The effects of anthracyclines on calcium handling and contractility in sheep ventricular myocytes; role of oxidative stress

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List of Contents

Table of figures

Table of Tables

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Confirmation of ethical approval

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6 December 2019

Amy Foster

Dear Amy,

RE: ETHICS APPLICATION STR1920-05 – The cellular basis and prevention of anthracycline-induced late cardiac toxicity following childhood cancer treatment

Based on the information you provided, I am pleased to inform you that your application STR1920-05 has been approved.

If there are any changes to the project and/ or its methodology, please inform the Panel as soon as possible by contacting S&T-ResearchEthics@salford.ac.uk

Yours sincerely,

List of Abbreviations

AC	Adenylyl cyclase
ALL	Acute lymphoblastic leukaemia
ATP	Adenosine triphosphate
AVN	Atrioventricular node
BDM	2,3 – Butanedione monoxime
BPM	Beats per minute
BSA	Bovine serum albumin
Ca	Calcium
$CaCl_2$	Calcium chloride
CaMKII	Calmodulin-dependent protein kinase II
CaM	Calmodulin
cAMP	Cyclic AMP
CDI	Calcium-dependent activation
CICR	Calcium-induced calcium release
CRUs	Calcium release units
CSQ2	Calsequestrin 2
DAUN	Daunorubicin
DCFDA	2,7-dichlordihydrofluorescein
DEX	Dexrazoxane
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
DPHs	Dihydropyridines
ECC	Excitation-contraction coupling
FC	Flow cytometry
GPx	Glutathione peroxidase
GSH	Glutathione
H_2O_2	Hydrogen peroxide

HF	Heart failure
HIF – 1	Hypoxia-inducible factor 1
HR	Heart rate
JPH2	Junctophilin - 2
K	Potassium
KCl	Potassium chloride
LTCC	L-type calcium channels
LVEF	Left ventricular ejection fraction
Mg	Magnesium
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
Na	Sodium
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	Monosodium phosphate
NCX	Sodium-calcium exchanger
NETs	Neutrophil extracellular traps
NO	Nitric oxide
NOX	NADPH oxidase
O_2^-	Superoxide anion
OH	Hydroxyl radicals
OS	Oxidative stress
PLB	Phospholamban
PMCA	Plasma membrane calcium ATPase
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SACPs	Sinoatrial conduction pathways
SAN	Sinoatrial node

SERCA	Sarcoendoplasmic reticulum calcium-ATPase
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum
SV	Stroke volume
Tm	Tropomyosin
Tn	Troponin
Tn1	Troponin - 1
TnC	Troponin-C
TnT	Troponin-T
VDI	Voltage-dependent inactivation

Abstract

Anthracyclines such as doxorubicin (DOX) and daunorubicin (DAUN) are effective chemotherapeutics and contribute to improved cancer survival rates in children and adults. However, anthracyclines exhibit acute and chronic cardiotoxicity which can produce heart failure in cancer survivors. While the cellular basis remains unclear, limited previous studies show DOX perturbs certain aspects of excitation-contraction coupling and increases production of reactive oxygen species (ROS). Fewer studies have investigated the effects of DAUN and the effects of either anthracycline on excitation-contraction coupling (ECC) in a large animal model has yet to be demonstrated. Furthermore, the extent to which altered ECC is dependent on anthracycline-induced ROS production remains ambiguous. This is compounded by the fact that no studies have investigated whether elevated ROS production produces oxidative stress in cardiac myocytes. To address these gaps in our understanding, we performed the first integrative investigation of the effects of DOX and DAUN in sheep ventricular myocytes. We also measured the effect of DOX and DAUN on oxidative stress in these cells and further elucidated the underlying sources of ROS. Furthermore, we investigated the dependence of perturbed ECC on ROS thence oxidative stress elevations.

Sheep ventricular myocytes were enzymatically isolated in accordance with the Animals (Scientific Procedures) Act, UK, 1986 and used for all experiments. Intracellular calcium and contractility dynamics were measured using epi-fluorescent photometry and video sarcomere detection simultaneously. Cells were field stimulated at 0.5 Hz then acutely exposed to 1 nM DOX or DAUN. Rapid application of 10 mM caffeine was used to measure SR Ca content. Oxidative

stress was measured using CellROX red. Fluorescent images were captured using the cytation imaging system and cell fluorescence determined using ImageJ software.

DOX reduced the activity of SERCA and increased the activity of NCX resulting in a reduction in SR Ca content. DAUN also reduced SR Ca content however due to an interaction with

caffeine the mechanism could not be fully elucidated. The decrease in SR Ca content accounted for a decrease in systolic Ca which underpinned a decrease in systolic shortening. Both DOX and DAUN increased myofilament sensitivity to Ca, potentially offsetting the effect on contractility. DOX increased oxidative stress in a concentration and time-dependent manner. DAUN also increased oxidative stress, but only at relatively high concentrations (10 mM). Removal of oxidative stress by n-acetylcysteine (NAC) attenuated the effects of DOX on the majority of Ca handling and contractility parameters. For example, the effect of DOX on SR Ca content and Ca transient amplitude were reduced by approximately 50 %. Inhibition of the ROS producing enzymes NADPH oxidase (NOX) and xanthine oxidase (XO) reduced DOX-mediated oxidative stress by ~50 % and ~20 % respectively and attenuated the effects on ECC. In cells from sheep with heart failure, DOX reduced SR Ca content thence systolic Ca and contractility but had no effect on SERCA and NCX.

These findings suggest that in a large animal model, DOX and DAUN decrease SR Ca content leading to a reduction in systolic Ca thence contractility. In the case of DOX, decreased SERCA and increased NCX activity likely contribute to the decrease of SR Ca content. However, that this isn't the case in heart failure suggests a role for other mechanisms. These findings are also the first to show that DOX and DAUN increase intracellular oxidative stress in the heart and that NOX and XO are key enzymatic sources of ROS. Furthermore, these findings show this increase in oxidative stress is pathologically important as it accounts for approximately half of the effects of DOX on ECC. Collectively, these findings further elucidate the effects of anthracyclines on ECC and make important contributions to the understanding of the cellular basis of anthracycline-mediated cardiotoxicity. Furthermore, the dependence on and sources of oxidative stress reveal clinically relevant therapeutic targets.

Chapter 1
General Introduction

1.1 Cancer

Cancer is the uncontrollable growth and division of cells within the body. There are more than 100 types of cancer and subtypes may then be found within certain organs (Hanahan & Weinberg, 2011) with incident rates of the disease predicted to increase each year in the UK (Smittenaar *et al.*, 2016). Treatment for cancer has improved due to advances in research, however improvements are still required, not only to reduce lives lost to this disease but to improve the quality of life following treatment.

1.1.1 Childhood cancer

The probability of being diagnosed with a malignant tumour increases with age. This is likely due to prolonged exposure to carcinogens and other risk factors discussed in 1.1.2. However, certain cancer types are predominantly seen in children and include acute lymphoblastic leukaemia (Yamamoto & Goodman, 2008), cancers of the brain and spinal cord and lymphomas (Pesola *et al.*, 2017).

Between 1980 and 2006 almost 30,000 cases of childhood cancer were recorded in Great Britain, which equates to approximately 1150 cases per year, with leukaemia being the most common (Kendall *et al.*, 2013). Data from between 2016 and 2018 shows that incidence increased to around 1800 cases of childhood cancer per year (Cancer Research UK, 2022). 27 % of these cancer cases are leukaemia (Grabow *et al.*, 2018).

The type of cancer prevalent in children often differs to that suffered by adults. In adults, colon and rectum cancer are among the most prevalent whereas this is not the case for children (NHS digital, 2019). The aetiology of the cancer is probably an influencing factor in this difference.

1.1.2 Aetiology of cancer

1.1.2.1 Hallmarks of cancer

In 2000, Hanahan & Weinberg defined 6 hallmarks of cancer which are required in terms of cell physiology - for a malignant growth to develop, these are: self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, tissue invasion & metastasis, limitless replicative potential, and sustained angiogenesis. In essence, this means that cancer cells grow without an external stimulus and can avoid death via apoptosis. Furthermore, cancer cells can invade tissues and alter angiogenesis which aids metastasis, and insensitivity to antigrowth signals allow the cells to replicate indefinitely. The hallmarks have since been expanded to include characteristics that enable the other characteristics specified (see figure 1.1). These include genomic instability and tumour-promoting inflammation which aid the other hallmarks microenvironment (Hanahan & Weinberg, 2011). These hallmarks of cancer are universal regardless of age at diagnosis.

