles set to their optimal length represent two quite different mechanical conditions. Nevertheless the more rapid decay of the Ca^{2+} transient in male single myocytes reported in Chapter 3 would perhaps have been expected to be reflected in a faster time to 50% relaxation in the male papillary muscles as compared to the female papillary muscles. The reasons why no difference in relaxation time could be detected are not clear. It may be that other factors influencing the rate of tension decay in a complex multicellular preparation mask differences that can be detected in a single cell. It would be interesting to record intracellular Ca^{2+} transients from intact papillary muscles under the conditions employed in this study to see whether the gender-related difference in the time constant of decay of the Ca^{2+} transient seen in isolated myocytes is also evident in the multicellular preparation.

In an attempt to more adequately define gender based differences in intracellular Ca^{2+} handling in the heart and the possibility that these differences were being mediated through the L-type Ca^{2+} channel, the Ca^{2+} channel blocker nifedipine was employed. The action of nifedipine has been described as a "plugging" of the Ca^{2+} channels (Braunwald, 1982) and several studies have reported a negative inotropic action of nifedipine in cardiac muscle (Ruch *et al.*, 1992; Hattori *et al.*, 1996; Noguchi *et al.*, 1996; Fratea *et al.*, 1997). Both male and female papillary muscles were exposed to

increasing concentrations of nifedipine and contractile force was monitored. Contractile force significantly decreased with the addition of nifedipine in papillary muscles from both male and female animals. This would be as a result of a reduced influx of Ca^{2+} through the L-type Ca^{2+} channels into the muscle, which would reduce cellular Ca^{2+} content and also the amount of Ca^{2+} available for the calcium-induced calcium release mechanism. This in turn would lead to a smaller release of calcium from the SR and, therefore, a smaller amount of calcium available to activate the contractile machinery.

Papillary muscles from female rats appeared to be more sensitive to nifedipine than did those from male rats. At equivalent concentrations of nifedipine force was reduced proportionately more in the female muscles. ED_{50} values were not determined since it was not certain that the highest concentration of nifedipine used represented the maximum effect. Nevertheless it is clear from Figure 4.6 that a lower ED_{50} would be expected in the female papillary muscles. If estrogen is acting within the heart to reduce the number of L-type Ca^{2+} channels, then a greater sensitivity to nifedipine might be expected. At submaximal concentrations of nifedipine a greater number of Ca^{2+} channels would remain functional in the male papillary muscle allowing for greater Ca^{2+} influx upon excitation and consequently a greater force response.

In addition to the Ca^{2+} channel blocker nifedipine, another pharmacological agent, the Ca^{2+} channel agonist Bay K8644 was employed to ascertain if the L-type Ca^{2+} channel was a major site of gender based Ca^{2+} handling and contractile force differences. As the concentration of Bay K8644 was increased, the contractile force increased significantly in male, but not significantly in female papillary muscles. Bay K8644 is known to promote prolonged openings of single L-type Ca^{2+} channels within the cardiac membrane as well as bursting activity which results in an increased influx of Ca^{2+} into the heart (Rampe *et al.*, 1993). The increased influx would be expected to result in greater Ca^{2+} release from the SR and hence a larger amount of Ca^{2+} would be available to act at the level of the contractile machinery and result in greater cardiac contraction.

At concentrations of Bay K8644 above 3×10^{-6} M a substantial decrease in force was observed in both male and female papillary muscles. A reduction in force at high Bay K8644 concentrations has also been reported by others (Manso *et al.*, 1999). This is likely to result from the fact that while the (-)-enantiomer of this compound is a Ca^{2+} channel agonist, the (+)-enantiomer is an antagonist (Bellemann and Franckowiak, 1985). It was (±)-Bay K8644 that was used in the present study.

Whilst a significant positive inotropic action of Bay K8644 was observed in the male papillary muscles, no significant effect was detected in the female muscles, indicating that they were not as responsive to this Ca^{2+} channel agonist as were the male muscles. Again, this difference is consistent with a reduced number of Ca^{2+} channels in the female cardiac cells. An increased number of targets (i.e. L-type Ca^{2+} channels) for Bay K8644 action in male cardiac muscle may facilitate an increased responsiveness to this Ca^{2+} channel agonist. In addition, the greater susceptibility of the female papillary muscles to Ca^{2+} channel antagonism may also predicate against a strong positive inotropic action of (±) Bay K8644.

It is apparent from these experiments that the L-type Ca^{2+} channel may be a fundamental site through which gender based differences in intracellular Ca^{2+} handling and contractility of cardiac muscle could arise. Taken together with results from Chapter 3, these findings support the notion that expression of the L-type Ca^{2+} channel may be regulated by estrogen and that this gives rise to gender-based differences in changes in intracellular Ca^{2+} and contractility.

Chapter 5

Chapter 5

Effects of Ovariectomy and 17β-Estradiol Replacement on Intracellular Calcium

In Chapter 3, a gender-based difference in $[Ca^{2+}]_i$ handling between male and female cardiac myocytes was established. Gender based differences in the incidence of cardiovascular disease have been linked to estrogen and its effects on the vasculature and the heart. It could be assumed, therefore, that the reduced $[Ca^{2+}]_i$ observed in female cardiac myocytes maybe as a result of the long term actions of estrogen. The experiments in this chapter were designed to ascertain if the sex steroid estrogen is the underlying factor in the gender based difference in $[Ca^{2+}]_i$ handling. To do this, a series of experiments were undertaken where the main source of estrogen within the female rat, the ovary, was surgically removed. In a second series of experiments, ovariectomised rats were treated with exogenous estrogen to determine whether any changes resulting from estrogen removal could be reversed.

Previous studies involving ovariectomy and estrogen replacement have resulted in an inconsistent outcome. Early studies reported that ovariectomy resulted in depression of cardiac function, a reduction in myosin ATPase activity, and a transition from the V1 myosin isoenzyme form to the V3 isoform (Schaible *et al.*, 1984). These changes were prevented by administration of estrogen (Scheuer *et al.*, 1987). In addition, Bowling *et*

al. (1997) reported an increased binding density of cardiac L-type Ca^{2+} channels in response to oral treatment of ovariectomised rats with ethinyl estradiol. This increased binding was, however, not accompanied by increased calcium-mediated inotropic responses. Patterson *et al.* (1998) reported contradictory results showing that long-term estrogen administration after ovariectomy resulted in changes that favoured increased force development (e.g. increased cardiac mass, increased myosin ATPase activity), whilst a second subset of changes favoured decreased overall force generation (e.g. decreased number of nitrendipine binding sites and decreased isometric force). In addition to these studies, others have reported a Ca^{2+} hypersensitivity of rat myofilaments and a suppression of maximum myofibrillar ATPase activity after ovariectomy and reversal of these two phenomena when estrogen replacement is received (Wattanapermpool, 1998; Wattanapermpool and Reiser, 1999; Wattanapermpool *et al.*, 2000).

Given the contradictory outcomes of previous ovariectomy and estrogen replacement studies and our observation of higher intracellular $[Ca^{2+}]$ in male than female cardiac myocytes, the present experiments were undertaken to investigate the effects of estrogen, and its removal, on calcium movements in female cardiac myocytes.

5.1 Methodology

5.1.1 Experimental Groups

Two experimental studies were conducted using 3-month old female Wistar rats. In the first study animals underwent either a sham operation or ovariectomy. In the second study, all animals were ovariectomised and half received a pellet containing 17β-estradiol in oil, with the other half receiving oil alone. All rats were left for 28-31 days after surgery before experiments commenced.

5.1.2 Ovariectomy Surgery

Ovariectomy (OVX) or sham-ovariectomy surgery was performed under anaesthesia induced with ketamine (4.5 mg/100g i.m.) and xylazine (0.7 mg/100g i.m.). A single transverse incision was made in the lower back just below the last rib and the ovaries were visualised. For sham operations, the ovaries were replaced and the incision closed. For ovariectomy, the ovarian pedicle was clamped, tied off with silk and the ovary removed. This was repeated for the contralateral ovary prior to the incision being closed.

5.1.3 Estradiol Replacement

Pellets containing 17β-estradiol or oil-alone were made from Silastic medical grade tubing (0.0062 inches i.d. × 0.125 inches o.d.; Dow Corning Corporation, Midland, MI, USA) and were used at a length of 10mm per 100g body weight. The estradiol pellets contained 17β-estradiol estradiol at a concentration of 150μg/ml in sesame oil. Tubing was cut and one end was sealed with adhesive silicone. An appropriate amount of estradiol was added into the tubing, it was then cut to the appropriate length and the other end was also sealed. Once the ovaries were removed during surgery, a small incision was made at the base of the neck and the estradiol pellet was inserted subcutaneously into this region. Following surgery, all animals were given free access to food and water for the remainder of the study.

To confirm the success of the surgery and the estrogen replacement, the uterus was removed at the beginning of the experiment and weighed. The uterine and body weights of the animals in the different experimental groups are shown in Table 5.1.

Table 5.1: Body weight and uterine weight of female rats in the various experimental groups.

Experimental Group	Body Weight (g)	Uterine Weight (g)	Uterine:Body Weight Ratio (mg/g)
Sham Female (n=6)	294±5	0.52±0.04	1.76±0.15
OVX Female (n=7)	362±14*	0.16±0.004*	0.46±0.01*
OVX + Sham Pellet (n=6)	347±4†	0.13±0.008†	0.38±0.03†
OVX + E_2 Pellet (n=8)	274±4	0.50±0.02	1.82±0.07

* indicates that this value is significantly different ($p<0.01$) from sham female group. † indicates that this value is significantly different ($p<0.01$) from 17β-estradiol replaced OVX female group.

5.1.4 Experimental Protocols

All experiments reported in this Chapter involved recordings of intracellular Ca^{2+} and cell shortening from freshly isolated single myocytes. The procedures for cell isolation and the recording techniques were as described in Chapter 2. The principal approach taken was to compare the responses of myocytes from animals in the different experimental groups to changes in the extracellular Ca^{2+} concentration. The experimental protocol was as described in Section 3.1.2. except for a small change in the stimulus frequency which was not varied, but was kept at 0.5 Hz steady-state.

5.1.5 Statistics

All data are presented as mean ± standard error of the mean (SEM). All data presented in this chapter have been analysed using a one-way ANOVA with repeated measures.

5.2 Results

5.2.1 Study 1: Effects of Ovariectomy on Changes in Intracellular [Ca^{2+}] in Response to Varied Extracellular [Ca^{2+}]

Figure 5.1 illustrates an example of original Ca^{2+} transient recordings from a sham operated female and an ovariectomised female cardiac myocyte stimulated to contract at 0.5 Hz steady-state and exposed to four different Ca^{2+} concentrations. In the top panel recordings from a sham female cardiac myocyte in 0.5, 1.0, 1.5 and 2.0 mM Ca^{2+} are shown, whilst in the bottom panel recordings from an ovariectomised female cardiac myocyte under the same conditions are shown. It is apparent from this figure that an increase in extracellular [Ca^{2+}] resulted in an increase in $[Ca^{2+}]_i$ in both sham and ovariectomised female cells. In addition, it seems apparent that this rise is greater in OVX female cells when compared with sham female cells.

Figure 5.2 shows mean baseline $[Ca^{2+}]_i$ in sham (n=7, N=5) and ovariectomised female (n=8, N=6) cardiac myocytes at four different extracellular [Ca^{2+}]. As $[Ca^{2+}]_o$ was increased, baseline $[Ca^{2+}]_i$ did not change significantly within each group. The OVX female cardiac myocytes do, however, have significantly higher ($p<0.03$) baseline $[Ca^{2+}]_i$ when compared with sham female cardiac myocytes at all $[Ca^{2+}]_o$ measured.

Figure 5.3 illustrates mean peak $[Ca^{2+}]_i$ values in sham female and OVX female cardiac myocytes at four different $[Ca^{2+}]_o$. As $[Ca^{2+}]_o$ was increased, $[Ca^{2+}]_i$ significantly increased in both sham and OVX female groups ($p<0.01$). In addition, the sham operated female group had, on average, significantly lower ($p<0.02$) $[Ca^{2+}]_i$ when compared with the OVX female group at all $[Ca^{2+}]_o$.

Figure 5.4 shows average data for the amplitude of the $[Ca^{2+}]_i$ transient in sham and OVX female cardiac myocytes at varying $[Ca^{2+}]_o$. As the $[Ca^{2+}]_o$ was increased, the amplitude of the $[Ca^{2+}]_i$ transient also significantly increased ($p<0.01$) in both sham and OVX female groups. On average, the amplitude of the Ca^{2+} transient was significantly greater ($p<0.03$) in OVX female when compared to sham female cardiac myocytes.

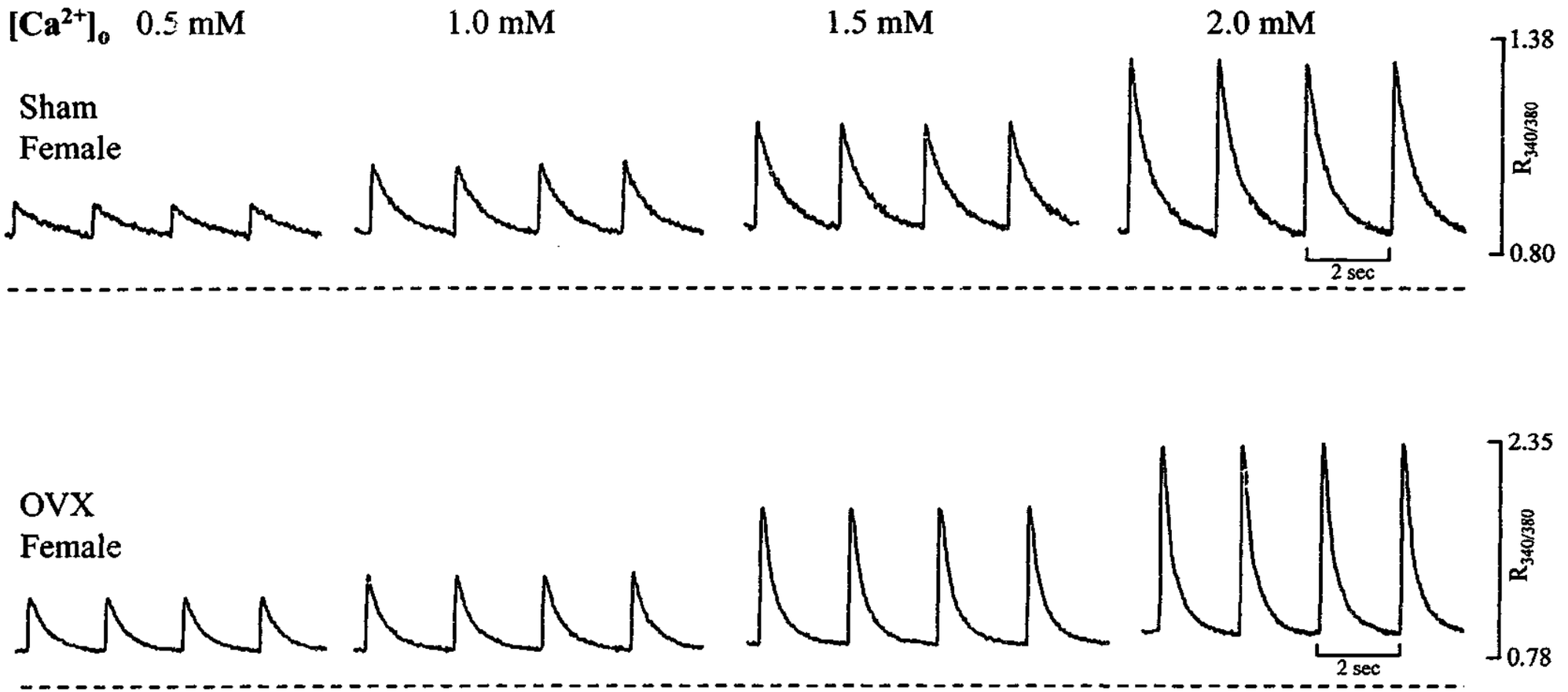


Figure 5.1: Examples of Ca^{2+} transients recorded from a sham female and an OVX female cardiac myocyte at 0.5 Hz steady-state and different extracellular [Ca^{2+}]. The top panel shows Ca^{2+} transients recorded from a sham female cell at 0.5, 1.0, 1.5 and 2.0 mM $[Ca^{2+}]_o$, whilst the bottom panel shows an OVX female cell under the same conditions.

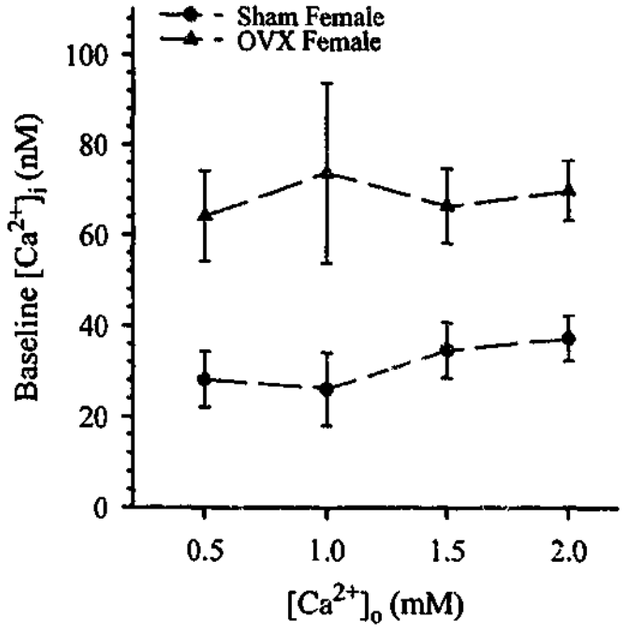


Figure 5.2: The effects of increasing extracellular $[Ca^{2+}]$ on baseline $[Ca^{2+}]_i$ in sham (n=7, N=5) and OVX (n=8, N=6) female cardiac myocytes. Values are mean ± SEM.

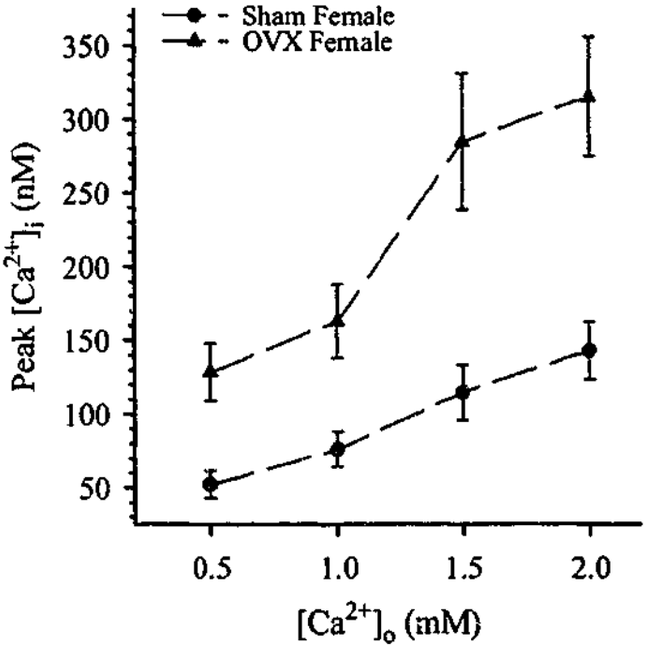


Figure 5.3: The effects of increasing extracellular $[Ca^{2+}]$ on peak $[Ca^{2+}]_i$ in sham (n=7, N=5) and OVX (n=8, N=6) female cardiac myocytes. Values are mean ± SEM.