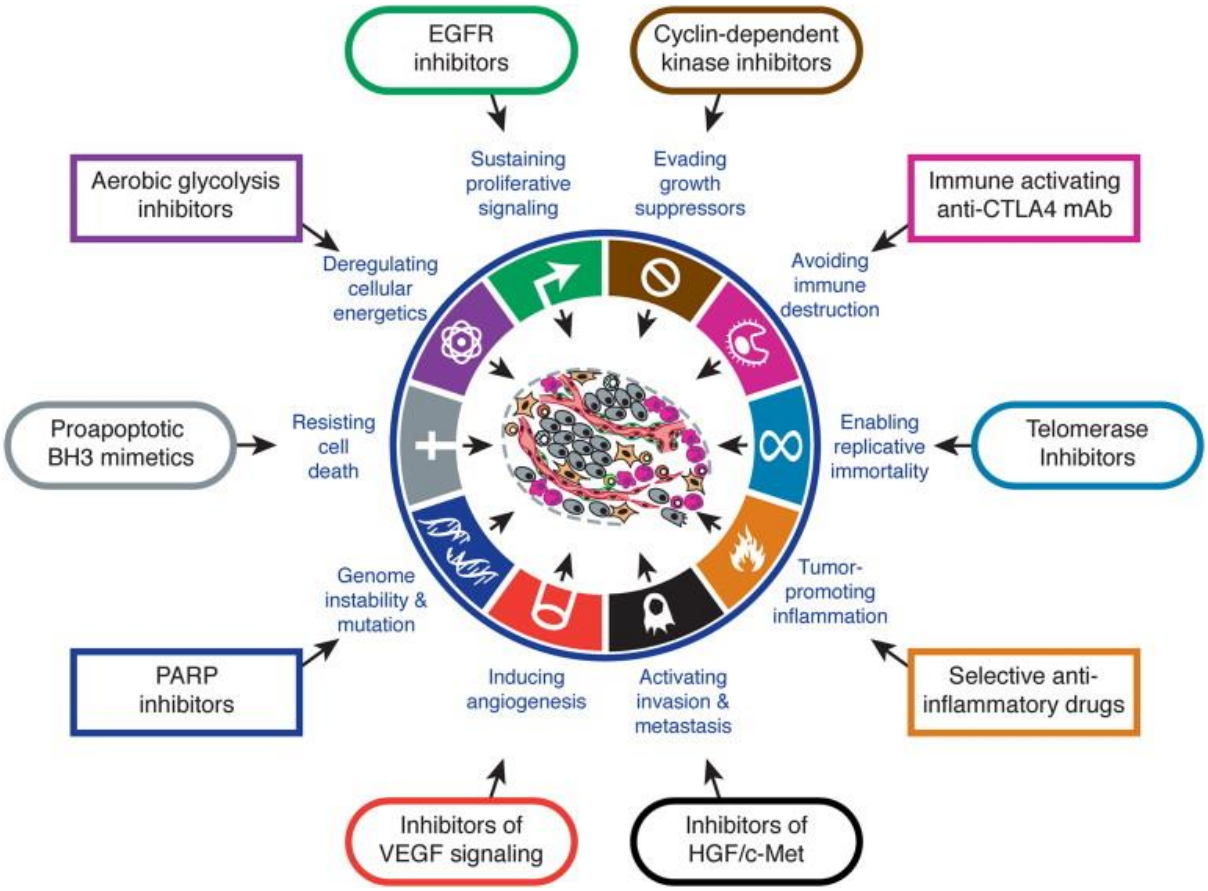


Figure 1.1: The Hallmarks of cancer and associated therapies (Hanahan & Weinberg, 2011)**.**

1.1.2.2 Aetiology of childhood cancer

The aetiology of childhood cancer is often unclear, but it is commonly accepted that genetic predisposition is a major contributing factor. Different types of childhood cancer are underpinned by genetic irregularities. For example, *TEL-AML1* chromosome translocation is the most common fusion gene (hybrid of two independent genes) present in acute lymphoblastic leukaemia (ALL). However, presence of this genetic translocation does not mean leukaemia is inevitable, therefore other factors must influence the progression of disease (Zuna *et al.*, 2011). This is where the other hallmarks of cancer such as sustaining proliferative signalling and resisting cell death may play a role.

This theory is also described in the two-hit model (also known as the two-stage model) which predicts if a person has a genetic mutation this alone is not enough to result in cancer. However, if a second genetic or environmental mutation occurs the risk of developing cancer increases considerably. This model was originally developed by Knudson when studying a retinal cancer, retinoblastoma (Knudson, 1971).

Identifying environmental and genetic risk factors for cancer is vital to reduce the number of people who develop the disease. However, it is also important to improve screening and treatment strategies.

1.1.3 Cancer diagnosis

Symptoms of cancer vary depending on the type and age of onset. In children these symptoms may not be specific and may present as more common or relatively benign illnesses. This often means that correct diagnosis takes longer by which time the disease has progressed to a more advanced stage (Shanmugavadivel *et al.*, 2022). Campaigns exist to increase general practitioners' awareness of childhood cancer in the UK. For example, the Grace Kelly childhood cancer trust has a diagnostic support tool which highlights some symptoms such as abdomen distension, recurring viral illnesses, headaches and vomiting (Walker, 2021). If a child presents to a health care professional with these symptoms at least three times in three months, a more thorough investigation may be conducted to ascertain if cancer is the cause. Symptoms such as lumps/swellings, pallor and fatigue further increase the likelihood that cancer may be present (Dommett *et al.*, 2013).

Once cancer is suspected various tests may be conducted. For example, in suspected cases of ALL bone marrow cytology including identifying the distribution of lymphoblasts and morphological examination of lymphocytes may be conducted (Brown *et al.*, 2020; Inaba *et al.*, 2013). Patients may then undergo further tests if cancer is detected to establish if any genetic abnormalities are present as this may alter treatment regimens (Nogrady, 2020).

1.1.4 Treatment of childhood cancer

Various treatment strategies exist depending on the type of cancer and age of the patient. Fundamental approaches often involve the use of anti-cancer drugs, radiation therapy and surgery, with treatment for children being given at lower doses due to the increased risk of long-term effects (Kattner *et al.*, 2019).

Anti-cancer drugs with different modes of action are often used in combination to reduce the risk of resistance and limit possible side effects (Tew, 2012). Examples of classes of anti-cancer drugs used in combination include steroids, anti-mitotic agents and anthracyclines (Schmiegelow *et al.*, 2010).

It is important to balance the concentration of the drug used to gain therapeutic effect without resulting in severe side effects, especially in children given the lifetime following treatment. Treatments for childhood cancer have improved considerably with 5-year survival reaching 80 %, however these survivors may then face various off-target effects (Armenian *et al.*, 2015). Cardiac complications are the most common cause of severe health issues in these survivors (Trachtenberg *et al.*, 2011). Deaths among childhood cancer survivors are 11 times more than that of the general population with 13 % of these attributed to cardiac causes (Reulen *et al.*, 2010). In particular, the anthracycline class of drugs have been associated with causing injury to cardiomyocytes which can induce heart failure as well as other complications (Henriksen, 2018). These drugs are commonly used to treat acute lymphoblastic leukaemia, acute myeloid leukaemia, Hodgkin lymphoma, Ewing sarcoma and osteosarcoma in children (Armenian & Bhatia, 2018). Anthracyclines are highly effective, however the cardiac implications need to be addressed. Therefore, it is important to understand how anthracyclines impact cardiac function on a cellular level. To do this we must first understand the cellular basis of cardiac function under normal physiological conditions.

1.2 Cardiac excitation-contraction coupling

1.2.1 The human heart

The human heart weighs between 250 - 350g and beats approximately 100,000 times a day (Shaffer *et al.*, 2014). The heart consists of 4 chambers: right atrium and ventricle and the left atrium and ventricle as shown in figure 1.2 (Whitaker, 2018). When functioning normally, the heart powers the circulation of enough oxygenated blood to meet metabolic needs of the body. The basic essential characteristics required for this are contractility and rhythmicity (Wilcken, 2012).

The fundamental cardiac cycle (a functional heart beat) consists of systole and diastole; during systole the ventricles contract increasing blood pressure while in diastole the ventricles relax allowing the chambers to refill with blood (Shaffer *et al.*, 2014). Within the chambers, heart muscle (the myocardium) is responsible for the generation of force required for pump function. Myocardial tissue is comprised mainly of contractile cells known as cardiac myocytes (Gupta & Poss, 2012). The generation of force at cell level is dependent on a process known as excitation-contraction coupling (ECC) which is a sequence of events from the electrical excitation of a given cardiac myocyte to that cardiac myocyte's contraction.

1.2.2 The role of calcium

Calcium (Ca) is essential in cardiac contraction (Ringer, 1883). As discussed below, at the cellular level Ca is involved in all stages of ECC; it contributes to the cellular action potential, links excitation to contraction (and controls inotropy), then acts as the ligand that activates the contraction itself.