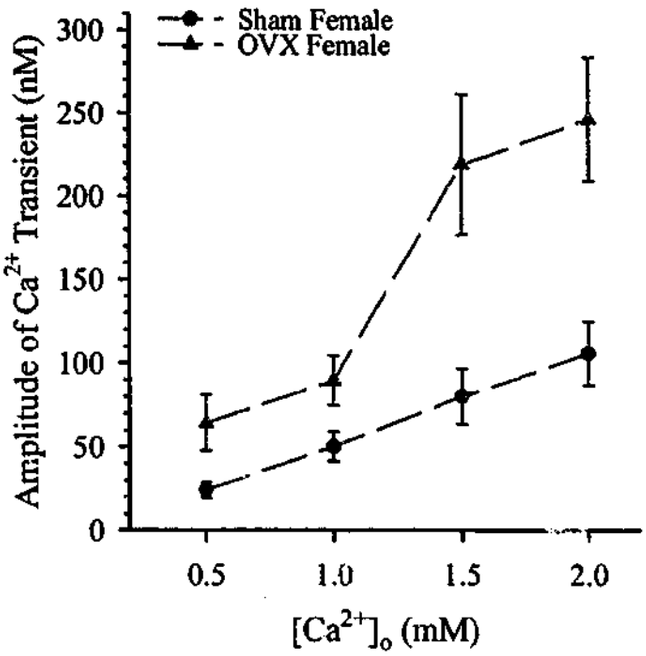


Figure 5.4: The effects of increasing extracellular $[Ca^{2+}]$ on the amplitude of the Ca^{2+} transient in sham (n=7, N=5) and OVX (n=8, N=6) female cardiac myocytes. Values are mean ± SEM.

5.2.2 Study 1: Effect of Ovariectomy on the Time Course of Decay of the Ca^{2+} Transient

Figure 5.5 shows the differences in the time course of decay of the Ca^{2+} transient between sham female (n=6, N=5) and OVX female (n=8, N=5) cardiac myocytes. In the top panel, transients from a sham female and an OVX female cell have been overlayed and normalised to facilitate comparison. Cells were stimulated to contract at 0.5 Hz steady-state in 1.5 mM Ca^{2+}. From these traces it can be seen that the time course of decay appears to be faster in the OVX female cells when compared with sham female cells. The average data for the time course of decay is shown in the bottom panel, with OVX female cardiac myocytes showing a significantly faster ($p<0.03$) time course of decay than sham operated female cardiac myocytes.

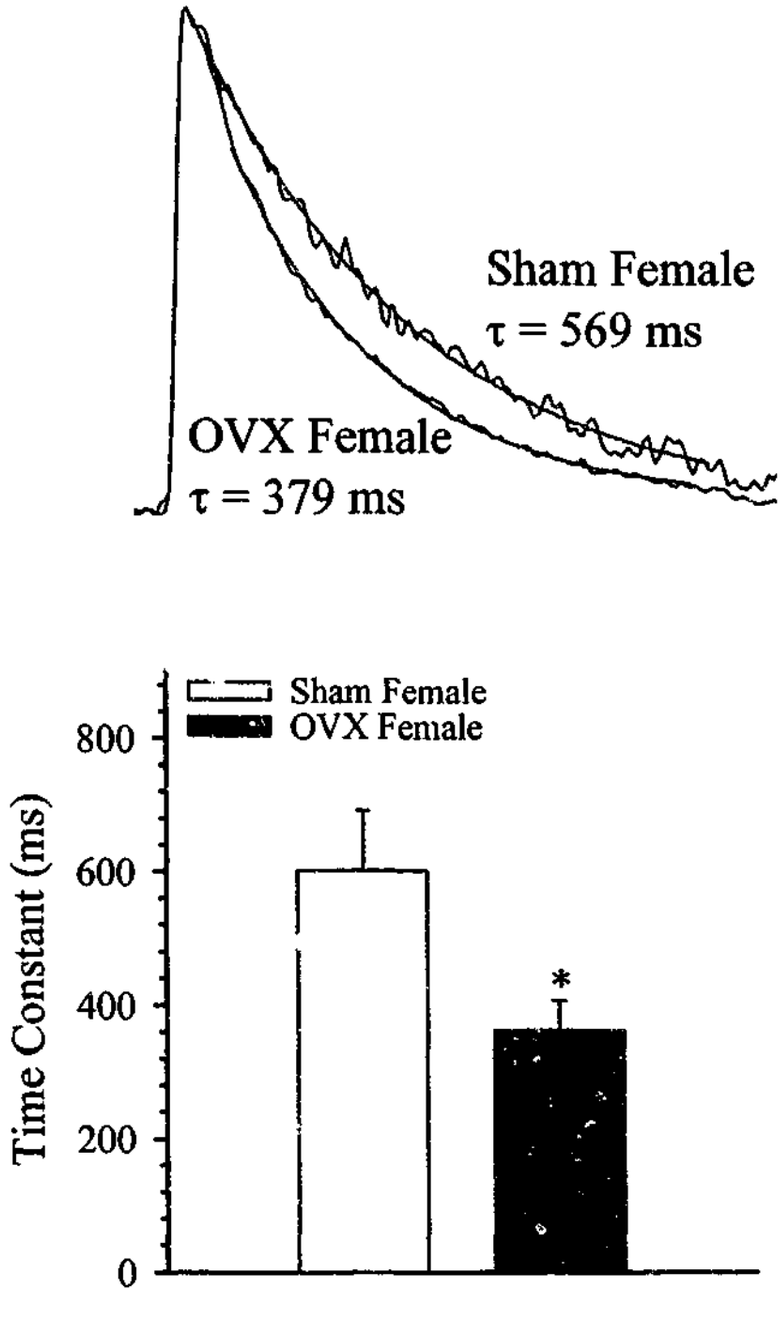


Figure 5.5: The effects of ovariectomy on the time course of decay of the Ca^{2+} transient. The transients in the top panel have been scaled to the same amplitude to facilitate comparison with a single exponential function fitted from which a time constant was derived. Average time constant values for sham (n=6, N=5) and OVX (n=8, N=5) female cardiac myocytes are shown in the bottom panel. Values are mean ± SEM. * denotes that the value is significantly different ($p<0.03$) from sham female cardiac myocyte.

5.2.3 Study 1: Effect of Ovariectomy on Myocyte Shortening

Figure 5.6 shows average extent of shortening data for sham female (n=7, N=5) and OVX female (n=6, N=5) cardiac myocytes stimulated to contract at 0.5 Hz steady-state and in 1.5 mM $[Ca^{2+}]_o$. The OVX female cardiac myocytes showed significantly greater ($p<0.03$) extent of shortening when compared with sham female cardiac myocytes.

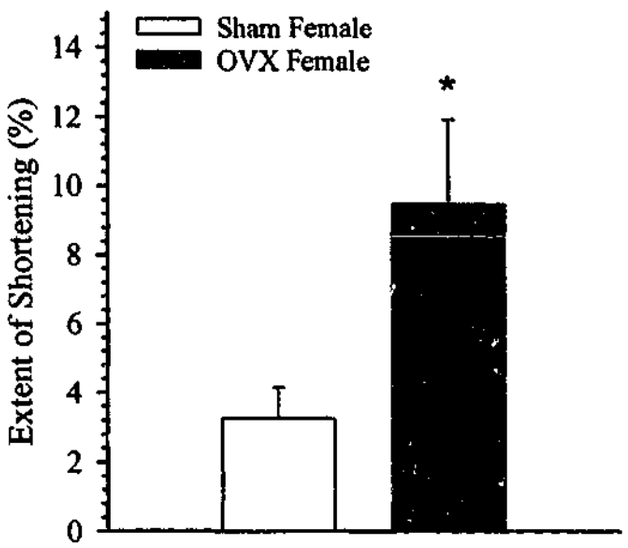


Figure 5.6: The average extent of cell shortening in sham (n=7, N=5) and OVX female (n=6, N=5) cardiac myocytes at 1.5mM extracellular $[Ca^{2+}]$ at 0.5 Hz steady-state. * denotes value is significantly different ($p<0.03$) from sham female group.

Figure 5.7 illustrates the differences in the time course of shortening of the myocyte from sham female (n=7, N=5) and OVX female (n=6, N=5) cells. The top panel shows original recordings from sham and OVX female rat cardiac myocytes which have been overlayed and normalised to the same peak amplitude to facilitate comparison. It is apparent from these traces that the time to 50% relaxation is faster in the OVX female cells when compared with the sham female cells. This is confirmed in the bottom bar graph which shows the mean time to 50% relaxation data for the two groups, and the significantly faster ($p<0.03$) rate in OVX female cells when compared with sham operated female cells.

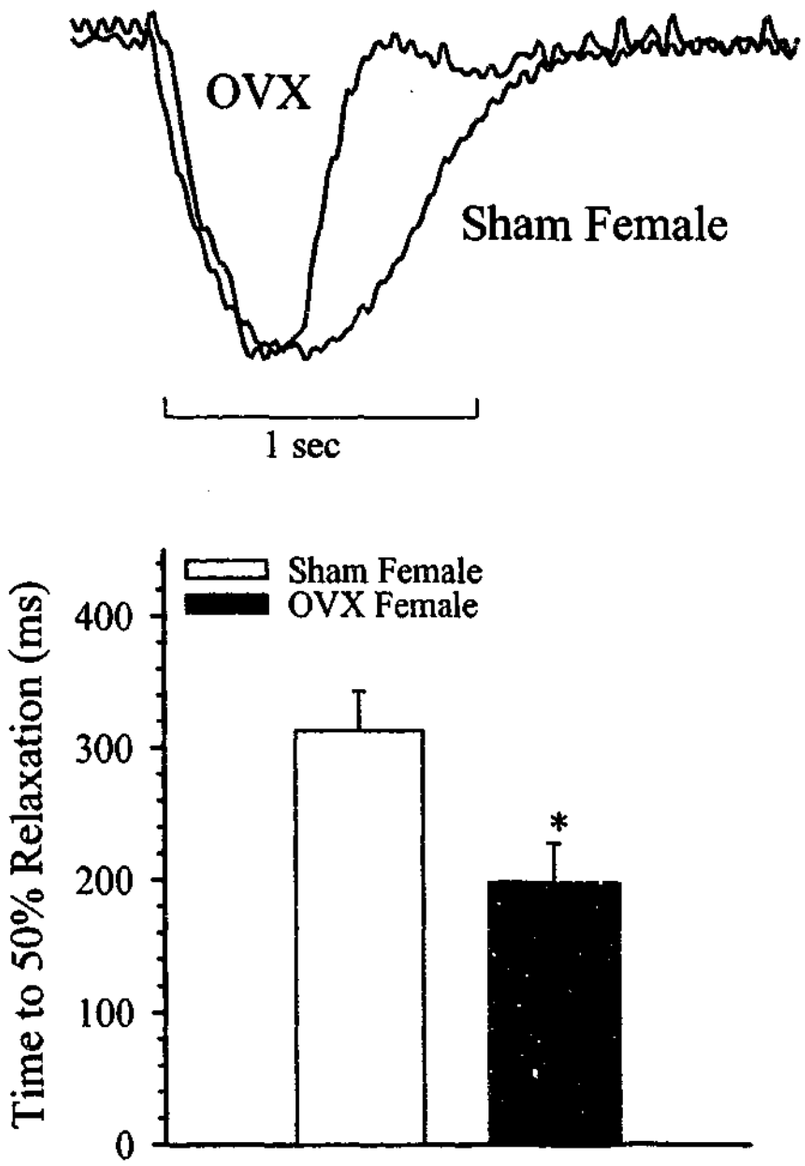


Figure 5.7: Original cell shortening recordings from sham and OVX female cardiac myocytes stimulated to contract at 0.5 Hz in 1.5 mM $[Ca^{2+}]_o$ are illustrated in the top panel. Traces are normalised to the same amplitude to facilitate comparison. From the traces the time to 50% relaxation was calculated in sham (n=7, N=5) and OVX (n=6, N=5) female cardiac myocytes. Values are mean ± SEM. * denotes that the value is significantly different ($p<0.03$) from sham female cardiac myocyte.

5.2.4 Study 2: Changes in Intracellular $[Ca^{2+}]$ in Response to Ovariectomy, 17β-Estradiol Replacement and Varied Extracellular $[Ca^{2+}]$

Figure 5.8 shows original recordings from sham pellet and 17β-estradiol pellet replaced OVX female cells. The top panel illustrates the effects of varying extracellular $[Ca^{2+}]$ (0.5, 1.0, 1.5 and 2.0 mM) on $[Ca^{2+}]_i$ in a sham pellet female cardiac myocyte stimulated to contract at 0.5 Hz. The bottom panel shows a 17β-estradiol replaced OVX female cardiac myocyte under the same conditions. As the extracellular $[Ca^{2+}]$ was increased it is apparent that the Ca^{2+} transients increased in both peak and amplitude in both sham pellet and 17β-estradiol replaced OVX female cells. At each $[Ca^{2+}]_o$, however, the Ca^{2+} transients appear to be larger in the sham pellet OVX cell.

Figure 5.9 illustrates mean baseline $[Ca^{2+}]_i$ in sham pellet (n=9, N=6) and 17β-estradiol replaced OVX female (n=15, N=8) cardiac myocytes at four different extracellular $[Ca^{2+}]$. As $[Ca^{2+}]_o$ was increased, baseline $[Ca^{2+}]_i$ remained at the same level, and although this level appeared to be greater in 17β-estradiol replaced OVX female cardiac myocytes when compared with sham pellet OVX female cells this difference was not statistically significant.

Figure 5.10 shows mean peak $[Ca^{2+}]_i$ values in sham pellet and 17β-estradiol replaced OVX female cardiac myocytes at four different $[Ca^{2+}]_o$. As the extracellular $[Ca^{2+}]$ was increased, the $[Ca^{2+}]_i$ increased significantly ($p<0.01$) in both groups. Peak $[Ca^{2+}]$ appeared to be greater in the sham pellet OVX female cardiac myocytes when compared with the 17β-estradiol replaced OVX female cardiac myocytes particularly at the higher $[Ca^{2+}]_o$. When compared over the entire $[Ca^{2+}]_o$ range (one way ANOVA), however, this difference was not statistically significant.

Figure 5.11 shows average Ca^{2+} transient amplitude data in sham pellet and 17β-estradiol replaced OVX female cardiac myocytes at varying $[Ca^{2+}]_o$. As the $[Ca^{2+}]_o$ was increased, the amplitude of the Ca^{2+} transient significantly increased ($p<0.01$) in both groups. On average, the amplitude of the Ca^{2+} transient was significantly greater ($p<0.01$) in the sham pellet OVX group when compared with the 17β-estradiol replaced OVX female group.

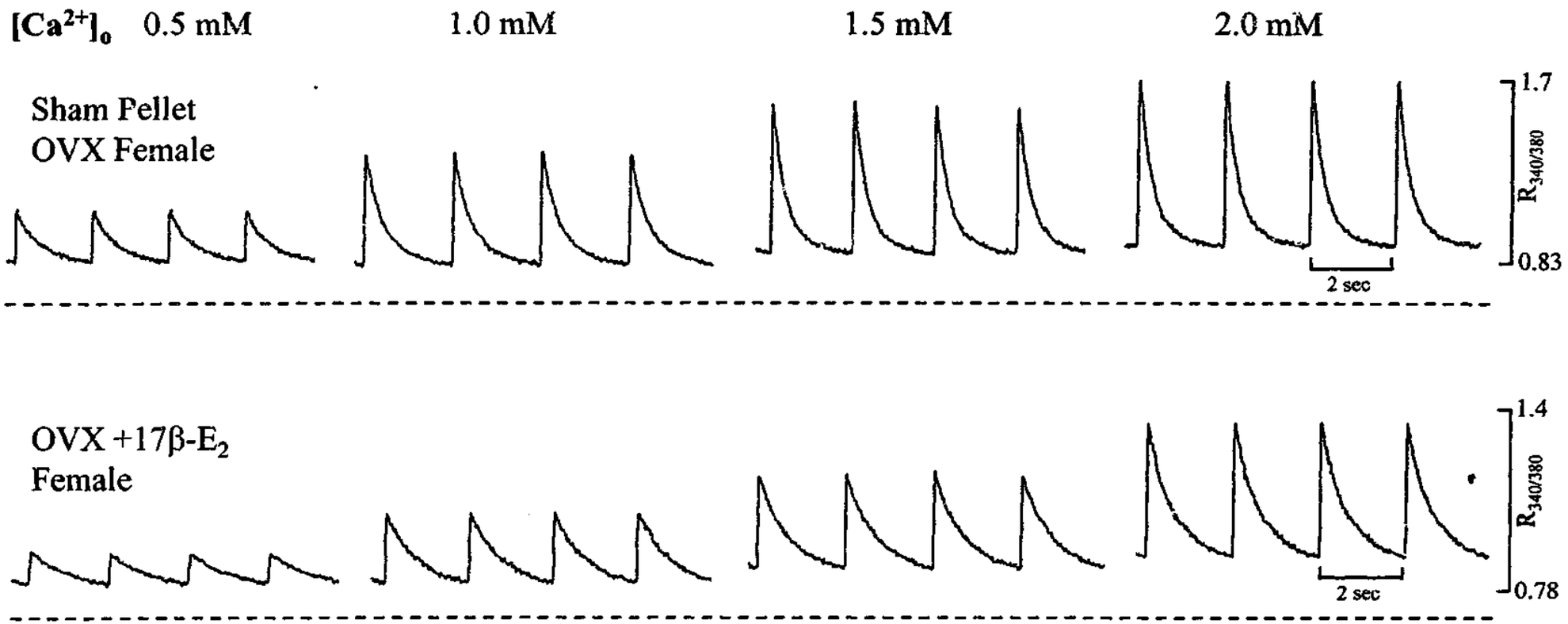


Figure 5.8: Examples of Ca^{2+} transients recorded from a sham pellet OVX female and a 17β-E_2 replaced OVX female cardiac myocyte at 0.5 Hz steady-state and different extracellular [Ca^{2+}]. The top panel shows Ca^{2+} transients recorded from a sham pellet OVX female cell at 0.5, 1.0, 1.5 and 2.0 mM $[Ca^{2+}]_o$, whilst the bottom panel shows a 17β-E_2 replaced OVX female cell under the same conditions.

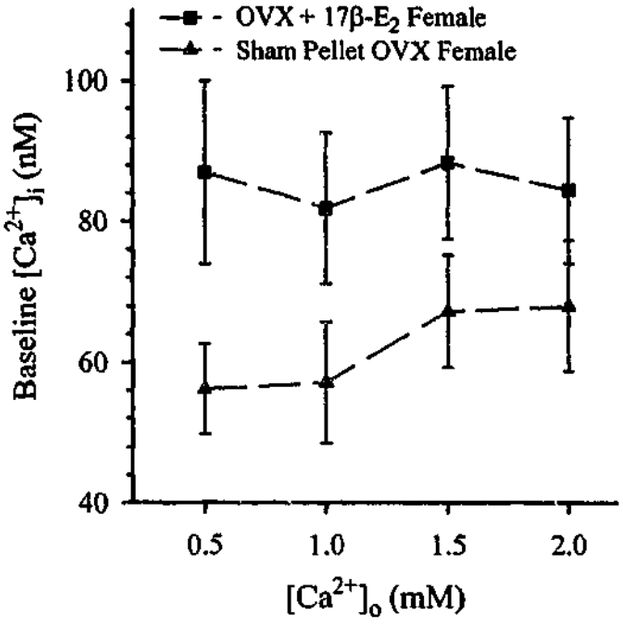


Figure 5.9: The effects of increasing extracellular $[Ca^{2+}]$ on baseline $[Ca^{2+}]_i$ in sham pellet (n=9, N=6) and OVX + 17β-estradiol replaced (n=15, N=8) female cardiac myocytes. Values are mean ± SEM.