Other signalling molecules exist, but unlike molecules such as phosphate, Ca is able to bind to and disassociate from sub-cellular structures rapidly. This is essential given the time course of one heart beat (Eisner, 2018). Ca is also ubiquitously involved in other cellular processes, such as gene expression and neurotransmitter release (Pantazis *et al.*, 2014). Ca is also a

regulatory co-factor for many intracellular enzymes, including those involved in the production of reactive oxygen species (ROS). Examples include xanthine oxidase and NADPH oxidases (Görlach *et al.*, 2015) which are discussed in 1.4.1.1.

1.2.3 Excitation

1.2.3.1 The source of excitation

Contraction of cardiac muscle is initiated by a wave of excitation originating from the sinoatrial node (SAN), the cells of which are capable of spontaneous depolarisation (Shih, 1994). In mammals the SAN is located within the wall of the right atrium (Baruscotti & Robinson, 2007) and is comprised of pacemaker cells which are surrounded by collagen and elastin fibres (Mitrofanova *et al.*, 2018). The SAN structure is extremely complex and has at least four sinoatrial conduction pathways (SACPs) which conduct electrical impulses to the myocardium of the atria (Fedorov *et al.*, 2012). In non-disease states excitation begins within one of the intranodal pacemakers in one of the SACP which then propagates through the other SACPs (Fedorov *et al.*, 2012). The internodal pathways connect thus excite the atrioventricular node (AVN). The AVN delays excitation permitting discrete atrial excitation before transducing excitation to the His-Purkinje system allowing the wave of excitation to spread across the ventricles (Hucker *et al.*, 2007). This wave of excitation results in contraction of the ventricles.

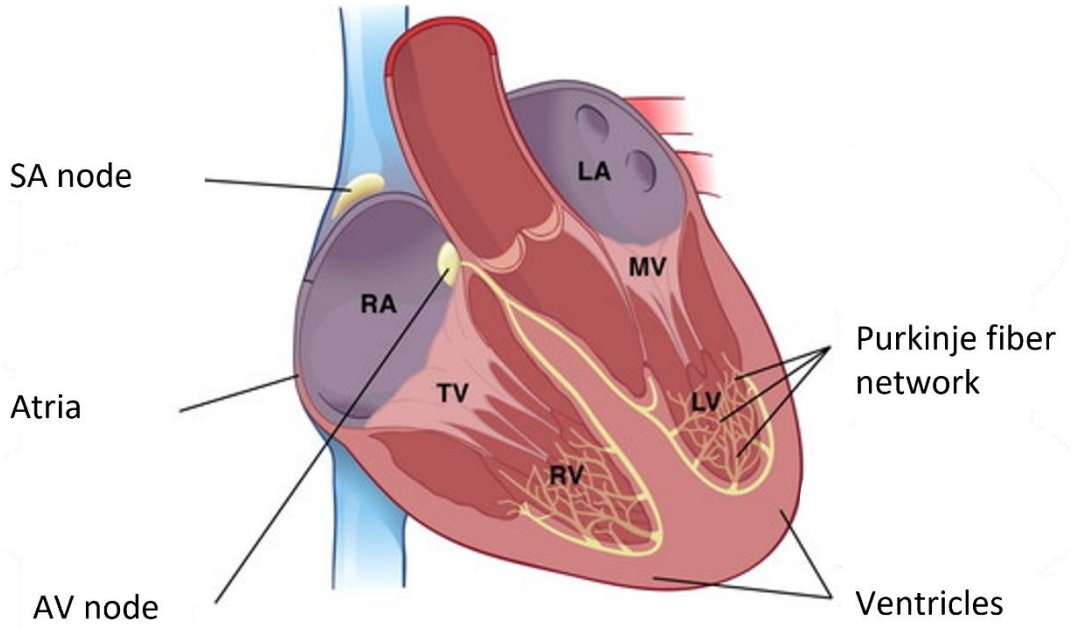


Figure 1.2: Anatomy of the cardiac conduction system. SA (sinoatrial), AV (atrioventricular). Adapted from Munshi (2012).

1.2.3.2 The cardiac cellular action potential

The wave of excitation described in 1.2.3.1 is produced by the sequential excitation thus depolarisation of each cell in the myocardium. This depolarisation manifests as the cardiac cellular action potential. The ventricular action potential has 4 distinct phases as shown in figure 1.3. A wave of excitation activates voltage gated sodium (Na) channels resulting in Na influx that depolarises the sarcolemma producing phase 0 of the action potential, the Na channels inactivate within a few milliseconds and result in the phase 1 notch (Catterall, 2018). As with neuronal tissue, potassium (K) channels open allowing K efflux thus repolarisation resulting in phase 3 (Catterall, 2018). However, cardiomyocytes (CMs) have longer duration of action potential depolarisation due to transient Ca influx that offsets K efflux resulting in a plateau phase (phase 2). As K efflux dominates repolarisation occurs producing phase 4 (Cooper *et al.*, 2010).

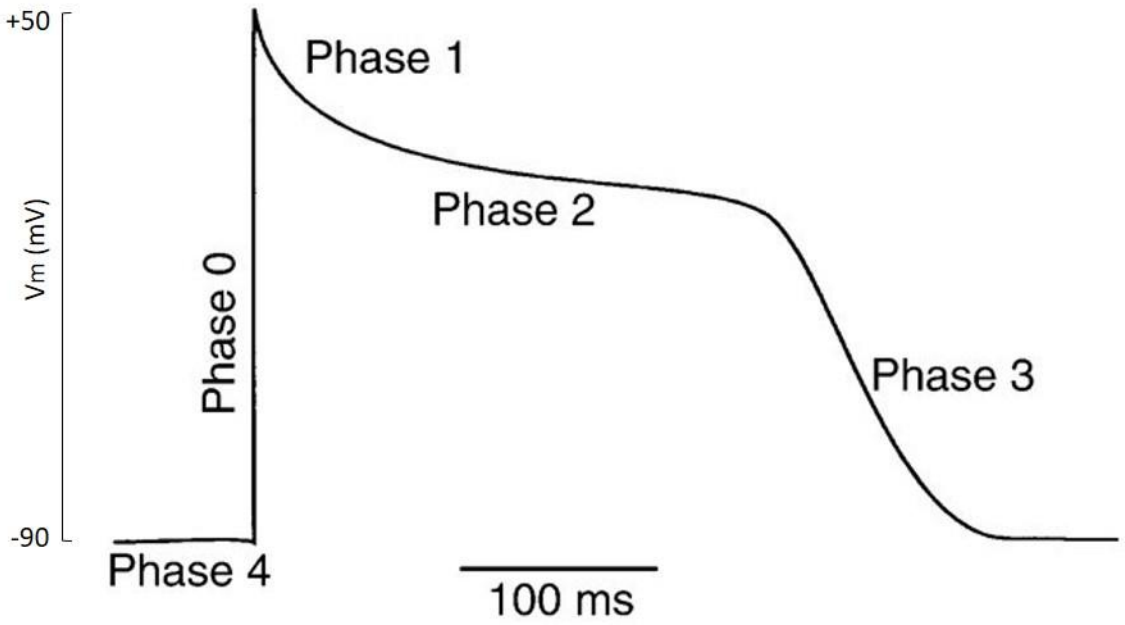


Figure 1.3: Phases of the cardiac action potential. Phase 0 is depolarisation followed by phase 1 which is termed the notch. Phase 2 is known as the plateau phase. Repolarisation is shown in phase 3. Phase 4 is resting phase. Figure modified from Nerbonne & Kass (2005).

1.2.4 Sarcolemma calcium entry

1.2.4.1 L-type voltage gated calcium channel activation

The primary pathway by which Ca enters the cardiac myocyte from the extracellular fluid via the L-type calcium channels (Lederer *et al.*, 1989). There are four subtypes of L-type calcium channels (LTCC) with $Ca_v1.2$ being mainly expressed in cardiac muscle (Lipscombe *et al.*, 2004). $Ca_v1.2$ are selective to Ca and are mainly located at the transverse tubules in close proximity to ryanodine receptors (RyR) forming a dyad as shown in figure 1.11 (Bers, 2001b).

LTCC consist of 3 different subunits that are tissue specific. $Ca_v1.2$ channels consist ofα_1 , $\alpha 2\delta_1$ and β sub-units as shown in figure 1.4 (Hu *et al.*, 2017; Wang *et al.*, 2004). The most common isoforms of α_1 and β within cardiac tissue are α_1c and β_2 respectively (Bers, 2001b). The α_1 subunit consists of twenty-four segments, four repeats of six transmembrane segments (S1-S6), four of which are responsible for the voltage-sensing domain while S5-S6 are associated

with the Ca conductive pore (Pantazis *et al.*, 2014). α_1 has binding sites for calcium channel blockers such as most dihydropyridines (DPHs) while, $\alpha 2\delta_1$ and β regulate the electrophysiological and pharmacological features of the LTCC (Hullin *et al.*, 2003).