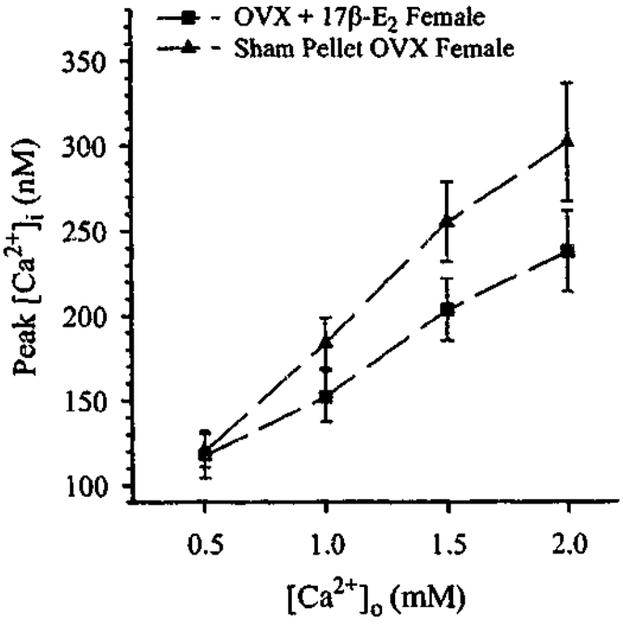


Figure 5.10: The effects of increasing extracellular $[Ca^{2+}]$ on peak $[Ca^{2+}]_i$ in sham pellet (n=9, N=6) and OVX + 17β-estradiol replaced (n=15, N=8) female cardiac myocytes. Values are mean ± SEM.

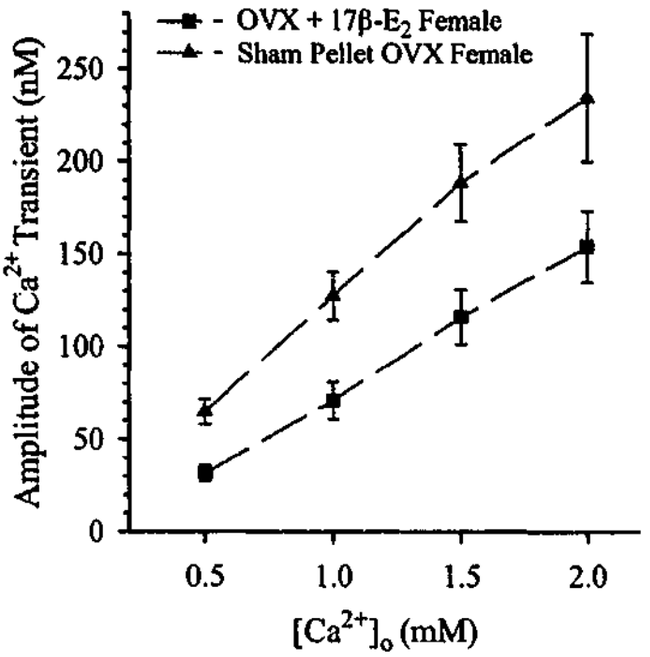


Figure 5.11: The effects of increasing extracellular [Ca^{2+}] on the amplitude of the Ca^{2+} transient in sham pellet (n=9, N=6) and OVX + 17β-estradiol replaced (n=15, N=8) female cardiac myocytes. Values are mean ± SEM.

5.2.5 Study 2: Effects of Ovariectomy and 17β-Estradiol Replacement on Ca^{2+} Transient Decay

The effect of ovariectomy and 17β-estradiol replacement on the time course of decay of the Ca^{2+} transient is shown in Figure 5.12. The top panel illustrates original transient recordings from sham pellet and 17β-estradiol replaced OVX female cardiac myocytes stimulated to contract at 0.5 Hz and in 1.5 mM Ca^{2+}. Transients have been normalised and overlayed to facilitate comparison. These transients show that the sham pellet OVX female (n=11, N=6) cells have a faster time course of decay than do the 17β-estradiol replaced OVX female (n=14, N=8) myocytes. This is confirmed in the bottom panel which shows the mean time constant values from both groups, and illustrates the significantly faster ($p<0.02$) rate of decay of the Ca^{2+} transient in sham pellet OVX female cardiac myocytes when compared with 17β-estradiol replaced OVX female cardiac myocytes.

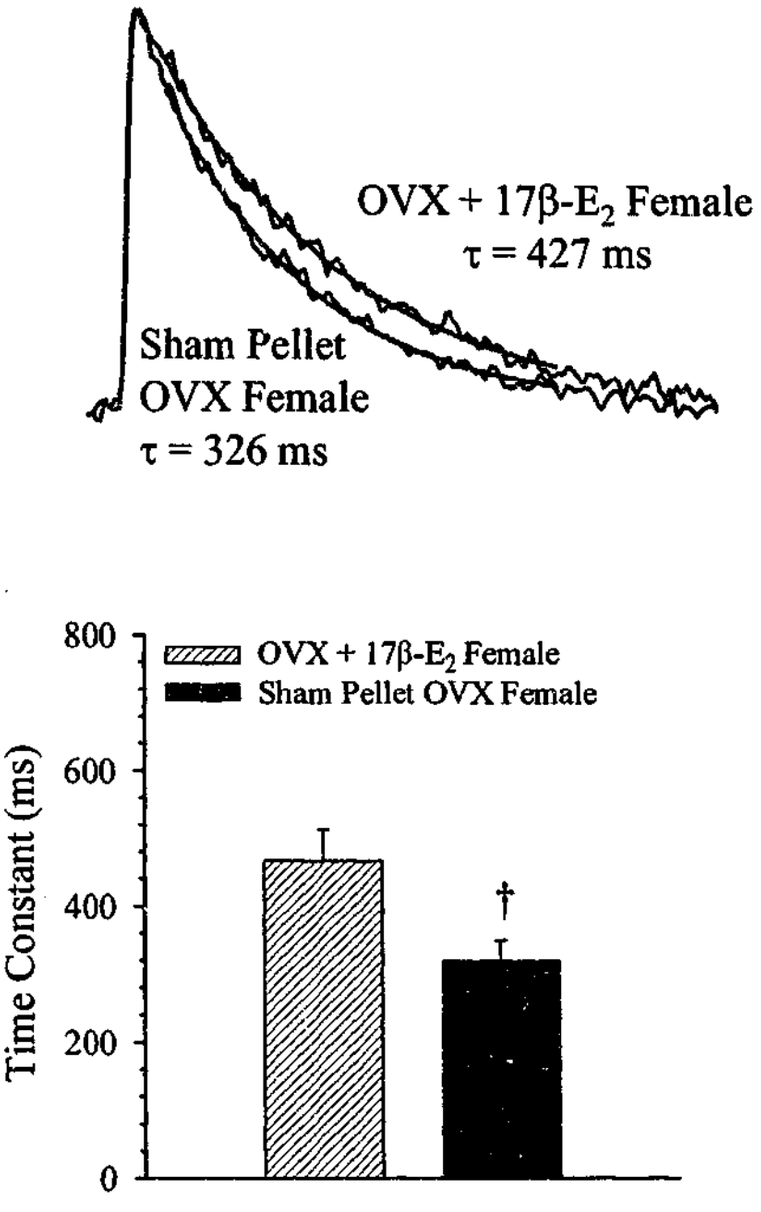


Figure 5.12: The effects of ovariectomy and 17β-estradiol replacement on the time course of decay of the Ca^{2+} transient. The transients in the top panel have been scaled to the same amplitude to facilitate comparison with a single exponential function fitted from which a time constant was derived. Average time constant values for sham pellet (n=11, N=6) and 17β-estradiol replaced OVX (n=14, N=8) female cardiac myocytes are shown in the bottom panel. Values are mean ± SEM. † denotes that the value is significantly different ($p<0.02$) from the 17β-estradiol replaced OVX female group.

5.2.6 Study 2: Effects of Ovariectomy and 17β-Estradiol Replacement on Myocyte Shortening

Figure 5.13 shows average extent of shortening data for sham pellet (n=9, N=5) and 17β-estradiol replaced OVX female (n=12, N=6) cardiac myocytes stimulated to contract at 0.5 Hz steady-state and in 1.5 mM Ca^{2+}. The sham pellet replaced OVX female cardiac myocytes showed significantly greater ($p<0.03$) extent of shortening when compared with 17β-estradiol replaced female cardiac myocytes.

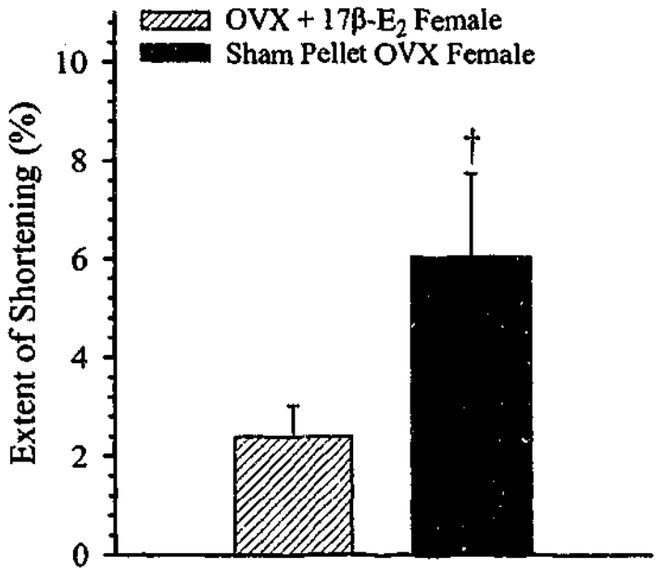
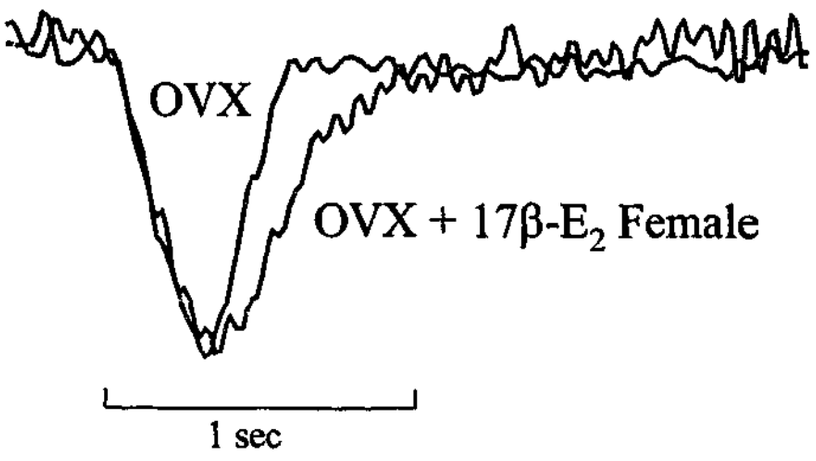


Figure 5.13: The average extent of cell shortening in sham pellet (n=9, N=5) and 17β-estradiol replaced OVX (n=12, N=6) female cardiac myocytes at 1.5 mM extracellular [Ca^{2+}] at 0.5 Hz steady-state. † denotes value is significantly ($p<0.03$) different from 17β-estradiol replaced OVX female group.

Figure 5.14 illustrates the differences in the time course of shortening of the myocyte between sham pellet (n=9, N=5) and 17β-estradiol replaced OVX female (n=12, N=6) rats. The top panel shows original shortening recordings from sham pellet and 17β-estradiol replaced OVX female cardiac myocytes which have been overlayed and normalised to facilitate comparison. These traces indicate that the time to 50% relaxation is faster in the sham pellet OVX female cells when compared with the 17β-estradiol replaced female cells. This is confirmed in the bottom bar graph which shows the mean time to 50% relaxation data for the two groups, and the significantly faster

($p<0.02$) rate in sham pellet OVX female cells when compared with 17β-estradiol replaced OVX female cells.



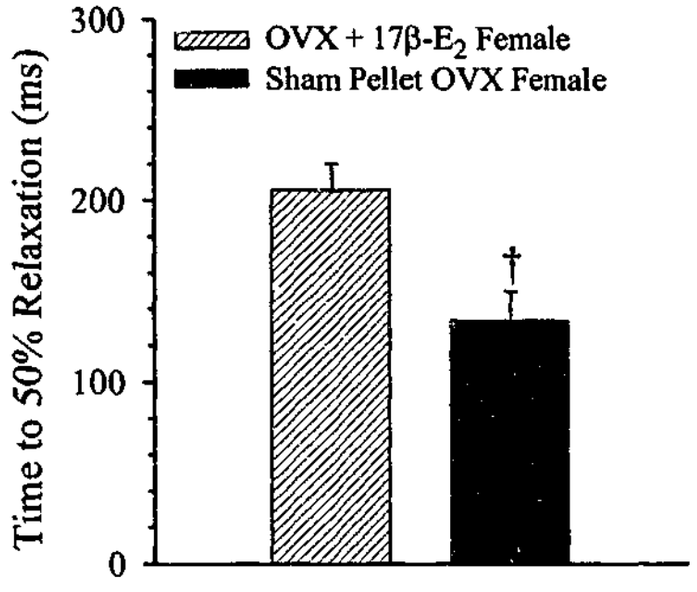


Figure 5.14: Original shortening recordings from sham pellet and 17β-estradiol replaced OVX female cardiac myocytes stimulated to contract at 0.5 Hz in 1.5 mM $[Ca^{2+}]_o$ are illustrated in the top panel. Traces are normalised to the same amplitude to facilitate comparison. From the traces the time to 50% relaxation was calculated in sham pellet (n=9, N=5) and OVX (n=12, N=6) female cardiac myocytes. Values are mean ± SEM. † denotes that the value is significantly different ($p<0.02$) from 17β-estradiol replaced OVX female group.

5.2.7 Comparison of Sham Operated, OVX and 17β-Estradiol Replaced OVX Female Cardiac Myocytes

In order to make a comparison between the two studies, the OVX female group from the first study was combined with the sham pellet OVX female group from the second study. A statistical analysis was undertaken to ensure that the two OVX female groups from the separate studies were in fact the same. No significant differences between the two groups could be detected in any of the parameters that were measured. Therefore the two groups were combined as being representative of ovariectomised, estrogen-deprived females.

5.2.8 Intracellular $[Ca^{2+}]$ Response to Varying Extracellular $[Ca^{2+}]$

Figure 5.15 shows mean baseline $[Ca^{2+}]_i$ data from sham operated (n=7, N=5), combined OVX (n=17, N=12) and 17β-estradiol replaced OVX female (n=15, N=8) cells. As $[Ca^{2+}]_o$ was increased, there was no significant change in $[Ca^{2+}]_i$ in any group. Resting $[Ca^{2+}]_i$ was, however, significantly greater ($p<0.01$) in the combined OVX female group when compared with the sham operated female cells. In addition, the 17β-estradiol replaced group had significantly higher ($p<0.01$) baseline $[Ca^{2+}]_i$ when compared to sham operated female cardiac myocytes. No difference was apparent between OVX female and 17β-estradiol replaced OVX female cardiac myocytes.

The effects of varying $[Ca^{2+}]_o$ on the peak $[Ca^{2+}]_i$ in sham operated, OVX and 17β-estradiol replaced OVX female cardiac myocytes is shown in Figure 5.16. As the $[Ca^{2+}]_o$ was increased, the $[Ca^{2+}]_i$ increased significantly in all three groups ($p<0.001$). On average, the peak $[Ca^{2+}]_i$ was significantly greater ($p<0.04$) in the OVX female cardiac myocytes when compared with both the sham operated and 17β-estradiol replaced groups. A significant difference ($p<0.01$) between the sham operated and 17β-estradiol replaced OVX female groups was also apparent.

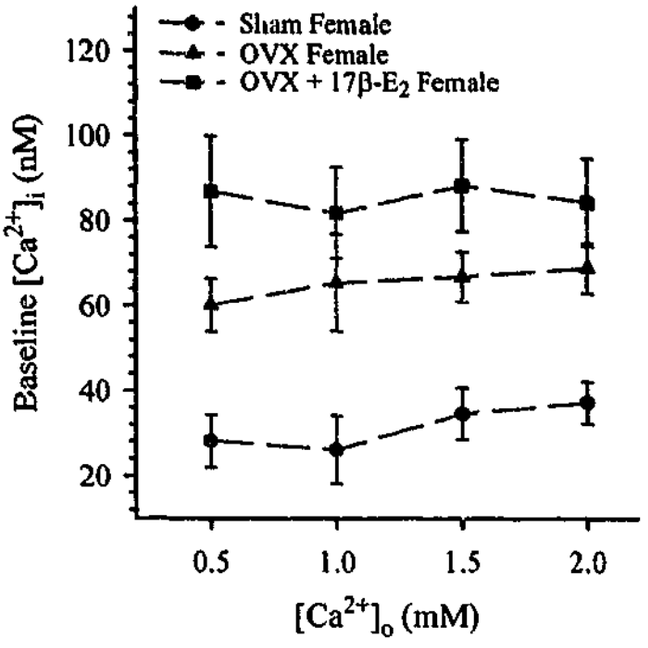


Figure 5.15: The effects of increasing extracellular $[Ca^{2+}]$ on baseline $[Ca^{2+}]_i$ in sham (n=7, N=5), combined OVX (n=17, N=12) and OVX + 17β-estradiol replaced (n=15, N=8) female cardiac myocytes. Values are mean ± SEM.

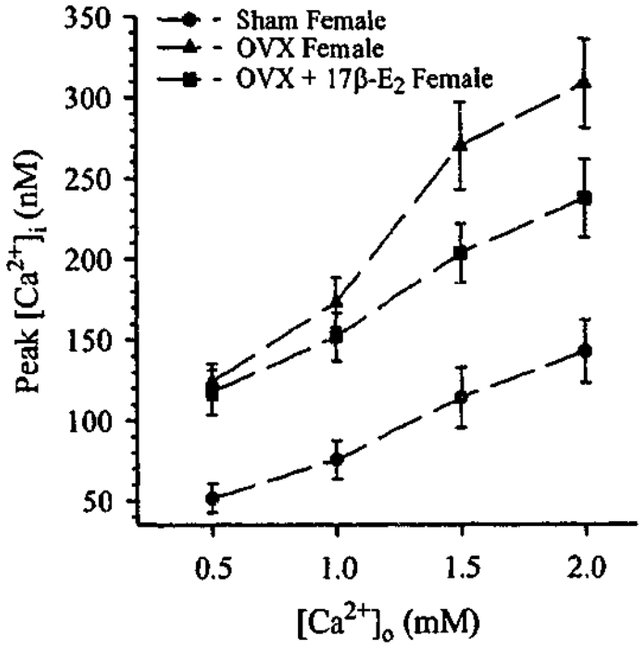


Figure 5.16: The effects of increasing extracellular $[Ca^{2+}]$ on peak $[Ca^{2+}]_i$ in sham (n=7, N=5), combined OVX (n=17, N=12) and OVX + 17β-estradiol replaced (n=15, N=8) female cardiac myocytes. Values are mean ± SEM.

Figure 5.17 shows the effects of varying $[Ca^{2+}]_o$ on the amplitude of the Ca^{2+} transient in sham operated, OVX and 17β-estradiol replaced OVX female cardiac myocytes. As $[Ca^{2+}]_o$ was increased, the amplitude of the Ca^{2+} transient also increased significantly in all three groups ($p<0.001$). The amplitude of the Ca^{2+} transient was significantly greater ($p<0.004$) in the OVX female cardiac myocytes when compared with both the sham operated and 17β-estradiol replaced OVX female cells. The amplitudes of the Ca^{2+} transient in the sham operated and the 17β-estradiol replaced groups were not significantly different.

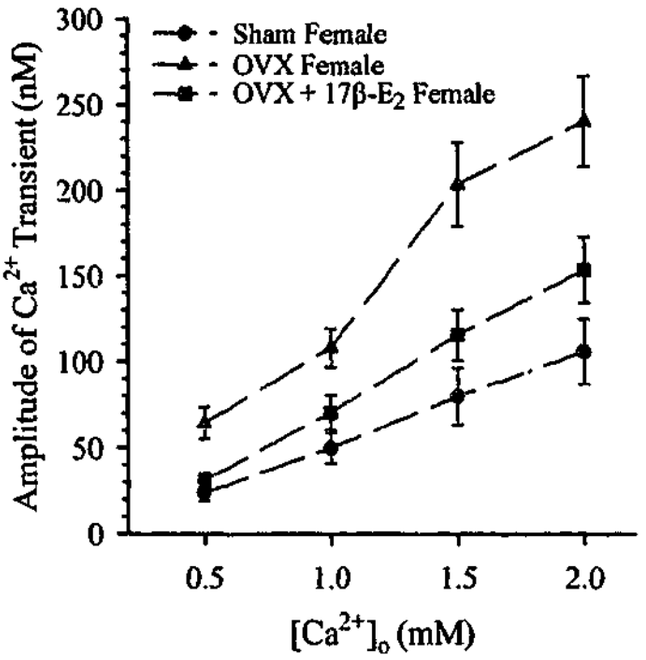


Figure 5.17: The effects of increasing extracellular $[Ca^{2+}]$ on the amplitude of the Ca^{2+} transient in sham (n=7, N=5), combined OVX (n=17, N=12) and OVX + 17β-estradiol replaced (n=15, N=8) female cardiac myocytes. Values are mean ± SEM.

5.2.9 Time Course of Decay of the Ca^{2+} Transient

The differences in the time course of decay of the Ca^{2+} transient in sham operated (n=6, N=5), combined OVX (n=19, N=11) and 17β-estradiol replaced OVX female (n=14, N=8) cardiac myocytes is shown in Figure 5.18. The rate of decay of the Ca^{2+} transient was significantly faster ($p<0.02$) in the combined OVX female group when compared with both the sham operated and 17β-estradiol replaced OVX female cells. No

difference in the time course of decay of the Ca^{2+} transient was apparent between the latter two groups.

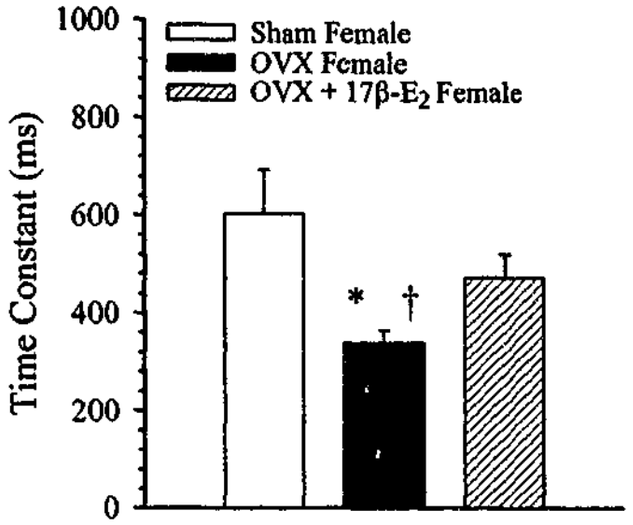


Figure 5.18: Average time constant of the decay of the Ca^{2+} transient in sham (n=6, N=5), combined OVX (n=19, N=11) and 17β-estradiol replaced OVX (n=14, N=8) female cardiac myocytes. Values are mean ± SEM. * denotes value is significantly ($p<0.02$) different from sham female group. † denotes value is significantly ($p<0.02$) different from 17β-estradiol replaced OVX group.

5.2.10 Shortening of the Myocyte

The extent of shortening in sham operated (n=7, N=5), OVX (n=15, N=10) and 17β-estradiol replaced OVX female (n=12, N=6) cardiac myocytes is shown in Figure 5.19. OVX female cardiac myocytes had significantly greater ($p<0.02$) extent of shortening when compared with both sham operated and 17β-estradiol replaced OVX female cells. No difference between the latter two groups was apparent.

Figure 5.20 illustrates the mean time to 50% relaxation values for sham (n=7, N=5), combined OVX (n=15, N=10) and 17β-estradiol replaced OVX female (n=12, N=6) cardiac myocytes. The combined OVX female cardiac myocytes had significantly faster ($p<0.05$) time to 50% relaxation when compared with both sham and 17β-estradiol replaced OVX female cardiac myocytes. A significant difference between the latter two groups was also apparent ($p<0.01$).

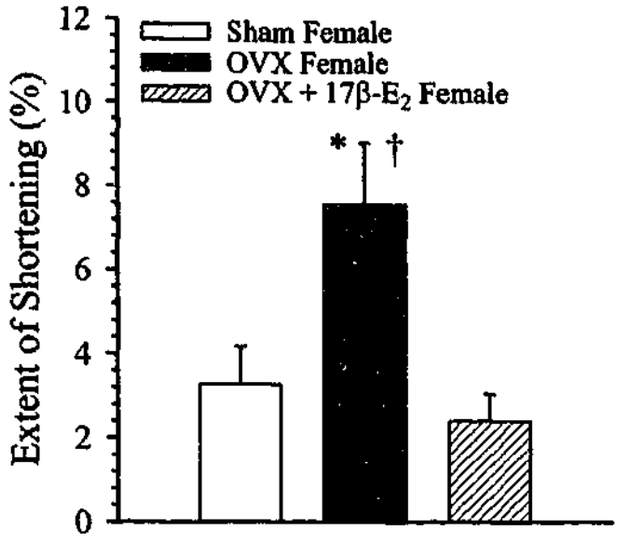


Figure 5.19: The average extent of shortening, expressed as percentage of resting cell length, in sham (n=7, N=5), combined OVX (n=15, N=10) and 17β-estradiol replaced OVX (n=12, N=6) female cardiac myocytes at 1.5mM extracellular [Ca^{2+}] at 0.5 Hz steady-state. * denotes value is significantly ($p<0.02$) different from sham female group. † denotes value is significantly ($p<0.02$) different from 17β-estradiol replaced OVX female group.

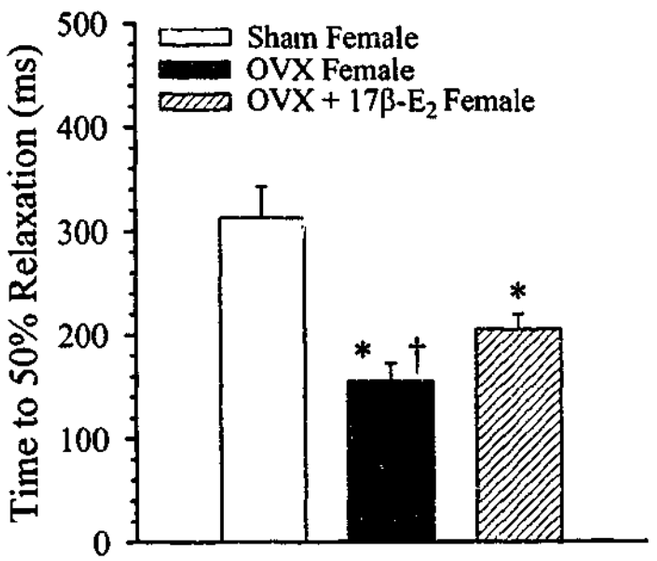


Figure 5.20: Average time to 50% relaxation for sham (n=7, N=5), combined OVX (n=15, N=10) and 17β-estradiol replaced OVX (n=12, N=6) female cardiac myocytes. Values are mean ± SEM. * denotes value is significantly ($p<0.05$) different from sham female group. † denotes value is significantly ($p<0.05$) different from 17β-estradiol replaced OVX group.

5.3 Discussion

The difference in the incidence of cardiovascular disease between men and pre-menopausal women is well recognised (Barrett-Connor and Bush, 1991; Stampfer *et al.*, 1991; Grady *et al.*, 1992; Chow, 1995; Gardin *et al.*, 1995), with the decreased risk in women attributed to the sex steroid estrogen and its actions on the vasculature, blood lipid profile (Barrett-Connor and Bush, 1991; Gerhard and Ganz, 1995; Farhat *et al.*, 1996; Pelzer *et al.*, 1997) and more recently on the heart itself (Grohe *et al.*, 1997; Meyer *et al.*, 1998). It has also been suggested that the beneficial effects of estrogen within the heart may be as a result of actions influencing Ca^{2+} movements (Jiang *et al.*, 1992; Collins *et al.*, 1993; Leblanc *et al.*, 1998; Meyer *et al.*, 1998). The present study sought to define the long-term effects of estrogen on Ca^{2+} movements within the heart, firstly by surgically removing the primary source of estrogen within the female rat, and secondly by replacing this source with an exogenous source of estrogen. The results demonstrate that cardiac myocytes from OVX female rats consistently have higher intracellular Ca^{2+} levels than those from intact female or 17β-estradiol replaced OVX female rats.

The peak $[Ca^{2+}]_i$ and amplitude of the Ca^{2+} transient both increased significantly with increasing extracellular $[Ca^{2+}]$ in cardiac myocytes from animals in all the experimental groups. This is consistent with what was reported in Chapter 3 and would be expected since as the extracellular $[Ca^{2+}]$ increased there would be an increased driving force for Ca^{2+} entry. Consequently, Ca^{2+} influx would increase as the extracellular $[Ca^{2+}]$ increased and this would increase both the Ca^{2+} content in the SR and the trigger Ca^{2+} for the Ca^{2+}-induced Ca^{2+} release mechanism. This in turn would result in larger amounts of Ca^{2+} released from the SR and hence greater $[Ca^{2+}]_i$ available to initiate contraction (Bassani *et al.*, 1994; Bers and Perez-Reyes, 1999). In contrast, baseline $[Ca^{2+}]_i$ did not change significantly with increasing extracellular $[Ca^{2+}]$ in any of the three groups. This is in contrast to what was found in the studies reported in Chapter 3, where resting $[Ca^{2+}]_i$ did increase significantly as the $[Ca^{2+}]_o$ was increased above 1.0 mM. The reasons for this are not clear. There was often a trend for resting $[Ca^{2+}]$ to increase with increasing $[Ca^{2+}]_o$ in individual cells (see Figures 5.1 and 5.8), however, with variability seen between cells no significant effect of extracellular $[Ca^{2+}]$ on resting $[Ca^{2+}]_i$ could be detected.

At all extracellular [Ca^{2+}], peak [Ca^{2+}]$_i$ and the amplitude of the Ca^{2+} transient were, on average, significantly greater in OVX female cardiac myocytes when compared with sham operated and 17β-estradiol replaced OVX female cardiac myocytes. In addition, no difference between sham operated and 17β-estradiol replaced animals in the amplitude of the Ca^{2+} transient was apparent indicating that removal of estrogen via ovariectomy results in an increase in the amplitude of the Ca^{2+} transient, whilst replacement of this estrogen brings the amplitude back to levels close to those observed in the intact female.

Baseline [Ca^{2+}]$_i$ was higher in myocytes from the OVX groups when compared to those from the sham operated group, but not when compared to those from the estrogen-replaced OVX group. The reasons behind why baseline [Ca^{2+}]$_i$ remained relatively high in this latter group are not clear, however, this observation would appear to be the reason why peak [Ca^{2+}]$_i$ remained significantly higher in the estrogen-replaced group than the intact group, while the amplitude of the Ca^{2+} transient was not significantly different between these two groups.

The results presented in this chapter indicate that long-term exposure to estrogen is an influencing factor on Ca^{2+} movements in the female heart. Estrogen has been shown to have acute inhibitory, non-genomic effects on the L-type Ca^{2+} current (Jiang *et al.*, 1992; Sitzler *et al.*, 1996; Meyer *et al.*, 1998) and has also been linked to genomic regulation of the L-type Ca^{2+} channel with an increased expression of the cardiac L-type Ca^{2+} channel being found in estrogen receptor α deficient mice (Johnson *et al.*, 1997). This would be expected to result in a larger influx of Ca^{2+} into the cell, larger SR Ca^{2+} release and greater contraction. Such genomic regulation of the L-type Ca^{2+} channel by estrogen would provide a plausible explanation for the increased Ca^{2+} transient amplitude levels that are apparent in OVX female animals in this study.

The differences between OVX female cardiac myocytes and intact and estrogen replaced OVX female cardiac myocyte in peak [Ca^{2+}]$_i$ and the amplitude of the Ca^{2+} transient are also reflected in the shortening of the myocyte. The extent of shortening was significantly greater in the OVX female cardiac myocyte when compared with the sham operated female and 17β-estradiol replaced OVX female groups. No difference between the latter two groups was apparent suggesting greater calcium entry and mobilisation of intracellular calcium in OVX female cells led to increased shortening in

these cells. This data on cell shortening is also consistent with that of Patterson *et al.* (1998) who reported that, in female rabbits, ovariectomy increased, and estrogen replacement decreased, isometric force associated with increased extracellular Ca^{2+}. This would be consistent with similar differences in the amplitude of the Ca^{2+} transient as those found in the present study.

Although reduced Ca^{2+} influx through the L-type Ca^{2+} channels has been linked to the beneficial effects of estrogen within the heart, the actions of estrogen on cellular Ca^{2+} movements may not be limited to this one site. Other studies have suggested that another possible site of action for estrogen is at the level of the SR (Penpargkul *et al.*, 1981; Leblanc *et al.*, 1998). The increased amplitude of the Ca^{2+} transient in the estrogen deficient OVX female animals may also be as a result of an increased SR Ca^{2+} content which would result in an increased Ca^{2+}-induced Ca^{2+} release and therefore a greater cardiac contraction. The rate of Ca^{2+} uptake into the SR during relaxation was assessed by measuring the time course of decay of the Ca^{2+} transient. As the SR is thought to be responsible for approximately 90% of the Ca^{2+} removal during relaxation in the rat ventricular myocyte (Bassani *et al.*, 1994), the time course of decay of the Ca^{2+} transient should give a good estimation of the rate of SR Ca^{2+} uptake. A significantly faster rate of decay in OVX female cardiac myocytes when compared with sham operated and 17β-estradiol replaced female cardiac myocytes was apparent. In accordance with this, a faster time to 50% relaxation was also observed in the OVX female cells. The faster rate of decay in the OVX female cells suggests an increased SR Ca^{2+}-ATPase activity which would result in faster rates of decay and possibly increased SR Ca^{2+} content. A previous study has found lower enzymatic activities in SR from female hearts when compared with male hearts (Penpargkul *et al.*, 1981) which would provide a plausible explanation for the faster rate of decay in OVX female rats which are not dissimilar in estrogen levels to male rats. Although this explanation for the increased rate of decay of the Ca^{2+} transient in OVX female rats seems convincing, the possibility that the Na^{+}/Ca^{2+} exchanger may also be playing a role cannot be discounted.

This study has shown clear differences in peak $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} transient in OVX female cardiac myocytes when compared with sham operated and 17β-estradiol replaced OVX female cardiac myocytes. The increased levels of intracellular Ca^{2+} within the estrogen deficient OVX female group when compared to

the estrogenised intact and 17β-estradiol replaced OVX groups suggests estrogen may play a long term role in limiting Ca^{2+} entry into the cardiac myocyte.

Chapter 6

Chapter 6

Effects of Gonadectomy and Testosterone Replacement on Intracellular Calcium

In the previous chapters, a gender-based difference in intracellular Ca^{2+} handling has been demonstrated. The importance of the sex steroid estrogen in this has also been addressed, however, the effects of the male steroid hormone testosterone have not been investigated. Whilst it seems likely that estrogen is acting cardioprotectively in the female, little research has been undertaken to investigate the role that testosterone may play in gender related differences in cardiac function.

Testosterone has numerous potential sites of action and plays a key role in a variety of processes in the male including spermatogenesis, development of secondary sexual characteristics and skeletal muscle growth. The discovery of functional androgen receptors within the heart has lead to speculation that testosterone may also be acting directly on the heart (Krieg *et al.*, 1978; McGill Jr *et al.*, 1980; Marsh *et al.*, 1998), however, very few studies have investigated the possible actions of testosterone on the heart itself.

Epidemiological studies have shed no clear light on any possible association between testosterone levels and the development of cardiovascular disease (Barrett-Connor and Khaw, 1988; Rosano and Panina, 1999; Rosano, 2000). In addition there is only a small

body of experimental evidence relating to possible actions of testosterone on the heart. Studies involving gonadectomy and testosterone replacement in rats found a decrease in contractile function of the heart in gonadectomised male rats when compared with control rats. This was associated with a reduction in myosin ATPase activity that accompanied a shift from the active V1 myosin isoenzyme to the less active V3 form (Schaible *et al.*, 1984). When testosterone was replaced, contractile function was increased back to, or in some cases slightly above, the control groups (Scheuer *et al.*, 1987).

In addition to these gonadectomy studies, acute Ca^{2+} antagonistic properties of testosterone on coronary vasoconstriction have also been investigated. In coronary arteries of castrated male pigs and male rats, testosterone was shown to cause coronary relaxation by inhibiting Ca^{2+} entry, and also by inhibiting other mechanisms independent of this pathway (Crews and Khalil, 1999a; Crews and Khalil, 1999b). This effect has also been shown in isolated coronary smooth muscle cells, with the relaxation of these cells and decrease of $[Ca^{2+}]_i$ mainly attributed to inhibition of Ca^{2+} entry from the extracellular space, but not inhibition of Ca^{2+} release from intracellular stores (Murphy and Khalil, 1999). Testosterone has also been shown to have a negative inotropic action in isolated guinea-pig papillary muscle, but this action was only demonstrated at supraphysiological concentrations of testosterone (Zhang *et al.*, 1998).

Given the relative lack of experimental evidence pertaining to possible long term effects of testosterone on the heart, and the earlier demonstration of a possible role for estrogen in modulating intracellular Ca^{2+} handling in the female heart, it was the aim of the current study to examine changes in $[Ca^{2+}]_i$ in male cardiac myocytes from sham operated, gonadectomised (GDX) and testosterone replaced GDX male rats.

6.1 Methodology

6.1.1 Experimental Groups

Two experimental studies were conducted using 3-month old male Wistar rats. In the first study animals underwent either a sham operation or gonadectomy. In the second study, the first group of animals was sham operated and implanted with an empty control pellet, the second group was gonadectomised and implanted with an empty control pellet and the third group was gonadectomised and implanted with a testosterone filled pellet. All rats were left for 14-18 days after surgery before experiments commenced.

6.1.2 Gonadectomy Surgery

Gonadectomy (GDX) or sham-gonadectomy was performed under anaesthesia induced with ketamine (4.5 mg/100 g i.m.) and xylazine (0.7 mg/100 g i.m.). Two separate incisions were placed in the scrotum and in each case the testicle was visualised. For sham operations, the testes were replaced and the incisions closed, while for gonadectomised animals each testicle was isolated from surrounding tissue and was removed.

6.1.3 Testosterone Replacement

Pellets containing testosterone were made from Silastic medical grade tubing (1.98 mm i.d. × 3.18 mm o.d.; Dow Corning Corporation, Midland, MI, USA) and were used at a length of 3 cm. The pellets were made by packing testosterone powder (Sigma, St Louis, MO) into the correctly sized pre-cut tubing and then sealing the ends with adhesive silicone. Once the testes were removed during surgery, a small incision was made at the base of the neck and the testosterone pellet was inserted subcutaneously into this region. Following surgery, all animals were given free access to food and water for the remainder of the study.

To confirm the success of the surgery and the testosterone replacement, the prostate was removed at the beginning of the experiment and weighed. The prostate and body weights of the animals in the different experimental groups are shown in Table 6.1.

Table 6.1: Body weight and prostate weight of male rats in the various experimental groups.