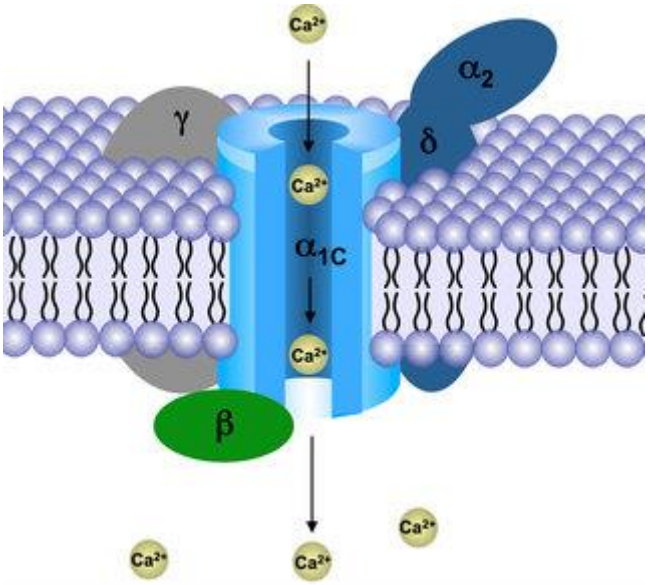


Figure 1.4 – Structure of the L-type calcium channel $Ca_v1.2$ from Hu *et al* (2017).

L-type calcium channels are voltage dependent, so are activated by the depolarisation of the membrane during the action potential (see 1.2.3.2.) (Sato *et al.*, 2018). In the α_1 subunit S4-S6 are involved in the voltage dependent activation of the channel, and the S3-S4 linker has been shown to have a role in the kinetics of the activation (Nakai *et al.*, 1994). LTCC activate at around -40 mV with peak activation being at approximately +10 mV, taking in the region of 5 msec (Pelzmann *et al.*, 1998). Once activated, Ca channels open allowing an influx of Ca. This Ca influx is due to the extracellular Ca concentration being greater than intracellular.

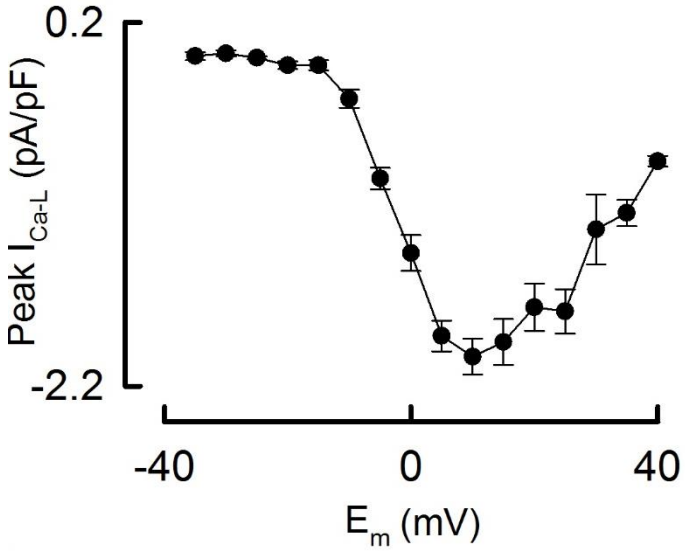


Figure 1.5: Voltage dependence of L-type Ca current; the current – voltage relationship. Adapted from Bers (2001).

1.2.4.2 L-type voltage gated calcium channel inactivation

Inactivation of LTCC is important in normal cardiac function. LTCC can be inactivated by calcium-dependent and voltage-dependent mechanisms. 80 % of LTCC inactivation is calcium dependent (Lacinová & Hofmann, 2005). It is well understood that calcium-dependent inactivation (CDI) is initiated when Ca binds to the α_1c subunit of the LTCC (Peterson *et al.*, 1999). This inactivation process involves amino acid sequences in the cytoplasmic segments of the α_1c subunit (Zühlke & Reuter, 1998). An EF-hand motif and an IQ calmodulin binding motif are present in the α_1c subunit that have been identified as being important in calcium-dependent inactivation (Zühlke & Reuter, 1998).

Calmodulin (CaM) is a protein involved in altering protein-protein interaction when there are changes in $[Ca^{2+}]$ (Chazin & Johnson, 2020). CaM is able to both inactivate and facilitate LTCC (Zühlke *et al.*, 1999). The influx of Ca, and CaM interacting with the IQ motif in LTCC leads to inactivation (Pitt *et al.*, 2001).

Voltage-dependent inactivation (VDI) is a relatively slower process compared to CDI (Findlay, 2002) and is facilitated by the current decay. During phase 3 of the ventricular action potential the efflux of K drives repolarisation leading to a decrease in the membrane potential which results in more LTCC being inactivated.

The influx of Ca due to activation of LTCC does not raise intracellular Ca to levels required for contraction. Rather it results in a mass release of Ca from the sarcoplasmic reticulum in a process is known as calcium-induced calcium release (CICR). To understand this, we must first consider the sarcoplasmic reticulum and associated structures.

1.2.5 The sarcoplasmic reticulum

The sarcoplasmic reticulum (SR) is a specialised form of endoplasmic reticulum and in the heart cells acts as an intracellular Ca store (Rossi *et al.*, 2008). In mammals, most of the Ca that activates contraction (see 1.2.7) is released from the SR in a process known as calcium induced calcium release.

1.2.6 Calcium-induced calcium release

Influx of Ca via the LTCC does not elevate cytoplasmic Ca to levels required for the activation of contraction. Rather, in a process known as calcium induced calcium release (CICR) it triggers mass Ca release from the SR which then allows contraction to occur (Hanna *et al.*, 2014). To fully understand this process, first, the structure and arrangement of proteins involved need to be considered.

1.2.6.1 The ryanodine receptor

On the surface of the SR are specialist Ca release channels. As these channels have a high affinity to ryanodine, they are termed ryanodine receptors (RyR) (Bers, 2001b).

With a molecular mass of more than 2MDa RyRs are the largest known ion channels and have a homotetrameric structure (Peng *et al.*, 2016). Three RyR isoforms are found in mammals,

though the isoform primarily expressed in the heart is RyR2 (Peng *et al.*, 2016). RyRs are found in clusters on the surface of the SR of cardiac myocytes near the plasma membrane (Sato & Bers, 2011). These clusters are known as Ca release units (CRUs), and release discrete units of Ca known as Ca sparks (Núñez-Acosta & Sobie, 2014). Approximately 14 RyR are in each cluster and each CRU is made up of 3 clusters at a minimum (Baddeley *et al.*, 2009; Zahradníková & Zahradník, 2012). However, this number can vary from a few to several hundred (Sato *et al.*, 2016).

RyR spontaneously and stochastically open in diastole however the open probability is low resulting in a small, short lived release of cytoplasmic Ca termed the Ca spark (Dulhunty *et al.*, 2012). However, certain factors regulate the open probability of RyR such as pH, magnesium (Mg), adenosine triphosphate (ATP) and Ca (hence CICR). A decrease in pH to 6.6 reduces the open probability of RyR by more than 50 % (Rousseau & Pinkos, 1990). Mg has also been shown to reduce the open probability of RyR and increase the decline in open probability when there is a rapid increase in [Ca] such as in CICR (Valdivia *et al.*, 1995). Due to the fact ATP and Mg do not change over the time course of a single cycle of excitation-contraction coupling (i.e. heart beat) it is thought that these molecules regulate the sensitivity of RyR to Ca; the ligand that gates RyR during ECC (Bers, 2001b).

This means in the context of ECC, *the* most important influence of RyR activity is the concentration of Ca in the cytosol (specifically, the dyadic cleft, see below) and SR [Ca] (Bers, 2001b).

1.2.6.2 The dyad

The intracellular junction between the sarcoplasmic reticulum cisterna and the sarcolemma is termed the dyadic junction (Franzini-Armstrong *et al.*, 1999). The dyadic junction is between 12-15 nM wide (Pinali *et al.*, 2013). The dyad is formed by a cluster of RyR in close proximity

to a given LTCC across the dyad which increases the open probability of the channels when Ca enters via LTCC.

Junctophilin-2 (JPH2) is a protein present in the dyad that contributes to the stabilisation of the junction (Takeshima *et al.*, 2000). Loss of this protein in the dyad can lead to suppression of CICR and therefore impacts ECC, this is likely due to the changes in dyadic junctional distances and loss of t-tubules (Oort *et al.*, 2011). The dysregulation of JPH2 has been implicated in various cardiac diseases which highlights the importance of the dyad in ECC (Beavers *et al.*, 2014).

1.2.6.3 The process of calcium induced calcium release

The local dyadic Ca regulates CICR (Greenstein *et al.*, 2006). While influx of Ca via LTCC is relatively small the local increase of Ca in this microdomain is high and given that cytosolic Ca is the primary determinant of RyR gating (see section 1.2.6.1) this leads to increased open probability of RyR. The stochastic opening of RyR is increased therefore spark frequency increases considerably and these sparks summate to produce mass Ca release (Winslow *et al.*, 2006), this is termed CICR.