Experimental Group	Body Weight (g)	Prostate Weight (g)	Prostate:Body Weight Ratio (mg/g)
Sham Male (n=5)	428±26	0.69±0.13	1.66±0.38
GDX Male (n=6)	436±13	0.02±0.001*	0.047±0.003*
Sham Male + Control Pellet (n=6)	509±23†	0.501±0.061	0.924±0.13
GDX Male + Control Pellet (n=6)	466±21	0.07±0.004*†	0.15±0.01*†
GDX Male + Testosterone Pellet (n=6)	421±13	0.624±0.028	1.49±0.072*

* indicates that this value is significantly different ($p<0.05$) from corresponding sham male group. † indicates that this value is significantly different ($p<0.05$) from the testosterone replaced GDX male group.

6.1.4 Experimental Protocols

All experiments reported in this chapter involved recordings of intracellular Ca^{2+} and cell shortening from freshly isolated single cardiac myocytes. The procedures for cell isolation and the recording techniques were as described in Chapter 2. The approach taken in this chapter was identical to that taken in Chapter 5, with responses of myocytes from animals in the different experimental groups to changes in extracellular Ca^{2+} concentration being recorded.

6.1.5 Statistics

All data are presented as mean ± standard error of the mean (SEM). All data presented in this chapter have been analysed using a one-way ANOVA with repeated measures.

6.2 Results

6.2.1 Study 1: Effects of Gonadectomy on Changes in Intracellular [Ca^{2+}] in Response to Varied Extracellular [Ca^{2+}]

Figure 6.1 illustrates an example of original Ca^{2+} transient recordings from sham male and gonadectomised male cardiac myocytes stimulated to contract at 0.5 Hz steady-state and exposed to four different Ca^{2+} concentrations. In the top panel recordings from a sham male cardiac myocyte in 0.5, 1.0, 1.5 and 2.0 mM Ca^{2+} are shown, whilst in the bottom panel recordings from a gonadectomised male cardiac myocyte under the same conditions is shown. It is apparent from this figure that an increase in extracellular [Ca^{2+}] resulted in an increase in $[Ca^{2+}]_i$ in both sham and GDX male cells. In addition, it seems apparent that this rise is greater in the sham male cells when compared with the GDX male cells.

Figure 6.2 shows mean baseline $[Ca^{2+}]_i$ levels in sham male (n=12, N=5) and GDX male (n=14, N=5) cardiac myocytes at four different extracellular [Ca^{2+}]. All cells were stimulated to contract at 0.5 Hz steady-state. As $[Ca^{2+}]_o$ was increased, baseline $[Ca^{2+}]_i$ did not change in the sham male group, but significantly increased in the GDX male group ($p<0.02$). No significant difference in baseline $[Ca^{2+}]_i$ between GDX male and sham male cardiac myocytes was apparent.

The mean peak $[Ca^{2+}]_i$ values in sham male and GDX male cardiac myocytes at four different extracellular [Ca^{2+}] is shown in Figure 6.3. As $[Ca^{2+}]_o$ was increased, peak $[Ca^{2+}]_i$ significantly increased ($p<0.001$) in both sham and GDX male cardiac myocytes. The rate at which this occurred was also significantly different ($p<0.02$) between the two groups. In addition, the sham operated male cardiac myocytes had, on average, significantly greater ($p<0.002$) peak $[Ca^{2+}]_i$ when compared with GDX male cardiac myocytes at all $[Ca^{2+}]_o$ investigated.

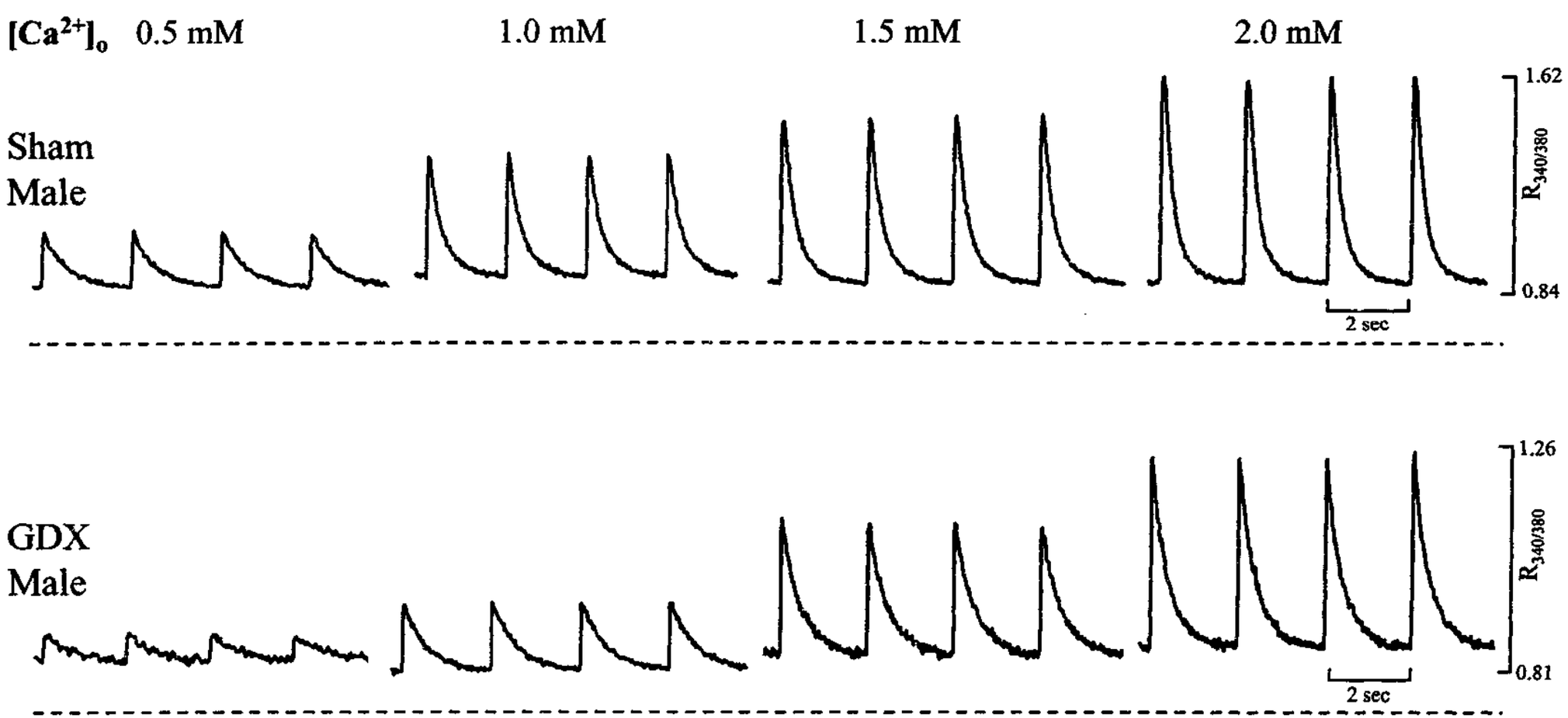


Figure 6.1: Example of Ca^{2+} transients recorded from male cardiac myocytes stimulated at 0.5 Hz steady-state in the presence of different extracellular $[Ca^{2+}]$. The top panel shows Ca^{2+} transients recorded from a sham male cell at 0.5, 1.0, 1.5 and 2.0 mM $[Ca^{2+}]_o$, whilst the bottom panel shows a GDX male cell under the same conditions.

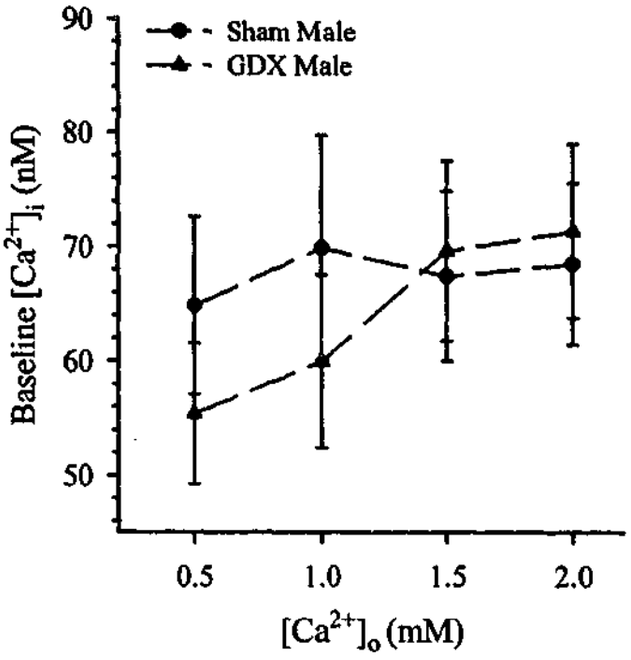


Figure 6.2: The effects of increasing extracellular $[Ca^{2+}]$ on baseline $[Ca^{2+}]_i$ in sham (n=12, N=5) and GDX (n=14, N=5) male cardiac myocytes. Values are mean ± SEM.

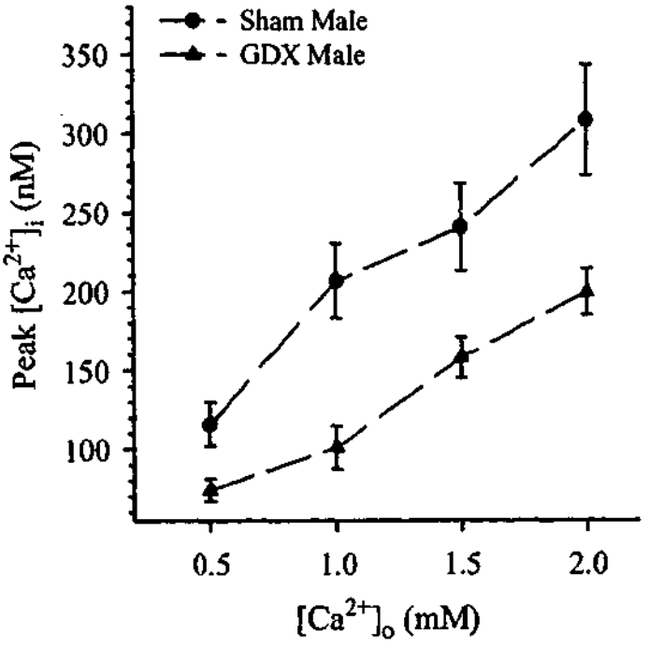


Figure 6.3: The effects of increasing extracellular $[Ca^{2+}]$ on peak $[Ca^{2+}]_i$ in sham (n=12, N=5) and GDX (n=14, N=5) male cardiac myocytes. Values are mean ± SEM.

Figure 6.4 shows the average amplitude of the Ca^{2+} transient in sham and GDX male cardiac myocytes stimulated to contract at 0.5 Hz steady-state and at varying extracellular [Ca^{2+}]. As the $[Ca^{2+}]_o$ was increased, the amplitude of the Ca^{2+} transient also significantly increased ($p<0.001$) in both sham and GDX male groups. Interestingly, the rate at which this increase occurred was also significantly different ($p<0.04$) between the two groups. On average, the amplitude of the Ca^{2+} transient was significantly greater ($p<0.001$) in sham male cardiac myocytes when compared with GDX male cardiac myocytes.

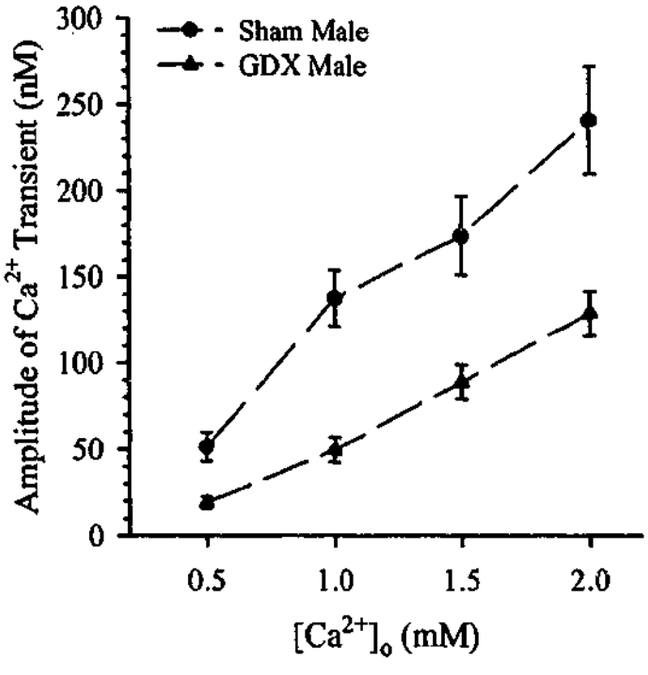
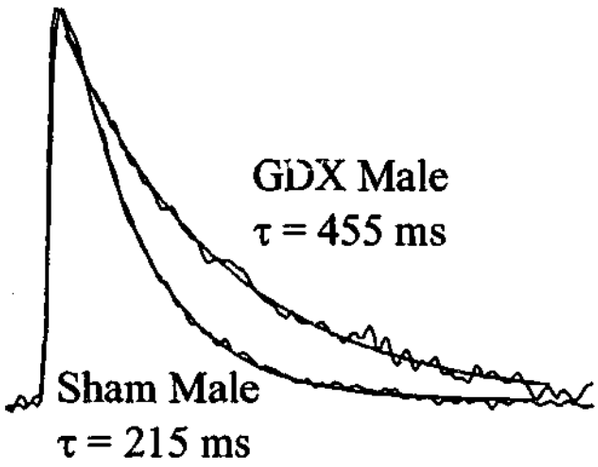


Figure 6.4: The effects of increasing extracellular [Ca^{2+}] on the amplitude of the Ca^{2+} transient in sham (n=12, N=5) and GDX (n=14, N=5) male cardiac myocytes. Values are mean ± SEM.

6.2.2 Study 1: Effect of Gonadectomy on the Time Course of the Ca^{2+} Transient Decay

The differences in the time course of decay of the Ca^{2+} transient between sham male (n=12, N=5) and GDX male (n=14, N=5) cardiac myocytes is illustrated in Figure 6.5. The top panel shows transients from a sham male and a GDX male cell which have been overlayed and normalised to the same amplitude to facilitate comparison. A single exponential function was fitted to each trace, from which a time constant was derived. Cells were stimulated to contract at 0.5 Hz steady-state in 1.5 mM Ca^{2+}. From these

traces it can be seen that the time course of decay appears to be faster in the sham male cardiac myocyte when compared with the GDX male cardiac myocyte. The average data for the time course of decay of the Ca^{2+} transient is shown in the bottom panel, with sham male cardiac myocytes showing a significantly faster ($p<0.05$) time course of decay than GDX operated male cardiac myocytes.



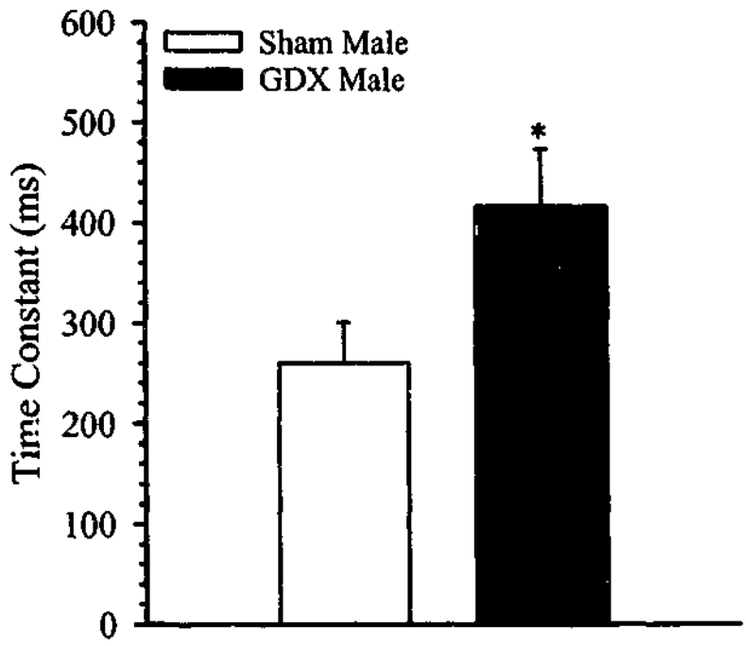


Figure 6.5: The effects of gonadectomy on the time course of decay of the Ca^{2+} transient. The transients in the top panel have been scaled to the same amplitude to facilitate comparison with a single exponential function fitted from which a time constant was derived. Average time constant values for sham (n=12, N=5) and GDX (n=14, N=5) male cardiac myocytes are shown in the bottom panel. Values are mean ± SEM. * denotes that the value is significantly different ($p<0.05$) from sham male cardiac myocyte.

6.2.3 Study 1: Effect of Gonadectomy on Myocyte Shortening

Figure 6.6 shows an example of original length recordings from sham male and GDX male cardiac myocytes stimulated to contract at 0.5 Hz steady-state and exposed to four different Ca^{2+} concentrations. In the top panel recordings from a sham male cardiac myocyte in 0.5, 1.0, 1.5 and 2.0 mM extracellular Ca^{2+} are shown, whilst in the bottom panel recordings from a GDX male cardiac myocyte under the same conditions are shown. It is apparent from this figure that an increase in $[Ca^{2+}]_o$ resulted in an increase in the extent of shortening of the cardiac myocyte in both sham and GDX groups. In addition, the extent of shortening of the sham male cardiac myocyte appears to be greater when compared with the GDX male cardiac myocyte.

The average extent of shortening data from sham male (n=12, N=5) and GDX male (n=14, N=5) cardiac myocytes stimulated to contract at 0.5 Hz steady-state and in 1.5 mM Ca^{2+} is shown in Figure 6.7. The sham male cardiac myocytes showed significantly greater ($p<0.003$) extent of shortening when compared with GDX male cardiac myocytes.

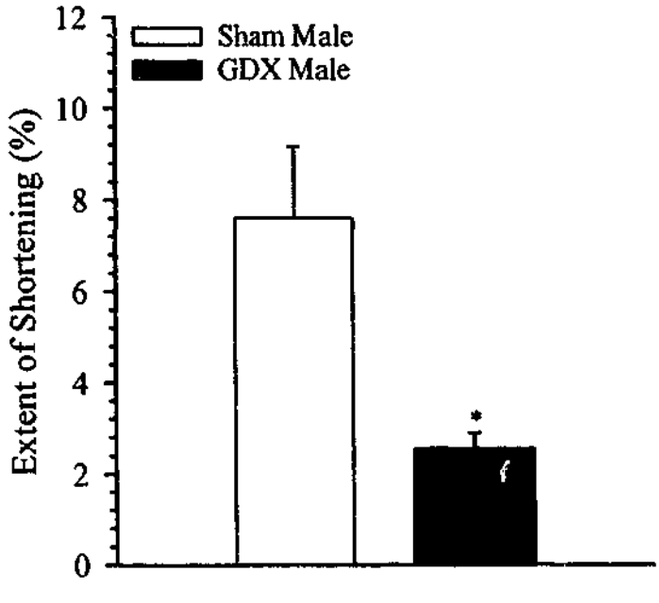


Figure 6.7: The average extent of shortening values in sham (n=12, N=5) and GDX (n=14, N=5) male cardiac myocytes in 1.5 mM extracellular $[Ca^{2+}]$ at 0.5 Hz steady-state. * denotes value is significantly different ($p<0.003$) from sham male group.

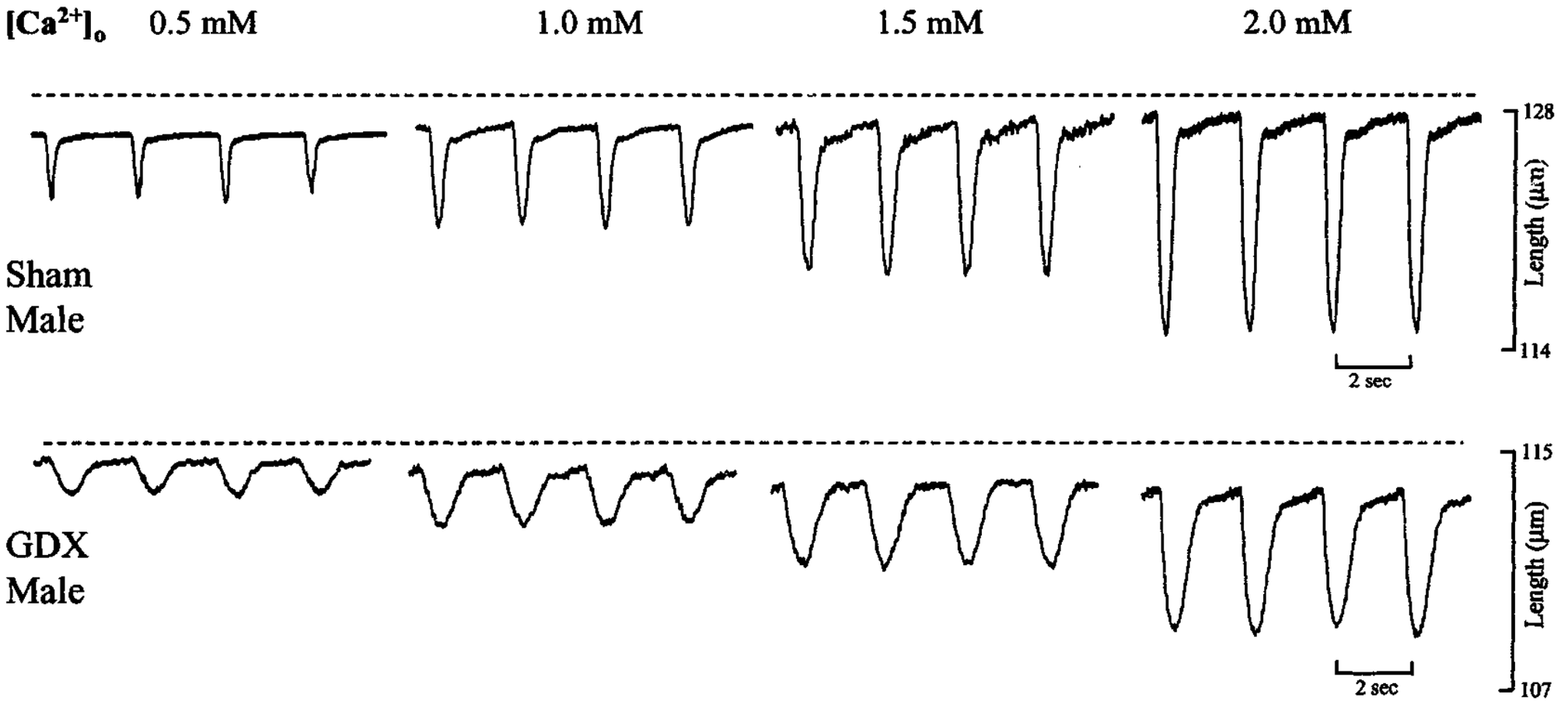


Figure 6.6: Example of shortening traces from a sham male and a GDX male cardiac myocyte stimulated to contract at 0.5 Hz steady-state in varying extracellular [Ca^{2+}]. The top panel shows shortening traces from a sham male cell which has been exposed to 0.5, 1.0, 1.5 and 2.0 mM $[Ca^{2+}]_o$, whilst the bottom panel shows a GDX male cell under the same conditions.

Figure 6.8 illustrates differences in the time course of relaxation of myocytes from sham male and GDX male cardiac myocytes. The top panel shows original recordings from sham and GDX male cells which have been overlayed and normalised to the same peak amplitude to facilitate comparison. It is apparent from these traces that the time to 50% relaxation is faster in the sham male cells when compared with the GDX male cells. This is confirmed in the bottom bar graph which shows the mean time to 50% relaxation data for the two groups, highlighting the significantly faster ($p<0.005$) rate in sham male (n=12, N=5) cardiac myocytes when compared with GDX male (n=14, N=5) cardiac myocytes.

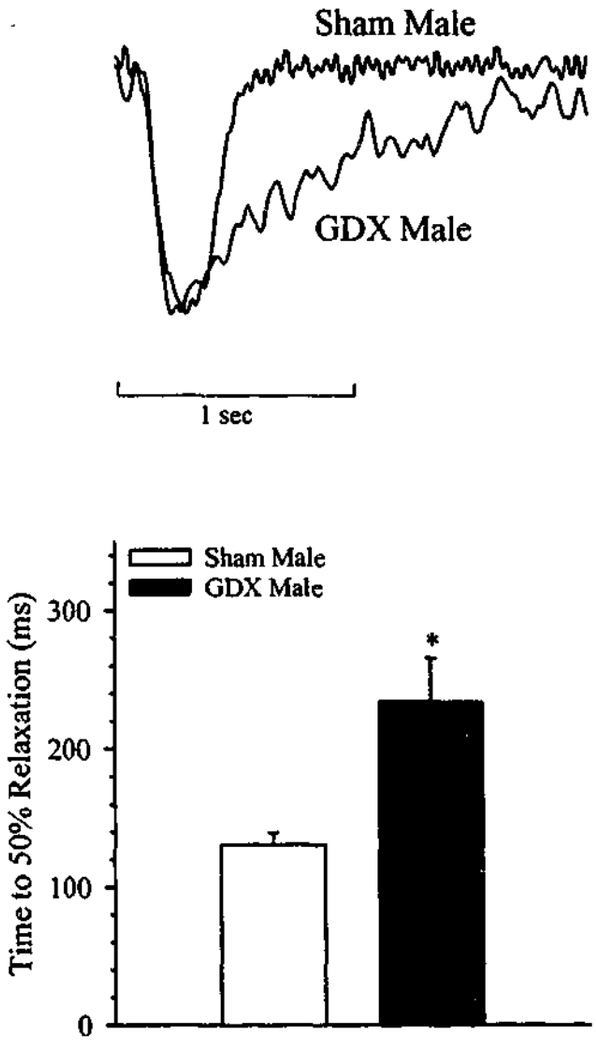


Figure 6.8: Original shortening recordings from sham and GDX male cardiac myocytes stimulated to contract at 0.5 Hz steady-state in 1.5 mM $[Ca^{2+}]_o$ are illustrated in the top panel. Traces are normalised to the same amplitude to facilitate comparison. From the traces the time to 50% relaxation was calculated. Average values for the time to 50% relaxation in sham (n=12, N=5) and GDX (n=14, N=5) male cardiac myocytes are shown in the bottom panel. Values are mean ± SEM. * denotes that the value is significantly different ($p<0.005$) from sham male cardiac myocyte.

6.2.4 Study 2: Changes in Intracellular [Ca^{2+}] in Response to Gonadectomy, Testosterone Replacement and Varied Extracellular [Ca^{2+}]

The mean baseline $[Ca^{2+}]_i$ values in sham male (n=12, N=6), GDX male (n=13, N=5) and testosterone replaced GDX male (n=15, N=6) cardiac myocytes at varying $[Ca^{2+}]_o$ are shown in Figure 6.9. As $[Ca^{2+}]_o$ was increased there was a significant increase ($p<0.02$) in baseline $[Ca^{2+}]_i$ in all three groups. In addition, a significant difference in baseline $[Ca^{2+}]_i$ between sham male cardiac myocytes and GDX male cardiac myocytes was apparent ($p<0.05$). There was no significant difference in baseline $[Ca^{2+}]_i$ between the sham male and testosterone replaced GDX male groups. In addition, whilst the baseline $[Ca^{2+}]_i$ in the testosterone replaced GDX group tended to be higher than that in the GDX group, this difference was not statistically significant.

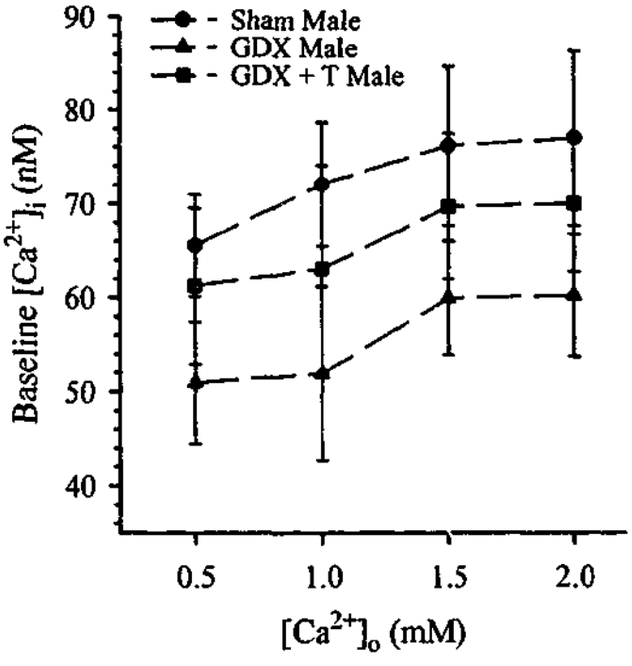


Figure 6.9: The effects of increasing extracellular $[Ca^{2+}]$ on baseline $[Ca^{2+}]_i$ in sham (n=12, N=6), GDX (n=13, N=5) and testosterone replaced GDX (n=15, N=6) male cardiac myocytes, Values are mean ± SEM.

Changes in peak $[Ca^{2+}]_i$ in response to varying $[Ca^{2+}]_o$ in sham, GDX and testosterone replaced GDX male cardiac myocytes is shown in Figure 6.10. As $[Ca^{2+}]_o$ was increased, peak $[Ca^{2+}]_i$ increased significantly ($p<0.001$) in all three groups.

Interestingly, the rate at which this increase occurred was significantly different in the GDX male group when compared with both the sham male and the testosterone replaced GDX male groups ($p<0.005$ for each). No difference was apparent between the sham and testosterone replaced GDX male groups. In addition, peak $[Ca^{2+}]_i$ was signif.cantly lower in the GDX male cardiac myocytes when compared with both sham and testosterone replaced GDX male cardiac myocytes ($p<0.001$). No significant difference in peak $[Ca^{2+}]_i$ was apparent between the sham and testosterone replaced GDX male cells.

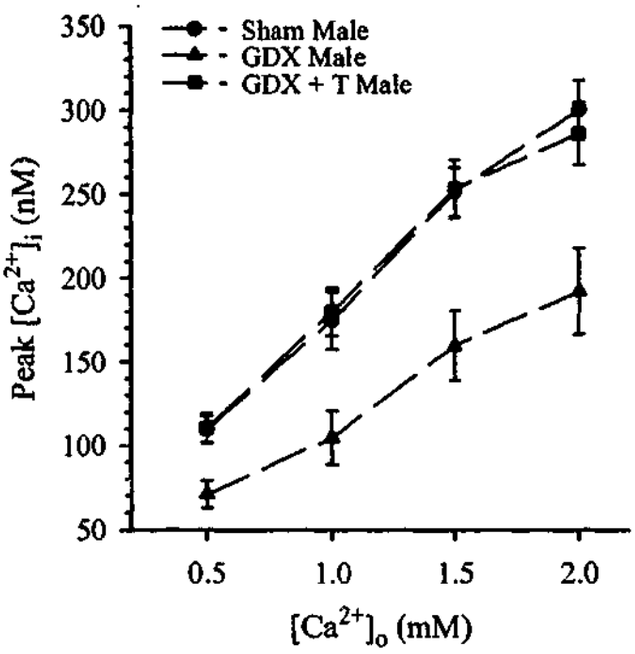


Figure 6.10: The effects of increasing extracellular $[Ca^{2+}]$ on peak $[Ca^{2+}]_i$ in sham (n=12, N=6), GDX (n=13, N=5) and testosterone replaced GDX (n=15, N=6) male cardiac myocytes. Values are mean ± SEM.

A similar trend was observed in the amplitude of the Ca^{2+} transient which is shown in Figure 6.11. A significant increase in the amplitude of the Ca^{2+} transient ($p<0.001$) was apparent in all three groups when extracellular $[Ca^{2+}]$ was increased. This increase also occurred at a significantly different rate in the GDX male cardiac myocytes when compared with both sham and testosterone replaced GDX male cardiac myocytes ($p<0.003$ for each). No difference between the latter two groups was apparent. In addition the amplitude of the Ca^{2+} transient was significantly smaller in the GDX male cells when compared with both sham ($p<0.002$) and testosterone replaced GDX

($p<0.001$) male cardiac myocytes. No significant difference between the sham and testosterone replaced GDX male cells was apparent.

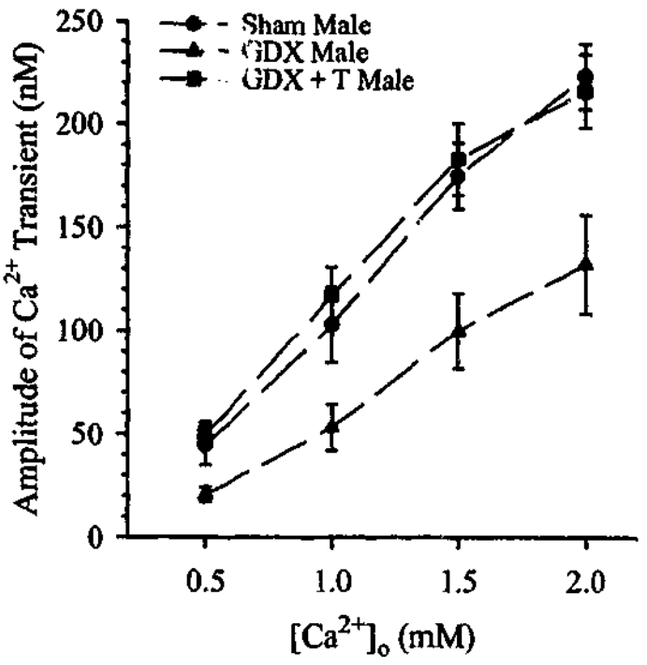


Figure 6.11: The effects of increasing extracellular [Ca^{2+}] on the amplitude of the Ca^{2+} transient in sham (n=12, N=6), GDX (n=13, N=5) and testosterone replaced GDX (n=15, N=6) male cardiac myocytes. Values are mean ± SEM.

6.2.5 Study 2: Effects of Gonadectomy and Testosterone Replacement on the Time Course of the Ca^{2+} Transient Decay

Figure 6.12 shows the average data for the time course of decay of the Ca^{2+} transient in myocytes from sham (n=12, N=6), GDX (n=12, N=5) and testosterone replaced GDX (n=14, N=6) male cardiac myocytes. GDX male cardiac myocytes displayed significantly slower Ca^{2+} transient decay when compared with both sham ($p<0.05$) and testosterone replaced GDX ($p<0.03$) male cardiac myocytes. No significant difference between sham and testosterone replaced GDX male cardiac myocytes was apparent.

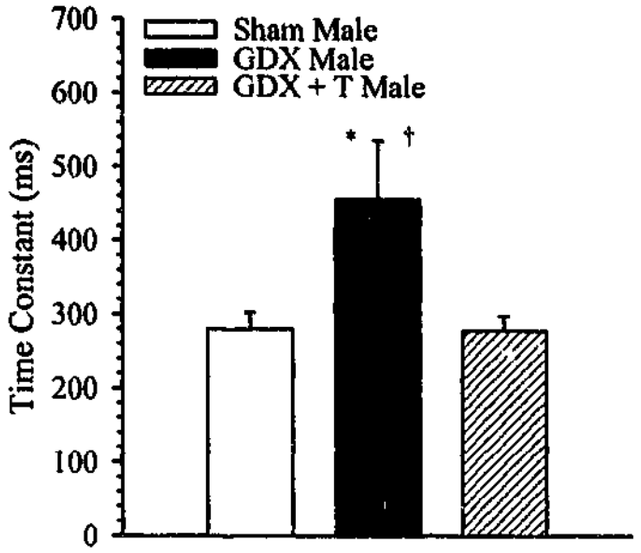


Figure 6.12: Average time constant for the decay of the Ca^{2+} transient in sham (n=12, N=6), GDX (n=12, N=5) and testosterone replaced GDX (n=14, N=6) male cardiac myocytes. Values are mean ± SEM. * denotes value is significantly different ($p<0.05$) from sham male group. † denotes value is significantly different ($p<0.03$) from testosterone replaced GDX male group.

6.2.6 Study 2: Effects of Gonadectomy and Testosterone Replacement on Myocyte Shortening

Figure 6.13 illustrates the extent of shortening of cardiac myocytes from sham (n=12, N=6), GDX (n=12, N=5) and testosterone replaced GDX male (n=15, N=6) rats. The extent of shortening was significantly smaller in the GDX group when compared with both the sham ($p<0.02$) and testosterone replaced GDX ($p<0.01$) male groups. No significant difference between the sham and testosterone replaced GDX male cardiac myocytes was evident.

Figure 6.14 illustrates the mean data for the time to 50% cell re-lengthening in myocytes from sham (n=12, N=6), GDX (n=12, N=5) and testosterone replaced GDX (n=15, N=6) male cardiac myocytes. GDX male cardiac myocytes exhibited significantly slower time to 50% relaxation when compared with sham ($p<0.02$) and testosterone replaced GDX ($p<0.03$) male cardiac myocytes. No significant difference between the latter two groups was apparent.

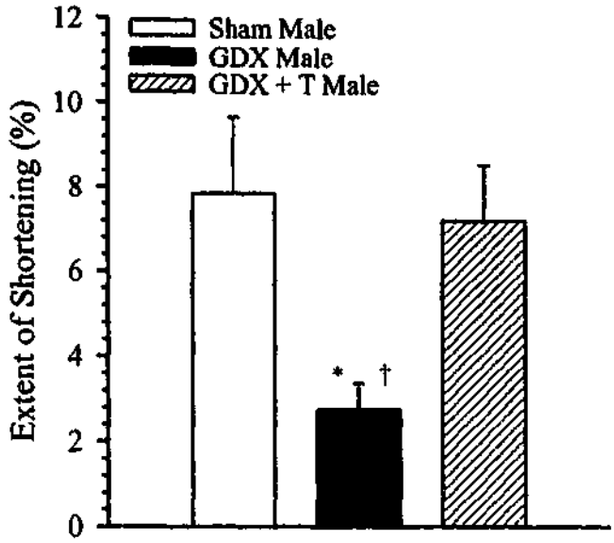


Figure 6.13: The average extent of shortening expressed as a percentage of resting cell length in sham (n=12, N=6), GDX (n=12, N=5) and testosterone replaced GDX (n=15, N=6) male cardiac myocytes in 1.5 mM extracellular [Ca^{2+}] at 0.5 Hz steady-state. * denotes value is significantly different ($p<0.02$) from sham group. † denotes value is significantly different ($p<0.01$) from testosterone replaced GDX male group.

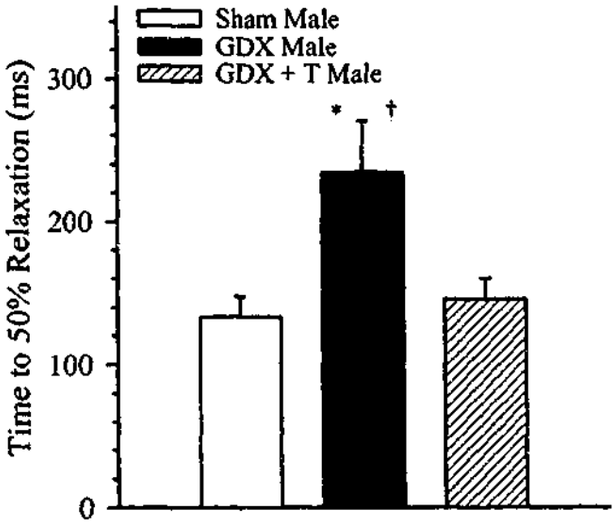


Figure 6.14: The average time to 50% relaxation values for sham (n=12, N=6), GDX (n=12, N=5) and testosterone replaced GDX (n=15, N=6) male cardiac myocytes. Values are mean ± SEM. * denotes value is significantly different ($p<0.02$) from sham male group. † denotes value is significantly different ($p<0.03$) from testosterone replaced GDX male group.