1.2.7 Contraction

Mechanically, for cardiac myocytes to contract Ca must trigger the alteration of the conformation of myofilaments within the sarcomeres (Schwan & Campbell, 2015).

1.2.7.1 Contractile apparatus

Myofilaments are the contractile proteins involved in cardiac cellular contraction. The structure of the sarcomere and myofilaments are depicted in figure 1.6. The contractile apparatus is comprised of thick (myosin) and thin (actin) filaments (Solaro & Rarick, 1998). These myofilaments are arranged in an overlapping structure that form contractile units termed sarcomeres. Using X-ray diffraction the organisation of the filaments in the sarcomere were identified with alternating A and I bands forming a striated appearance (Hanson &

Huxley, 1953). The Z-line depicts the end of each sarcomere and anchors the structures together. Actin protrudes from the Z-line towards the centre of the sarcomere (see figure 1.6). Actin filaments are double stranded helical structures that overlap with myosin in regions except the I band (Huxley & Niedergerke, 1954).

The interaction between myosin and actin is the fundamental basis of sarcomere shortening and is regulated by troponin and tropomyosin (Vanburen & Palmer, 2010) as illustrated in figure 1.6. Troponin (Tn) is a complex of three proteins: troponin-C (TnC), troponin-T (TnT) and cardiac troponin-1 (Tn1) (de Tombe, 2003). Tropomyosin (Tm) is a protein that is located between actin subunits, and every seventh troponin is bound to Tm which prevents myosin from binding to actin and therefore prevents contraction (Bers, 2001b). Tm dissociates from Tn when Ca binds to TnC subunit of Tn, this binding strengthens the interaction of TnC to Tn1 which is the subunit bound to actin (Bers, 2001b). Therefore, the interaction between Tn1 and actin is less stable and allows myosin to interact with actin.

The attachment of myosin to actin is termed the cross bridge. In the presence of ATP and Ca cross bridge cycling occurs and the actin slides alongside myosin (towards the z-line) shortening the sarcomere, thus myofibril, thus myocytes. Upon removal of Ca these contractions cease.

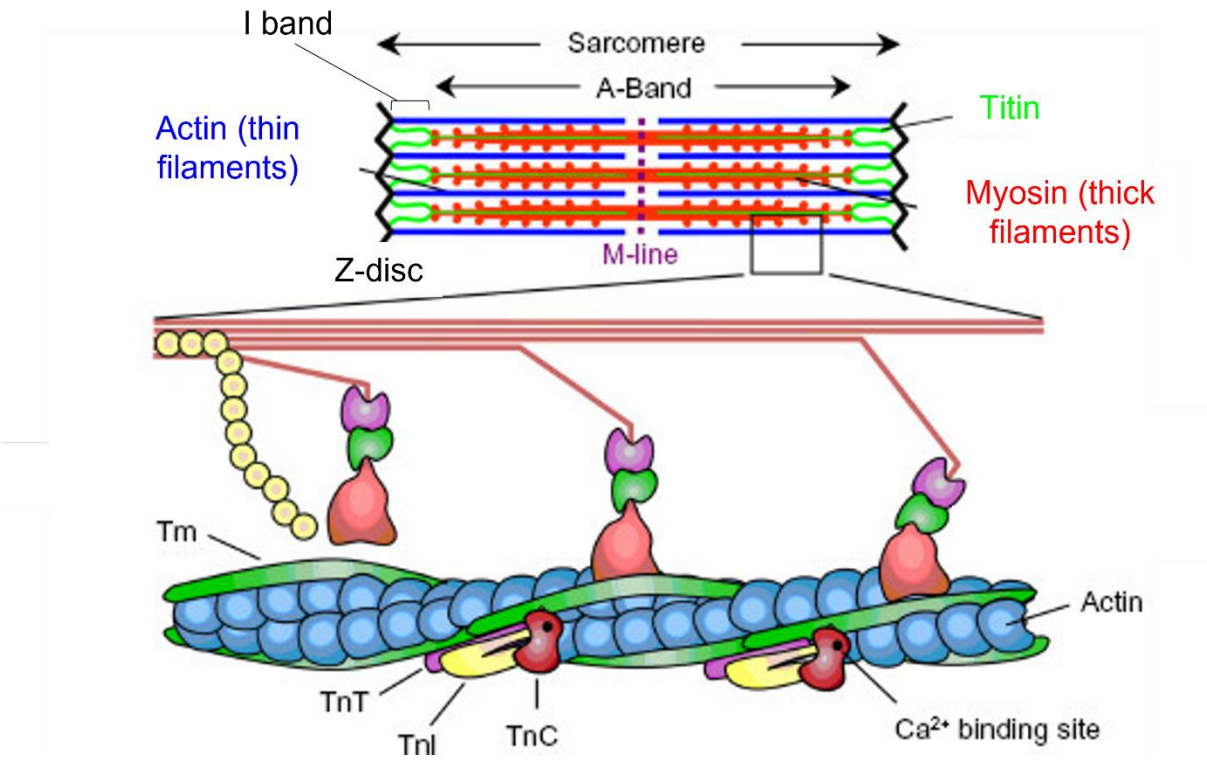


Figure 1.6: Contractile apparatus that comprises the sarcomeres in cardiac myocytes adapted from Schwan & Campbell (2015).

1.2.8 Relaxation

For the chambers of the heart to be filled before the next contraction, relaxation must occur, if this is impaired then the cardiac output would be reduced, and the oxygenated blood circulated would be insufficient (Biesiadecki *et al.*, 2014). The combined activity of the Ca removal mechanisms allows restoration of diastolic Ca. Here, Ca disassociates from troponin and thus the myocytes therefore the heart relaxes.

1.2.8.1 The need for calcium removal

As Ca is integral in cardiac contraction, for relaxation to occur the free Ca must be removed from cytosol. Four transport systems compete for cytoplasmic Ca during relaxation; the sarco-endoplasmic reticulum calcium transport ATPase (SERCA), sarcolemmal sodium-calcium exchanger (NCX), sarcolemmal Ca-ATPase (PMCA) and mitochondrial Ca uniport system

(Bassani *et al.*, 1992). By blocking each channel separately Bassani *et al* (1992) were able to determine the relative contribution of each transport system to Ca removal. In mammals SERCA and NCX account for 95 % of calcium removal (Bers, 1997).

1.2.8.2 Sarco-endoplasmic reticulum calcium transport ATPase

SERCA belongs to the P-type ion transporting ATPase family (Bers, 2001b). Three genes encode SERCA. *SERCA1* is expressed in fast twitch muscle while *SERCA2* is expressed in slow-twitch skeletal and cardiac muscle, the third gene *SERCA3* is present in various tissues that are not muscles (Lytton *et al.*, 1992). The transmembrane region of SERCA contains ten α-helices (M1-M10), with M4-M6 being thought to be responsible for the Ca binding sites (Toyoshima *et al.*, 2000). In the heart, SERCA is responsible for 70 – 90 % of systolic Ca removal (Bers, 2001b). It also replenishes SR Ca in readiness for the next beat.

Transport of Ca back into the SR via SERCA is always against a concentration gradient thus an active process. Two molecules of Ca bind to the cytoplasmic side of SERCA along with a molecule of ATP (Bers, 2001b). The transmembrane site M4-M6 have higher Ca affinity than those in the luminal side (M1-M2) due to the amino acid sequence (Clarke *et al.*, 1989). Phosphorylation leads to a change in conformation that prevents the bound Ca re-entering the cytoplasm and reduces the binding affinity, meaning the calcium molecules are released into the lumen of the SR (Bers, 2001b).

SERCA activity is regulated by phospholamban. Phospholamban (PLB) is a 6KDa pentamer protein that is found within the SR of cardiac, smooth and slow twitch skeletal muscles (Schmidt *et al.*, 2001). In the dephosphorylated basal state, PLB inhibits SERCA activity by decreasing the ability of SERCA to bind Ca (Akin *et al.*, 2013). When phosphorylated the effects of PLB are relieved and an increase in SERCA activity is observed (Kranias & Hajjar, 2012).

1.2.8.3 Sodium- calcium exchanger

The Sodium-calcium exchanger (NCX) is a membrane bound exchanger that removes 10-30% of systolic Ca (Bassani *et al.*, 1992) and is responsible for the majority of Ca removal out of the cell. The exchange mechanism has been found to occur at a ratio of approximately 3:1, in that for every 3 Na brought in to the cell, 1 Ca is removed (Pitts, 1979). Structure of the NCX can be seen in figure 1.7.