6.3 Discussion

Gender-specific differences in the incidence of cardiovascular disease are well recognised (Barrett-Connor and Bush, 1991; Stampfer *et al.*, 1991; Grady *et al.*, 1992; Chow, 1995; Gardin *et al.*, 1995) with the decreased risk in women mainly attributed to the sex steroid estrogen and its reported beneficial actions on the lipid profile and vascular system (Barrett-Connor and Bush, 1991; Gerhard and Ganz, 1995). The beneficial effects of estrogen within the female heart have been suggested to be as a result of actions influencing Ca^{2+} movements (Jiang *et al.*, 1992; Collins *et al.*, 1993; Leblanc *et al.*, 1998; Meyer *et al.*, 1998) and a small amount of evidence is gathering suggesting that testosterone may also be capable of influencing Ca^{2+} handling within the heart (Zhang *et al.*, 1998; Crews and Khalil, 1999a; Crews and Khalil, 1999b; Murphy and Khalil, 1999). These studies, however, were concerned with rapid effects of testosterone applied acutely to isolated tissues at relatively high concentrations. The present study was undertaken to investigate the long term effects of testosterone on Ca^{2+} movements within the heart, firstly by surgically removing the source of testosterone within the male rat, and secondly by replacing this with an exogenous source of testosterone. The results demonstrate that cardiac myocytes from GDX male rats consistently have lower $[Ca^{2+}]_i$ levels than those from sham operated or testosterone replaced GDX male rats.

Increases in $[Ca^{2+}]_o$ resulted in significant increases in the baseline $[Ca^{2+}]_i$, peak $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} transient in cardiac myocytes from sham operated, GDX and testosterone replaced GDX male rats. This is consistent with observations in female cells reported in both Chapters 3 and 5. As the extracellular $[Ca^{2+}]$ is increased the concentration gradient favouring Ca^{2+} entry increases. This would be expected to increase Ca^{2+} entry, which in turn would promote increased Ca^{2+} storage in the SR, as well as providing a greater trigger for Ca^{2+}-induced Ca^{2+}-release. The result would be a larger Ca^{2+} influx and a larger release of Ca^{2+} from the SR which would lead to greater calcium activation of the contractile machinery and, therefore, increased cardiac contraction (Bassani *et al.*, 1994; Bers and Perez-Reyes, 1999).

Peak $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} transient were, on average, significantly lower in GDX male cardiac myocytes when compared with both sham and testosterone replaced GDX male cardiac myocytes at all extracellular $[Ca^{2+}]$ studied. In addition, no

difference between the sham and testosterone replaced GDX male cardiac myocytes was apparent indicating that removal of testosterone via gonadectomy resulted in a decrease in the amplitude of the Ca^{2+} transient, whilst replacement of this testosterone with an exogenous source returned the amplitude of the Ca^{2+} transient to that seen in the sham operated animals. A decrease in cardiac contractile function in gonadectomised male rats, when compared with control rats, has been reported previously. This was accompanied by a reduction in myosin ATPase activity that was correlated with a shift from the more active V1 myosin isoenzyme to the less active V3 form (Schaible *et al.*, 1984). Administration of testosterone replacement to the gonadectomised animals prevented both the decrease in contractile function and the shift in myosin isoform pattern (Scheuer *et al.*, 1987). Whilst changes in myosin isoform expression could undoubtedly contribute to the differences in cardiac function observed in these studies, they are also consistent with the differences in the amplitude of the Ca^{2+} transient reported in this chapter.

The results indicate that the presence of the gonads is an influencing factor on intracellular Ca^{2+} movements in the male heart. Furthermore, since the changes in $[Ca^{2+}]_i$ that resulted from gonadectomy were reversed by the administration of testosterone, it is likely that it is this hormone that is responsible for the differences observed between the intact and GDX males. This would presumably reflect a long term action of testosterone, mediated through a genomic mechanism. The fact that the androgen receptor gene has been shown to be expressed specifically in cardiac myocytes, and that it has been determined to be functionally active (Marsh *et al.*, 1998), suggests a possible role for testosterone at this site, which may include regulation of $[Ca^{2+}]_i$ movements.

One possibility that would be consistent with the results reported in this chapter could be that testosterone is acting to modulate genomic regulation of the L-type Ca^{2+} channel, and hence increase expression of this channel. This would provide an explanation for the decreased peak $[Ca^{2+}]_i$ and Ca^{2+} transient amplitude seen in the testosterone deficient GDX male animals in this study. It would also be consistent with the lesser extent of increase in $[Ca^{2+}]_i$ with increasing extracellular $[Ca^{2+}]$ seen in these animals. It is, however, important to acknowledge that the present experiments do not provide any direct evidence for a long term effect of testosterone specifically on the expression of the L-type Ca^{2+} channel. There also do not appear to be any previous

studies that have addressed possible genomic modulation of the L-type Ca^{2+} channel by testosterone. Previous studies investigating the role of testosterone on Ca^{2+} movements have found testosterone to possess Ca^{2+} antagonistic properties, which on the face of it would appear to be quite opposite to the results presented in this investigation. These studies, however, were performed predominantly in vascular smooth muscle, and focussed on rapid, non-genomic actions of testosterone at supraphysiological levels (Rubio *et al.*, 1998; Zhang *et al.*, 1998; Murphy and Khalil, 1999).

Another possible reason for the decreased amplitude of the Ca^{2+} transient and peak $[Ca^{2+}]_i$ in the testosterone deficient GDX male animals may be a decreased SR Ca^{2+} uptake. This would result in smaller SR Ca^{2+} content, reduced Ca^{2+}-induced Ca^{2+}-release and, therefore, a smaller cardiac contraction. The rate of Ca^{2+} uptake into the SR during relaxation was assessed by measuring the time course of decay of the Ca^{2+} transient. As the SR is thought to be responsible for approximately 90% of the cytosolic Ca^{2+} removal during relaxation in the rat ventricular myocyte (Bassani *et al.*, 1994), the time course of decay of the Ca^{2+} transient should give a good estimation of the rate of SR Ca^{2+} uptake. The rate of decay of the Ca^{2+} transient was found to be significantly slower in GDX male cardiac myocytes, along with a slower time to 50% relaxation, when compared with sham and testosterone replaced GDX male cardiac myocytes. The slower rate of decay in the GDX male cells suggests a decreased SR Ca^{2+}-ATPase activity which would result in slower rates of decay and possible decreased SR Ca^{2+} content. Whilst this would provide a plausible explanation for the effects of testosterone on the time course of decay, it is also possible that testosterone could be acting at the level of the Na^+/Ca^{2+} exchanger or sarcolemmal Ca^{2+} pump to influence Ca^{2+} extrusion during relaxation. At present there appears to be no direct evidence available that relates to possible actions of testosterone on these Ca^{2+} handling pathways.

The differences between GDX male cardiac myocytes and sham operated and testosterone replaced GDX male cardiac myocytes in peak $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} transient are also reflected in the shortening of the myocyte. The extent of shortening was significantly less in the GDX male cardiac myocytes when compared with sham and testosterone replaced GDX male cardiac myocytes. No difference between the latter two groups was apparent. These results are consistent with reduced calcium entry and mobilisation of intracellular calcium in the testosterone deficient GDX male cells. These differences in cell shortening are compatible with the decreased

contractile function found in isolated perfused hearts of gonadectomised male rats, and the reversal of this by testosterone supplementation (Schaible *et al.*, 1984; Scheuer *et al.*, 1987)

It is interesting to note that abuse of androgenic anabolic steroids has been reported to be linked to incidences of sudden cardiac death, myocardial infarction, cardiac hypertrophy and ventricular remodelling, as well as altered serum lipoproteins (Melchert and Welder, 1995). Although the precise mechanisms leading to the development of cardiac pathologies associated with steroid abuse are currently unknown, direct actions on the heart itself may well contribute (Melchert and Welder, 1995; Shapiro *et al.*, 1999). Perhaps one of the best demonstrations of the capacity of androgens to have a direct, genomic action on cardiac myocytes in this context is the study of Marsh *et al.* (1998) showing that androgen receptors mediate hypertrophy in cardiac myocytes. In the intact animal, the effects of androgenic steroids on the heart are likely to be multifactorial, however, the results reported here raise the possibility that a contributing factor may be high levels of intracellular Ca^{2+} occurring in cardiac myocytes in a high androgenic steroid environment.

This study has shown clear differences in peak $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} transient between GDX male cardiac myocytes and sham and testosterone replaced GDX male cardiac myocytes. The decreased levels of intracellular Ca^{2+} within the testosterone deficient GDX male cardiac myocytes would favour reduced myocardial contractility, and limitation of Ca^{2+} entry may serve to protect the hearts of these animals against potentially damaging intracellular Ca^{2+} levels under some circumstances. On the other hand, high levels of testosterone, whilst favouring increased myocardial contractility, may also have the potential to play a deleterious role by promoting high levels of Ca^{2+} entry in the male heart.

Chapter 7

Chapter 7

General Discussion

The existence of gender based differences in cardiovascular disease are now well recognised with pre-menopausal women displaying a significantly reduced risk of developing cardiovascular complications than age-matched men (Barrett-Connor and Bush, 1991; Stampfer *et al.*, 1991; Grady *et al.*, 1992; Chow, 1995; Gardin *et al.*, 1995). After menopause, however, the risk of cardiovascular disease in women reaches levels similar to, or in some cases greater than, that in age-matched men unless hormone replacement therapy is given. This suggests that estrogen is an important factor in the protection against cardiovascular disease (Stampfer *et al.*, 1991; Grady *et al.*, 1992; Collins *et al.*, 1993; Chow, 1995). Estrogen has been shown to have a beneficial effect on both the lipid profile and vasculature in women, and for many years it was believed that these pathways were the only way in which estrogen was having its beneficial actions (Bush *et al.*, 1987; Sacks and Walsh, 1990; Grady *et al.*, 1992; Gerhard and Ganz, 1995). The discovery of estrogen receptors within the heart itself suggested that estrogen's beneficial actions may not be limited to the coronary circulation alone but may also extend to the myocardium as well (Stumpf *et al.*, 1977; Grohe *et al.*, 1997).

Since this discovery, a number of studies have been undertaken to elucidate the role of estrogen in the heart. A role for estrogen influencing Ca^{2+} movements has been suggested by several studies which investigated the acute effects of 17β-estradiol in various muscle types (Raddino *et al.*, 1986; Jiang *et al.*, 1992; Sitzler *et al.*, 1996; Meyer *et al.*, 1998). Although these studies revealed a non-genomic Ca^{2+} inhibitory action for 17β-estradiol, this was only evident at estradiol concentrations that far exceeded the physiological range. A small body of evidence also exists suggesting possible long-term genomic regulation of cardiac contractility by estrogen (Johnson *et al.*, 1997). In light of this, the current studies were undertaken to firstly clarify if a gender difference in Ca^{2+} handling was apparent in cardiac myocytes, and secondly, if a difference was found, to ascertain if the steroid hormones estrogen and testosterone were contributing to this difference.

The experiments undertaken in this thesis have shown a clear gender based difference in Ca^{2+} handling in the heart, with cardiac myocytes from intact male rats consistently demonstrating higher $[Ca^{2+}]_i$ than intact female cardiac myocytes. In addition, normalised force was significantly greater in male rat papillary muscle when compared with female rat papillary muscle. To ascertain if the steroid hormone estrogen was involved in this gender based difference, female rats underwent ovariectomy with or without subsequent 17β-estradiol replacement. Cardiac myocytes from female rats that underwent ovariectomy surgery consistently demonstrated significantly higher $[Ca^{2+}]_i$ than those from sham operated female rats. In addition, 17β-estradiol replacement in ovariectomised females reduced the $[Ca^{2+}]_i$ levels back to near sham operated female levels. The role of testosterone in long term modulation of intracellular Ca^{2+} handling in cardiac myocytes of male rats was also studied in an attempt to see if this sex steroid was contributing to the apparent gender differences. Cardiac myocytes from male rats that underwent gonadectomy surgery consistently demonstrated significantly lower $[Ca^{2+}]_i$ than those from sham operated male rats. Testosterone replacement in gonadectomised males restored the $[Ca^{2+}]_i$ levels back to sham operated male animal levels.

The key results obtained from these studies are summarised in Figures 7.1 and 7.2. The effects of varying extracellular $[Ca^{2+}]$ on the amplitude of the Ca^{2+} transient in the sham operated female, OVX female, 17β-estradiol replaced OVX female and the normal male

groups are summarised in Figure 7.1, which is a compilation of data shown in Figures 3.11 and 5.17. As has been mentioned previously, the removal of estrogen from the circulation of the female rats by ovariectomy resulted in a significant increase in the amplitude of the Ca^{2+} transient. Interestingly, this increase resulted in the ovariectomised female cardiac myocytes having Ca^{2+} transients of comparable amplitude to those seen in normal male cardiac myocytes, suggesting that estrogen is a major factor contributing to the differences in Ca^{2+} handling between the two genders. Addition of an exogenous source of 17β-estradiol resulted in a reduction of the amplitude of the Ca^{2+} transient back toward levels similar to those seen in normal female cardiac myocytes. The decrease in levels of estrogen seen in the ovariectomised animals is affecting the amplitude of the Ca^{2+} transient presumably through modulation of mechanisms involved in the excitation-contraction coupling process.

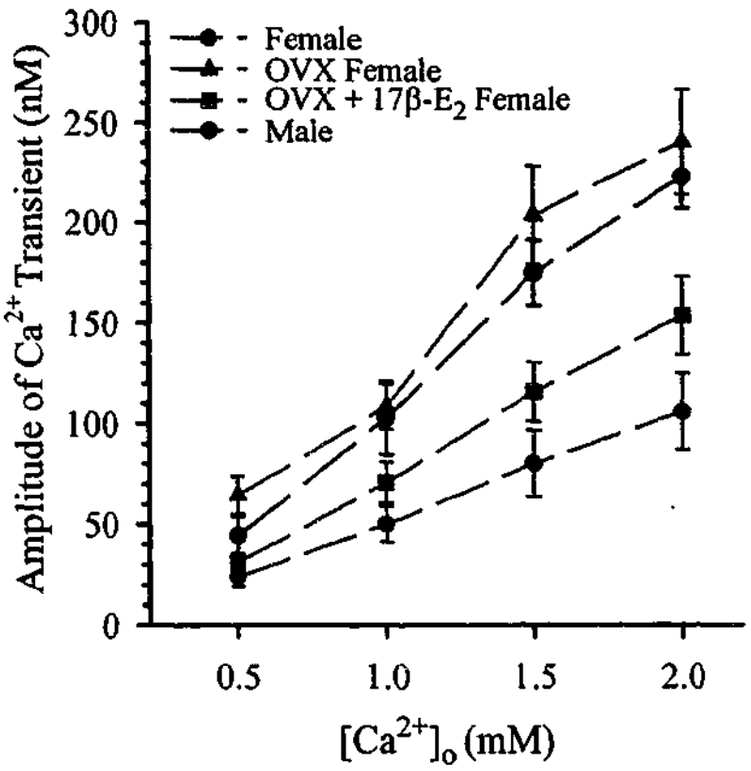


Figure 7.1: Effects of varying extracellular [Ca^{2+}] on the amplitude of the Ca^{2+} transient in female, OVX female, 17β-estradiol replaced OVX female and male cardiac myocytes stimulated to contract at 0.5 Hz steady-state. Values are mean ± SEM.

Figure 7.2 highlights the differences in the sham operated male, GDX male and testosterone replaced GDX male groups when compared with the normal female group. The removal of the majority of testosterone from the circulation of the male rats by gonadectomy resulted in a significant reduction in the amplitude of the Ca^{2+} transient. This brought the Ca^{2+} transient amplitude to levels similar to those seen in normal female cardiac myocytes. This suggests that testosterone also plays a significant role in the gender based difference that is apparent in Ca^{2+} handling in the heart. Addition of an exogenous source of testosterone increased the amplitude of the Ca^{2+} transient back to the same levels as seen in normal male cardiac myocytes.

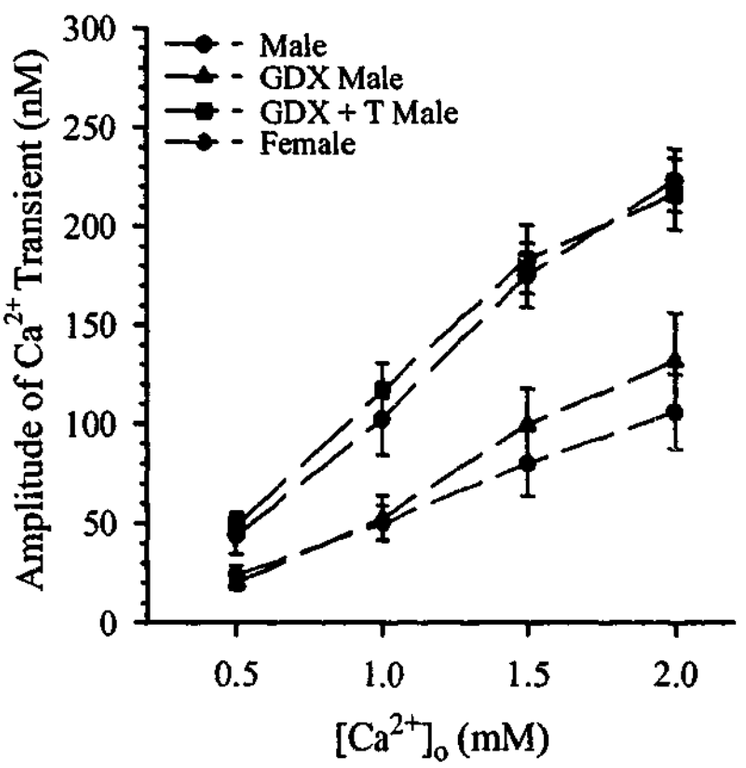


Figure 7.2: Effects of varying extracellular [Ca^{2+}] on the amplitude of the Ca^{2+} transient in male, GDX male, testosterone replaced GDX male and female cardiac myocytes stimulated to contract at 0.5 Hz steady-state. Values are mean ± SEM.

The fact that OVX resulted in the amplitude of the Ca^{2+} transient increasing to levels similar to those seen in the intact male cardiac myocyte suggests that estrogen is playing a role within the female heart to limit intracellular Ca^{2+} mobilisation. In contrast, the removal of the testes and, therefore, testosterone in the male resulted in a reduced Ca^{2+}

transient amplitude to levels similar to those seen in the intact female cardiac myocyte suggesting that the presence of the male sex steroid hormone testosterone results in enhanced intracellular Ca^{2+} mobilisation within the heart.

During the excitation-contraction coupling process many different ion transporters, pumps and proteins interact to allow contraction and relaxation to occur. Several different sites involved in the excitation-contraction coupling process may be implicated in the gender based difference that is seen in Ca^{2+} movements during contraction. Figure 7.3 illustrates the possible sites of action for both testosterone and estrogen on Ca^{2+} movements in the cardiac myocyte.