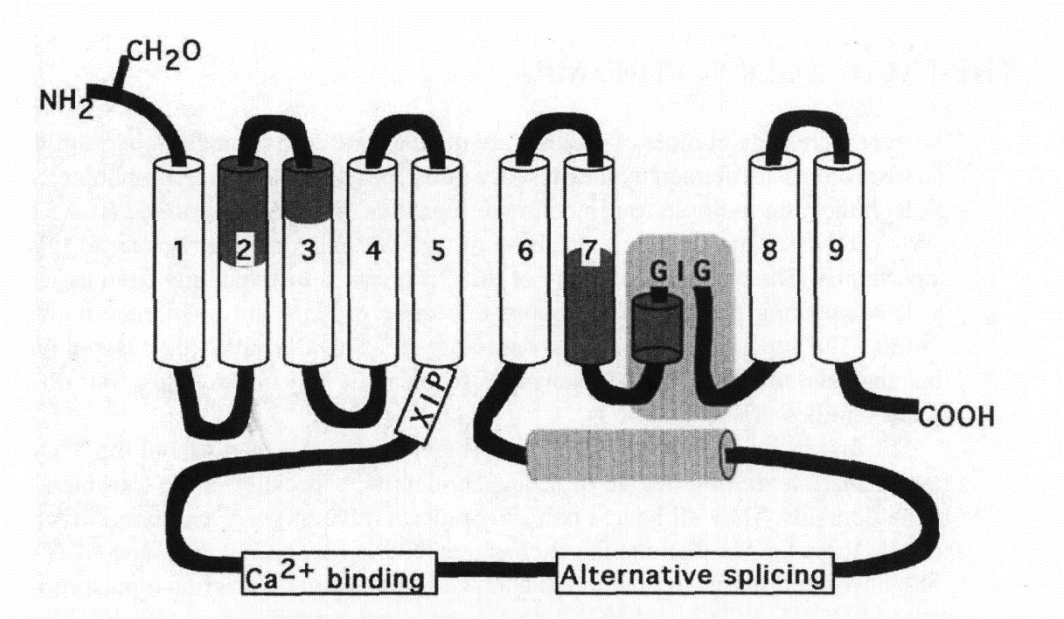


Figure 1.7: Na/Ca exchanger structure. The exchanger contains 9 transmembrane domains along with a large cytoplasmic loop from Philipson & Nicoll (2000).

The concentration of the substrate at which the speed of reaction is half its maximum is termed K_m. The K_m for intracellular Na is around 20 mM for the NCX, while for intracellular calcium it is 0.6 µM (Miura & Kimura, 1989). Miura & Kimura (1989) identified that external Ca is not required for NCX current however intracellular Ca is essential in activating this channel with the exchanger being fully activated within an intracellular Ca concentration range that is present physiologically at resting.

NCX is not only sensitive to the intracellular and extracellular concentrations of Na and Ca but also to the membrane potential (Bers, 2001b). Depolarisation of the membrane leads to Ca entry as described in 1.2.4.1, which enhances the activity of NCX.

NCX activity is also influenced by pH as a low pH reduces the exchange. Maximal exchange occurs at an extracellular pH of 7.6, with activity reducing to 70 % at pH 6 and a further reduction resulting in 10 % activity at pH 4 (Marcel & Ernst, 2000).

1.2.8.4 Other calcium removal processes

As mentioned in 1.2.8.1 the majority of Ca removal in mammals in attributed to SERCA and NCX with PMCA and mitochondrial Ca uptake accounting for less than 5 % of removal (Bers, 1997).

The PMCA is a P-type ATPase like SERCA (Bers, 2001b). This means that PMCA uses energy produced when ATP is converted to ADP and phosphate to transport ions. Structurally, PMCA has a long cytosolic C-terminal tail which binds calmodulin (Brini *et al.*, 2013). There are 4 isoforms of PMCA with isoforms 1 and 4 being found in multiple tissue types and have been found to be an important regulator within the heart (Mohamed *et al.*, 2013). At resting [Ca] the calmodulin binding motif in the C terminal interacts with the catalytic site which inhibits the activity of PMCA (Bruce, 2018). When the concentration of intracellular Ca increases it binds to the autoinhibitory motif in the C terminal along with calmodulin and causes a conformational change which increases the pump's ability to transport Ca out of the cell (Carafoli, 1994).

The mitochondria takes up Ca via a uniport system, meaning it travels down a concentration gradient caused by removal of a positively charged particle from the mitochondria (proton) (Bers, 2001b). This uptake is predominantly controlled by a protein complex known as the mitochondrial Ca uniporter (Nickel *et al.*, 2020).

1.2.8.5 The calcium transient

Cellular contraction causes an increase in cytoplasmic Ca concentration and produces a systolic Ca transient (Eisner, 2014). The Ca transient is a ratio of fluorescence emissions at 340 nm which increase with a rise in intracellular Ca concentration to fluorescence emissions at 380 nm which decrease with a rise in intracellular Ca concentration. The rising phase of the Ca transient indicates the increase in cytoplasmic Ca concentration while the decay phase of the Ca transient illustrates the decrease in intracellular Ca concentration (removal of Ca).

1.2.9 Control of inotropy

Previous sections described the fundamental process of ECC, however the heart is a dynamic organ and cardiac output is constantly modulated to meet changing metabolic demand. Cardiac output is the product of stroke volume (SV) x heart rate (HR) (Vincent, 2008). Therefore, to increase cardiac output the heart SV and HR must also increase (and *vice versa* for decrease). For example, during exercise the cardiac output is increased to meet the increased rate of oxygen consumption.

Changes in SV have a cellular basis as the greater the cell contracts, the greater the chamber contracts and therefore the greater SV will be. To understand how SV is regulated by the cells, we must understand the dependence of force on systolic Ca.

1.2.9.1 The dependence of contractile force on systolic Ca

The force of contraction of the sarcomere is determined by systolic Ca. A concentration of approximately 70 µmol of intracellular calcium is required to achieve half the maximum activation of contraction (Bers, 2001a). As the concentration of calcium increases (and therefore the Ca transient amplitude) the force of contraction increases up to a given point as shown in figure 1.8.

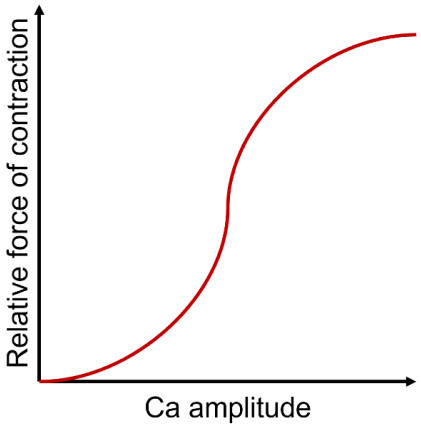


Figure 1.8: Relationship between calcium transient amplitude and force of sarcomere contraction in cardiac myocytes. Adapted from Sun & Irving (2009).

We must now consider what controls the levels of systolic Ca which results in a change of force to meet metabolic demand.

1.2.9.2 The dependence of systolic Ca on sarcoplasmic reticulum Ca content

The Ca content of the SR is directly related to systolic Ca (Trafford *et al.*, 1997). The amplitude of the Ca transient is dependent on the cube of SR Ca content as shown in figure 1.9 (Trafford *et al.*, 2000).

The steepness of this relationship is due to several factors. As per 1.2.6.1 RyR open probability is dependent on luminal/SR Ca content therefore, an increase in SR Ca content results in an increase in the basal open probability of RyR meaning for a given Ca trigger via LTCC the open probability of RyR increases further (Eisner, 2014). This means more Ca sparks occur and greater Ca release. The steepness of this relationship means that modest changes in SR Ca content will have a greater impact on systolic Ca. For example, a minor decrease in SR Ca content will result in a larger decrease in systolic Ca. To maintain Ca amplitude with each contraction the SR Ca content of the cell has to be precisely regulated. This makes SR Ca content a powerful physiological regulator of inotropy.

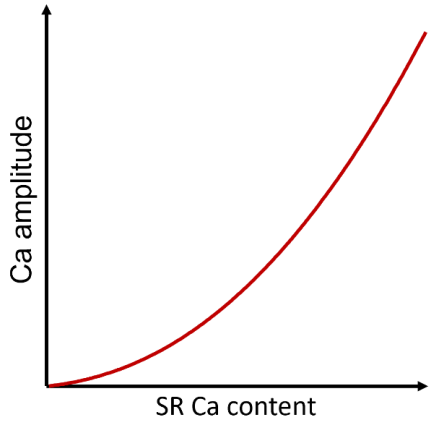


Figure 1.9: Cubic relationship between sarcoplasmic reticulum calcium content and calcium transient amplitude. Adapted from Trafford *et al* (2000).

1.2.9.3 The dependence of systolic Ca on trigger Ca

A rise may occur due to an increase in SR Ca release as discussed in 1.2.9.2, however may also occur via Ca influx (via LTCC). The two are not mutually exclusive as an increase in Ca influx triggers a greater release in SR Ca release. As discussed in 1.2.6.1 the open probability of RyR is also dependent on cytosolic [Ca] in the dyadic junction. Therefore, for all things being equal the greater the influx of Ca, the greater the open probability of RyR meaning spark frequency increases resulting in greater summation and a greater fractional SR Ca release.

1.2.9.4 Regulation of SR Ca content

SERCA is a powerful regulator of SR Ca content as evidenced in heart failure as decreased activity results in a decrease in SR Ca content (Eisner, 2014). NCX is also important in regulating SR Ca content and recent evidence suggest that is a far more important regulator than originally thought with this contributing to SR Ca content to a greater extent than SERCA (Bode *et al.*, 2011). We must now consider the signalling mechanisms that influence the aforementioned regulator of inotropy.

1.2.9.5 β-adrenergic stimulation

β-adrenergic stimulation is one of the main physiological mechanisms to modulate cardiac function (Bers, 2001b). Three β-adrenergic receptors have been identified with β_1 and β_2 receptors involved in positive inotropic reactions (Lee *et al.*, 2010). The dominant β receptor in the heart is β_1.

As shown in figure 1.10 stimulation of β-adrenergic receptors then stimulates adenylyl cyclase (AC) via Gs-coupled proteins which leads to an increase in production of cyclic AMP (cAMP) (Bers, 2001b). This then activates cAMP protein kinase A which phosphorylates various targets such as; troponin I, sarcolemmal Ca channels, SR Ca release channels and phospholamban (Bers, 2001b).

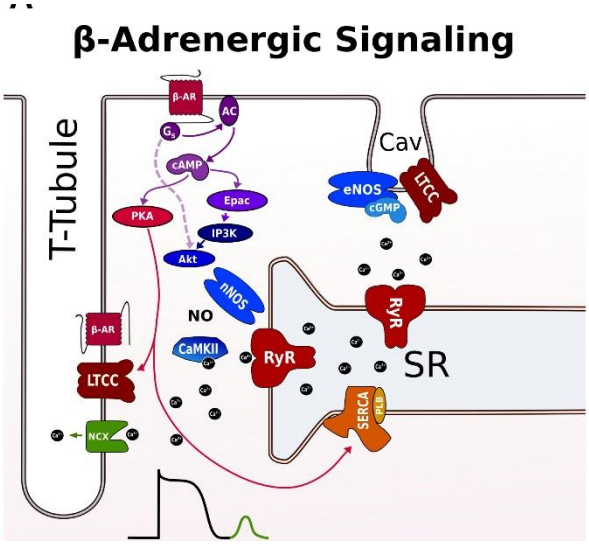


Figure 1.10: β-adrenergic signalling cascade adapted from Johnson & Antoons (2018).

Phosphorylation of Tn1 reduces the affinity of Ca to bind to the Ca specific site on TnC and increases the rate of removal of Ca from the troponin complex, therefore accelerating the time taken for relaxation (Robertson *et al.*, 1982). Furthermore, β-adrenergic stimulation leads to phosphorylation of protein kinase A (Bryant *et al.*, 2014). This then alters the voltage required for activation and inactivation of LTCC to a more negative membrane potential (Chase *et al.*, 2010). Therefore, increasing the Ca current which results in more Ca entering the cell at each excitation.

As previously discussed, phosphorylation of phospholamban allows SERCA to be stimulated (see section 1.2.8.2). β-adrenergic stimulation leads to phosphorylation of phospholamban which increases SERCA activity and thus SR Ca content. To summarise β-adrenergic stimulation provides an inotropic set point. However, this set point must be tightly regulated to prevent arrhythmogenic consequences.

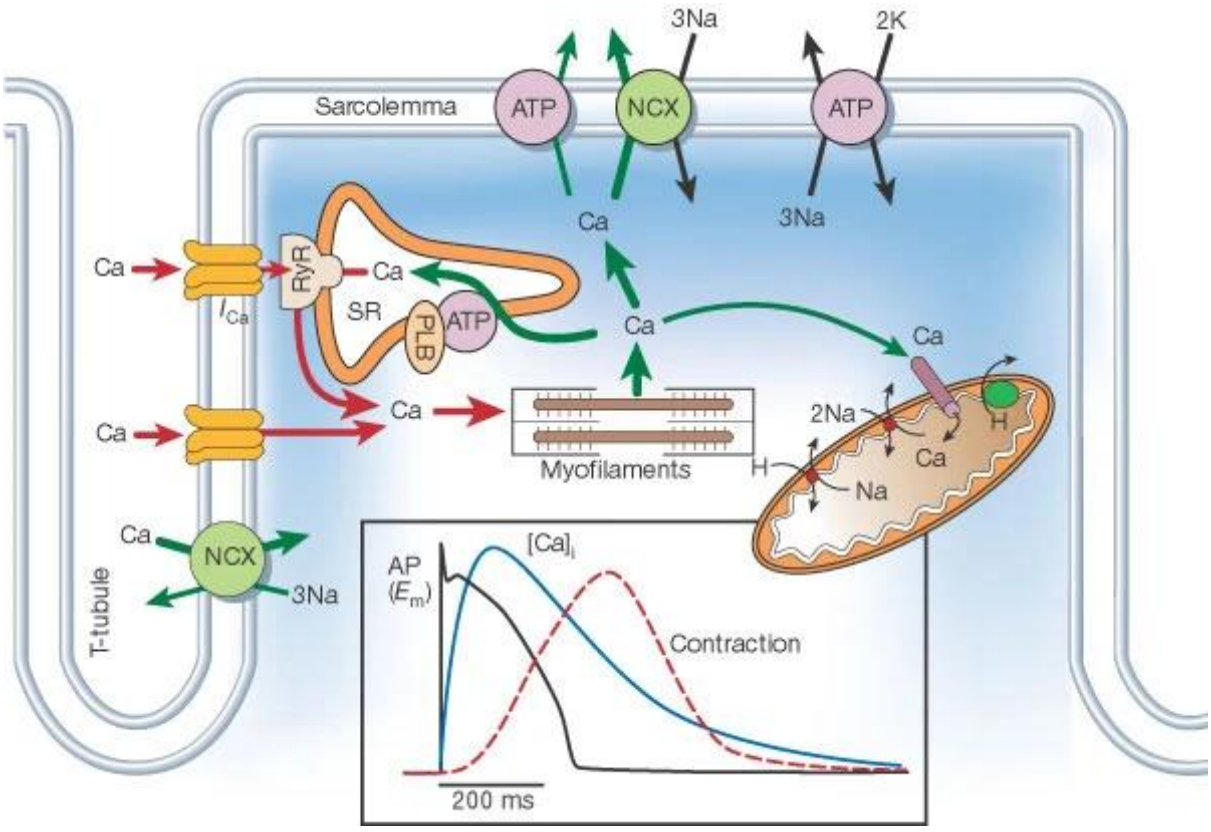
1.2.10 Autoregulation

Autoregulation ensures stability in Ca handling through a feedback mechanism. As discussed in section 1.2.9.2 regulation of SR Ca content is required to ensure Ca transient amplitude and therefore inotropy are kept consistent beat to beat. Autoregulation ensures this stability.
A change in activity of a single component such as RyR will not produce a prolonged effect on SR Ca release. Though not physiologically applicable a depletion in SR Ca content resulted in Ca transient progressively returning to steady state where Ca influx balanced efflux (Trafford *et al.*, 1997). This demonstrates how through the feedback autoregulation regulates demand. To further explain, an increase in the open probability of RyR will result in a brief increase in SR Ca release therefore increasing systolic Ca. On the next beat LTCC inactivation is enhanced due to the dependence on Ca. The increase in systolic Ca will increase the forward-mode of NCX activity resulting in an increase in Ca efflux. Ca efflux exceeds influx which leads to reduced intracellular Ca for subsequent beats, therefore less Ca is taken up into the SR via SERCA and SR Ca content is reduced. A reduction in SR Ca content and reduced Ca trigger compensate for the increase in open probability of RyR and restores the Ca transient amplitude (Eisner *et al.*, 2000; Greensmith *et al.*, 2014).

In disease states autoregulation may be impacted which can lead to variation between beats because of the imbalance in Ca influx/efflux. A change in a single component of Ca handling will not lead to a long-term effect on inotropy and the effect may be acute due to the other mechanisms in place. However, if more than one of these components are impacted issues with autoregulation will occur resulting in heart problems. The metabolic demand of the cell may vary in different conditions which will therefore alter the threshold for autoregulation. Cellular inotropy is important in setting these thresholds.

1.3 Summary of Excitation-contraction coupling

The action potential results in depolarisation of the sarcolemma which activates LTCC and a small amount of Ca influx occurs. This then increases the open probability of RyR leading to a large magnitude of Ca release from the SR in a process termed CICR. The release of Ca from the SR binds to the myofilaments and facilitates contraction. For relaxation to occur this Ca must be removed. In mammals this is mainly achieved by NCX and SERCA. SERCA actively uptakes the Ca back into the SR, while NCX is responsible for sarcolemmal efflux of Ca. This is summarised in figure 1.11.