The L-type Ca^{2+} channel is the primary site of Ca^{2+} influx into the cell during contraction and has been shown to be affected by both genomic and non-genomic actions of estrogen. The increase in the density of the L-type Ca^{2+} channels which was reported in ERKO mice (Johnson *et al.*, 1997) suggests that the L-type Ca^{2+} channel is a strong contender for the site at which estrogen acts in female rats and, therefore, a potential site for gender based differences. An increase in Ca^{2+} channel density would lead to an increase in Ca^{2+} current into the cell, and hence a larger trigger for Ca^{2+} release from the SR. A difference in cardiac L-type Ca^{2+} channel expression between males and females could certainly potentially account for the gender differences seen in the amplitude of the Ca^{2+} transient. Figure 7.4 shows the experimental data comparing the Ca^{2+} transient amplitude in male and female cardiac myocytes at different extracellular $[Ca^{2+}]$ together with a simple simulation of how total Ca^{2+} entry would vary with differing amounts of Ca^{2+} entry per Ca^{2+} channel in two situations where there are different numbers of channels. Increasing Ca^{2+} entry per channel is what might be expected as the extracellular $[Ca^{2+}]$ is raised, and although there are several simplifying assumptions in the simulated situation shown in the right hand panel of Figure 7.4 it does suggest that the gender related difference in the amplitude of the Ca^{2+} transient could be explained by a difference in the number of Ca^{2+} channels in myocytes of male and female rats.

No specific evidence is available regarding possible long term effects of testosterone on the L-type Ca^{2+} channel in cardiac myocytes, however, it could be speculated to potentially be a site of action for testosterone. Once again, the diminished amplitude of the Ca^{2+} transient seen in cardiac myocytes from gonadectomised male rats could be

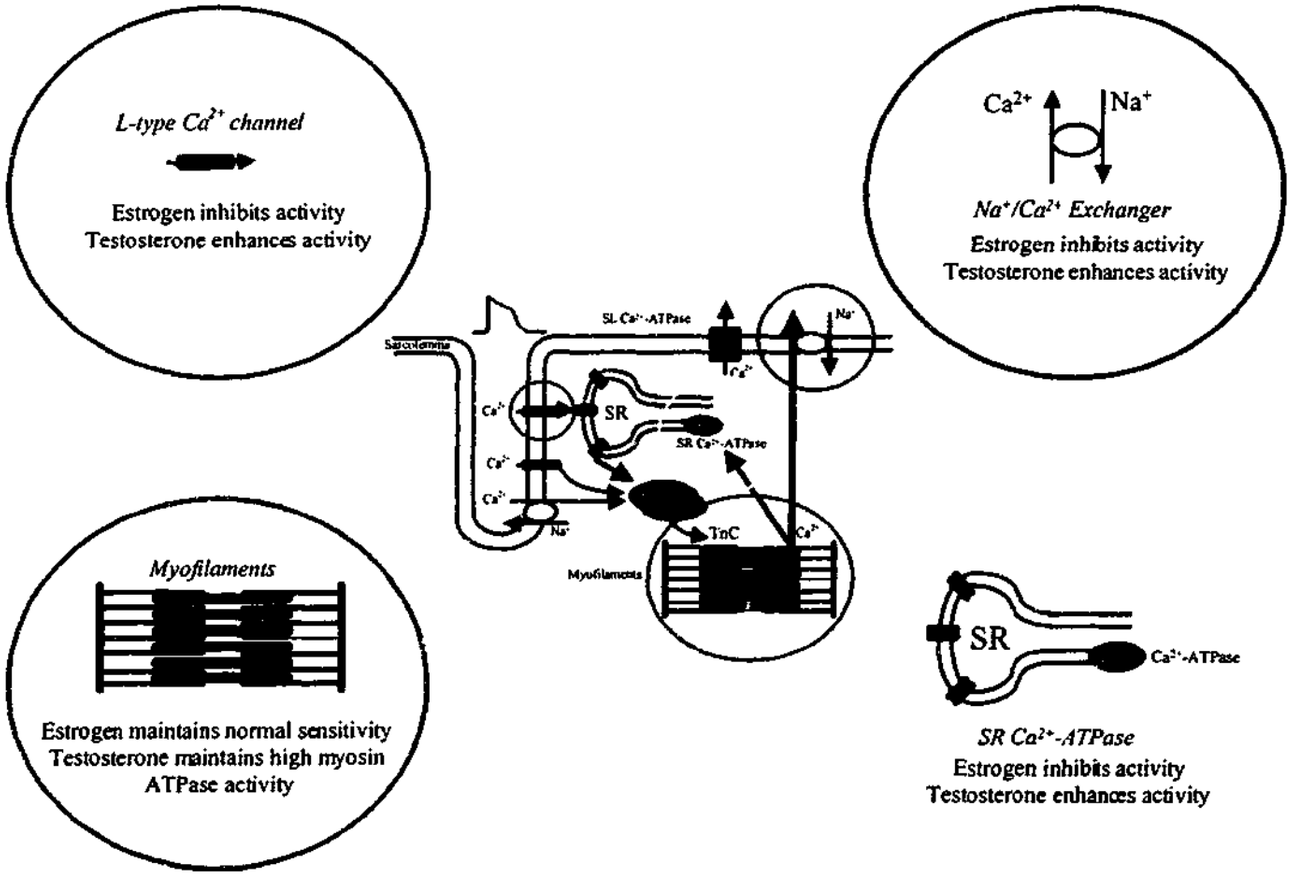


Figure 7.3: Possible sites of action within the cardiac myocyte for testosterone and estrogen during the excitation-contraction coupling process.

accounted for by a decrease in expression of the L-type Ca^{2+} channel. It is intriguing that removal of the respective primary sex steroid hormone in the female and the male results in precisely the opposite outcome in terms of the subsequent changes in intracellular Ca^{2+} handling.

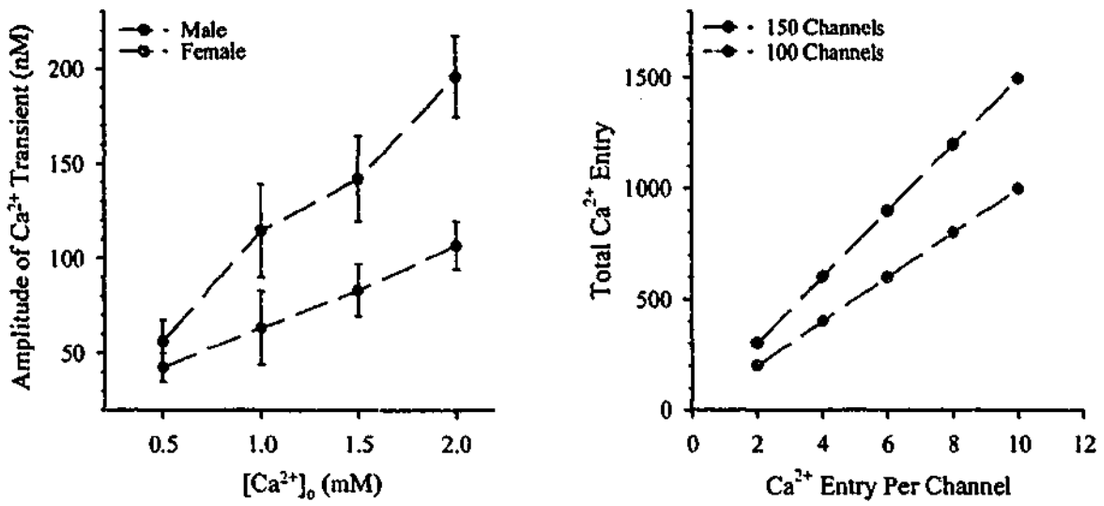


Figure 7.4: Experimental data (left) demonstrating the effects of varying extracellular $[Ca^{2+}]$ on the amplitude of the Ca^{2+} transient in male and female cardiac myocytes. A simple simulation (right) of how total Ca^{2+} entry would vary in two myocytes with different numbers of Ca^{2+} channels with increasing Ca^{2+} entry per channel.

It is important to recognise that the observations reported in this thesis do not provide direct evidence that changes in L-type Ca^{2+} channel expression underlie the gender based differences in intracellular Ca^{2+} handling. Future experiments directed at L-type Ca^{2+} current measurements in different surgically and hormonally replaced groups would provide additional knowledge regarding changes to Ca^{2+} influx in cardiac myocytes in response to gender and changing levels of sex steroid hormones. Levels of mRNA for the L-type Ca^{2+} channel could be assessed under similar circumstances together with determination of actual Ca^{2+} channel numbers by ligand binding studies. This would provide further insight into the possibility of genomic regulation of the L-type Ca^{2+} channel by the sex steroid hormones.

Another important site of Ca^{2+} regulation during cardiac muscle contraction is the sarcoplasmic reticulum. An increase in both SR Ca^{2+} release and SR Ca^{2+} uptake would also lead to a greater amplitude of the Ca^{2+} transient and a higher peak $[Ca^{2+}]_i$.

Increased SR Ca^{2+} uptake would result in a larger load of Ca^{2+} within the SR and, therefore, a greater available pool of releasable Ca^{2+} when triggered by extracellular Ca^{2+} influx. Together these would cause an increased mobilisation of Ca^{2+} into the cytoplasm of the cell. A clear difference in the time constant of decay of the Ca^{2+} transient between the genders was found, and changes in this parameter were also observed following gonadectomy and hormone supplementation in both genders. This suggests that the rate of Ca^{2+} uptake by the SR is influenced by the gender and sex hormone status of the animal. Consistently throughout the studies reported in this thesis the experimental group, whether it be male or female, that had the larger peak $[Ca^{2+}]_i$ and amplitude of the Ca^{2+} transient always had the faster rate of decay. It may be that in the groups with the large amplitude Ca^{2+} transients (i.e., intact male and OVX female) the fractional SR Ca^{2+} release is greatest due to larger trigger influx of Ca^{2+}. Consequently the SR would be more depleted following the Ca^{2+} release. If the rate of filling of the SR is greatest at lower SR Ca^{2+} levels, becoming slower the fuller the SR gets, this may account for the differences observed. For example the female SR would not be depleted as much as the male and, therefore, would not fill as fast. Alternatively, estrogen and testosterone may influence the activity of the SR Ca^{2+}-ATPase, or indeed the activity of the Na^+/Ca^{2+} exchanger, to result in the observed differences in the rate of decay of the Ca^{2+} transient. Direct measurements of the expression and activity of these Ca^{2+} transport proteins are required to elucidate whether they are modulated in response to altered hormonal status in both male and female rat hearts.

The contractile machinery is another possible site of action for both estrogen and testosterone. Previous studies have implicated the myofilaments in gender based differences in the heart. A reduction in myosin ATPase activity associated with a shift from the active V1 myosin isoenzyme to the less active V3 form was found by Schaible *et al.* (1984) in GDX male rats when compared with sham operated animals. In addition, when testosterone was replaced the myosin isoform shift was reversed and contractile function increased back to, or in some cases slightly above, the control groups (Scheuer *et al.*, 1987). In the OVX female rat, however, both reduced (Bowling *et al.*, 1997) and increased (Patterson *et al.*, 1998) myosin ATPase activity have been reported. In addition a Ca^{2+} hypersensitivity of rat myofilaments in OVX female rats and reversal of this when estrogen replacement was given has also been reported (Wattanapermpool, 1998; Wattanapermpool and Reiser, 1999; Wattanapermpool *et al.*,

2000). Although no direct measurements of myosin ATPase activity were made during the course of this thesis, this would be another area for future study since modulation of certain characteristics of the myofilaments by sex steroid hormones could play a role in gender based differences in cardiac function.

The way in which estrogen may be exerting a protective action within the heart is still not fully understood. Whilst most studies undertaken thus far have concentrated on either long term genomic or short term non-genomic actions of estrogen separately in an attempt to clarify its mode of action, a recent study has suggested that a combination of these two factors may in fact explain estrogen's beneficial actions within the heart (Dubey and Jackson, 2001). Dubey and Jackson (2001) believe that estrogen and its metabolites are acting within the heart in both a genomic and non-genomic fashion to bring about cardiovascular protective effects. Although non-genomic effects of estrogen have only been shown at supraphysiological levels, the idea that both genomic and non-genomic actions of estrogen are interacting to bring about beneficial effects is very appealing. It seems logical that the existence of both modes of action for estrogen are present for a particular purpose, thereby possibly combining to bring about estrogens overall Ca^{2+} antagonistic activity.

It is difficult to speculate on the mode of action for testosterone within the heart, as very few studies have been performed to assess this. The presence of the testosterone receptor in cardiac myocytes suggests a potential genomic role for testosterone within the heart. In addition a small amount of evidence supporting a non-genomic pathway for testosterone has been reported (Rubio *et al.*, 1998; Zhang *et al.*, 1998). It is possible that the way in which testosterone is bringing about its actions within the heart is also via a combination of both genomic and non-genomic actions. This is schematically illustrated in Figure 7.5. The combination of the two modes of action would seem ideal, as fast actions of estrogen or testosterone could be carried out in the heart via non-genomic pathways, whilst longer term, slower changes could be effected by genomic pathways.

The results obtained when measuring intracellular $[Ca^{2+}]$ in each group were also reflected in the shortening of the myocyte. A video edge detection system was used to measure shortening of the isolated myocytes. This technique has received some criticism due to the fact that shortening is being measured from an unloaded myocyte. It can be argued that the extent of shortening of each individual myocyte is unclear, as the

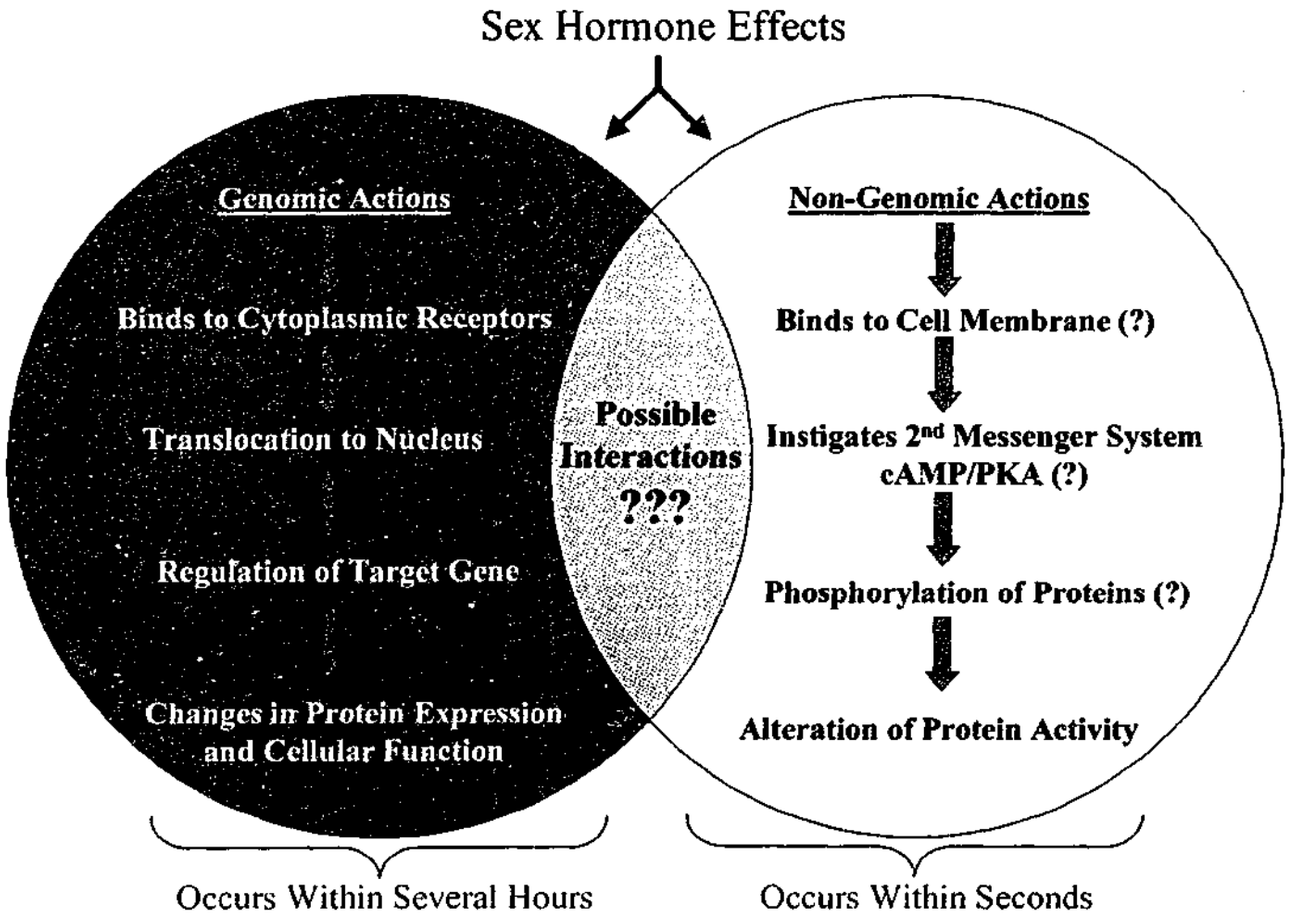


Figure 7.5: The two possible modes of action for estrogen and testosterone within the heart.

potential length change the cell could undergo may depend on the fraction of the myocyte that is attached to the coverslip base of the chamber. A myocyte that is fully attached to the coverslip along its entire length, for example, would not be expected to visibly shorten much, and therefore the edge detection system would not detect and record much shortening. On the other hand, it would be expected that a myocyte that is attached only at one end would have a higher degree of shortening. Cardiac myocytes have consistently been shown to be an excellent model when measuring intracellular [Ca^{2+}] levels, but due to technical difficulties, as mentioned above, have been considered to be not as good for measuring mechanical parameters of contractility. In saying this, however, the shortening data that was determined from each experiment conducted for this thesis was surprisingly consistent, in that within each group a similar trend was always seen, coupled with the fact that the shortening data always consistently followed the corresponding $[Ca^{2+}]_i$ data.

Throughout the course of this thesis the rat was used as the animal model for all experiments. Rats have been shown to have a very tight coupling between the L-type Ca^{2+} current and the SR because rat cardiac myocytes are so reliant on SR Ca^{2+} for activation (i.e. approximately 92% of Ca^{2+} removed from the cytosol during relaxation is sequestered into the SR). It would, therefore, be very interesting to repeat certain aspects of this study in another species, such as the rabbit which is not as reliant on SR Ca^{2+}. Such studies could help elucidate possible alternate sites of action for estrogen and testosterone.

In conclusion, this thesis has revealed distinct gender differences in $[Ca^{2+}]_i$ and contractility in single cardiac myocytes and intact, multicellular cardiac muscle. In extension to this, the effects of ovariectomy and estrogen replacement, along with gonadectomy and testosterone replacement were studied in rat cardiac myocytes. Significant effects of the animal's sex hormone status on $[Ca^{2+}]_i$ movements were demonstrated, with estrogen reducing $[Ca^{2+}]_i$ in OVX female cardiac myocytes while testosterone, on the other hand, increased $[Ca^{2+}]_i$ in GDX male cardiac myocytes. The data suggest that changes in $[Ca^{2+}]_i$ in male and female rats as a result of hormonal influences may play a role in gender based differences in cardiovascular disease. It appears, from these results, that estrogen could play a beneficial role within the heart whilst testosterone has the potential to play a deleterious role. As raised $[Ca^{2+}]_i$ has been associated with pathological responses in a range of cell types, it is highly probable that

a portion of the increase in cardiovascular morbidity and mortality seen in response to changing sex steroid environments is associated with changes in excitation-contraction coupling and cardiac myocyte physiology. It is important to realise, however, that these results, whilst convincing, by no means represent the sole reason for the differences in the incidence of cardiovascular disease between the genders, and it is imperative that the many other systemic and vascular related factors be taken into consideration. An interaction between many different factors is undoubtedly responsible for the gender differences present in the incidence of cardiovascular disease. These studies have provided an insight into one of those factors.

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