1.11: Schematic of excitation-contraction coupling from Bers (2002).

1.4 Reactive oxygen species and oxidative stress

1.4.1 Reactive oxygen species

Reactive oxygen species (ROS) include oxygen free radicals such as superoxide anion (O_2^-) and hydroxyl radicals ($OH^\bullet$) as well as oxidising agents such as hydrogen peroxide (H_2O_2), they are formed due to partial reduction of oxygen (Ray *et al.*, 2012). While ROS are implicated in a variety of disease states there is also evidence that they are involved in normal physiological functions, such as intracellular signalling pathways (Finkel, 2011) and the antimicrobial action of neutrophils (Winterbourn *et al.*, 2016).

1.4.1.1 Physiological sources of reactive oxygen species

Enzymatic and non-enzymatic processes are involved in ROS generation including the mitochondrial electron transport chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, xanthine oxidase (XO) and myeloperoxidase (Sugamura & Keaney, 2011b). The mitochondria produce ATP and are involved in maintaining homeostasis for example, cardiac myocytes rely on the mitochondria to produce 95 % of the ATP (Ventura-Clapier *et al.*, 2011). However, they also have a role in generating ROS as a by-product of the respiration chain (Jensen, 1966). Approximately 2 – 3 % electron leakage occurs to form $O^{\bullet-}$ (Chance *et al.*, 1979). ROS may then leak out of the mitochondria and cause ROS-induced ROS release from neighbouring mitochondria (Zorov *et al.*, 2006).

Enzymatically, ROS may be produced via NADPH oxidase (NOX) that use NADPH as an electron donor (García *et al.*, 2023). There are seven isoforms of NOX and these enzymes are transmembrane oxidoreductases with 6 α helices (Chen *et al.*, 2012a; Sugamura & Keaney, 2011a). NOX2 is the most-studied and is primarily expressed in phagocytic cells but is also present in cardiac myocytes (Noreng *et al.*, 2022). NOX2 activation involves phosphorylation of an adaptor protein which leads to ROS production (Brandes *et al.*, 2014). This ROS production aids in killing microorganisms as part of the immune defence (Bayir, 2005).

Furthermore, xanthine oxidoreductase produces ROS as a biproduct of uric acid production as part of hypoxanthine metabolism (Kang & Ha, 2014). This enzyme exists in various mammalian tissues, with particularly high expression in capillary endothelium (Jarasch *et al.*, 1981). The enzyme exists in two interconvertible forms in mammals; xanthine dehydrogenase and xanthine oxidase (Chen & Meng, 2022). Both forms of the enzyme convert hypoxanthine to xanthine and xanthine to uric acid, however XO has an important role when cells are under stress as it generates $O^{\bullet -}$ and H_2O_2 (Harrison, 2002).

1.4.1.2 The physiological fate of ROS

Various antioxidants exist naturally within the human body to ensure a balance in ROS and avoid oxidative stress. These may be enzymatic such as catalase and glutathione peroxidase (GPx) or external molecules such as certain vitamins, flavonoids and carotenoids which are ingested as part of the diet (He *et al.*, 2017). Antioxidant enzymes scavenge ROS, without this ROS production may lead to oxidative stress. Superoxide species produced via enzymes and the mitochondrial electron transport chain are converted to H_2O_2 by superoxide dismutase (SOD), H_2O_2 is then neutralised to $H_2O + O_2$ by catalase and GPx as shown in figure 1.12 (Bayir, 2005).

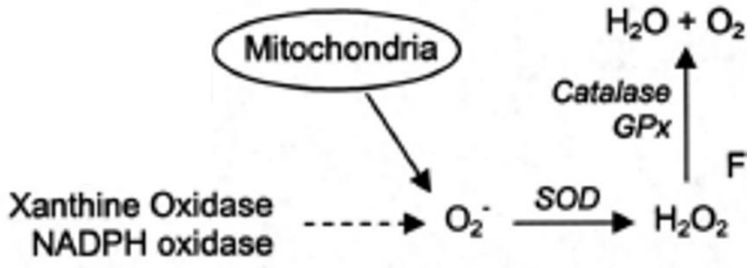


Figure 1.12: Enzymatic processes that result in neutralisation of ROS adapted from Bayir (2005).

Oxidative stress occurs when there is an imbalance in the generation of reactive oxygen species (ROS) and the antioxidant defences (Burton & Jauniaux, 2011). ROS can be produced in many tissues including the heart by various mechanisms such as the uncoupling of NO synthase, the action of cytokines, or by NADPH oxidases (Giordano, 2005). To prevent the

overproduction of ROS certain systems are in place that may be either enzymatic or nonenzymatic. Antioxidant enzyme systems to combat ROS include superoxide dismutase (SOD), catalase and glutathione (GSH), with GSH being the most abundant intracellular thiol-based antioxidant (Nordberg & Arnér, 2001). Issues occur if there is an over production of ROS or under activity of the systems to remove ROS.

If superoxide and therefore H_2O_2 production overwhelms SOD and GPx/catalase this may result in further ROS production by reacting with transition metals (see figure 1.13). The reaction of H_2O_2 with iron (II) results in $OH^{\bullet}$ and further reactions may then occur with iron (III) to generate $O_2^{\bullet -}$ and reduce iron (III) to iron (II) to allow self-propagation (Bayir, 2005). When ROS production exceeds the antioxidant capacity this results in oxidative stress (OS).

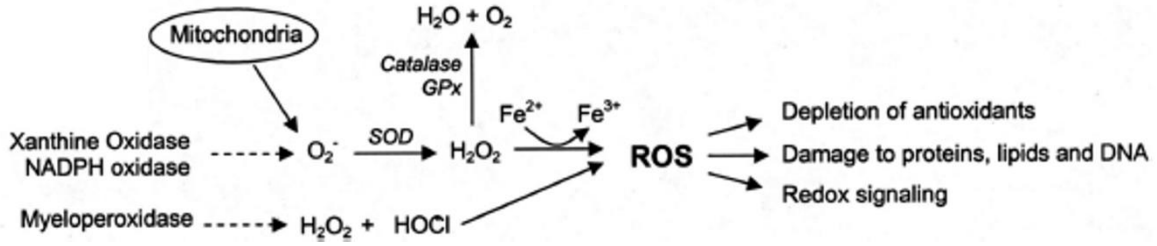


Figure 1.13: The Fenton reaction resulting in ROS production from Bayer (2005).

1.4.2 Oxidative stress and disease

As previously stated, OS is implicated in a range of diseases. One area of interest is the implication of OS in heart failure and cardiovascular events. OS is correlated to increased endothelial dysfunction which can be linked to coronary artery disease (Heitzer *et al.*, 2001). Endothelial dysfunction results in the endothelium losing its anti-inflammatory properties and its ability to maintain vascular homeostasis (Ana María *et al.*, 2015) which may then give rise to states such as coronary artery disease.

Furthermore, patients with congenital heart failure have a higher level of oxygen free radicals (Keith *et al.*, 1998). One theory to explain this is that the chronic activation of renin-

angiotensin-aldosterone systems is associated with heart failure (Münzel *et al.*, 2017). Research has shown the vasoconstrictor angiotensin II, activates monoamine oxidase which is an enzyme that produces H_2O_2 while deaminating compounds such as serotonin (Manni *et al.*, 2013). As previously mentioned, hydrogen peroxide is a ROS. Other sources of ROS in heart failure include the enzymes xanthine oxidase and uncoupled nitric oxide synthases (Borchi *et al.*, 2010). Angiotensin II also activates NADH/NADPH oxidase which stimulates the production of superoxides (Ushio-Fukai *et al.*, 1996).

It is evident that cardiovascular disease is associated with OS, this cardiac dysfunction is often underpinned by perturbed ECC.

1.4.3 The effects of oxidative stress of excitation contraction coupling

ROS have a role in normal signalling as discussed in 1.4.1 however the heart has lower levels of antioxidant enzymes in comparison to other organs (Costa *et al.*, 2013) with catalase activity being particularly lower in the heart (Thayer, 1986). This means that while under normal physiological conditions the heart has the coping mechanisms to neutralise ROS, in states of pathology that result in increased ROS production these mechanisms may not be sufficient and reactions such as those discussed in 1.4.1.2 may occur. This will result in a state of OS.

ROS are known to perturb ECC through a wide range of effects on ECC components (D'Oria *et al.*, 2020). The Ca transient amplitude and contractility in cardiac myocytes have been shown to be reduced by ROS (Goldhaber & Liu, 1994; Greensmith *et al.*, 2010; Kuster *et al.*, 2010) and may be explained by reduction in SR Ca content (Boraso & Williams, 1994; Greensmith *et al.*, 2010; Kuster *et al.*, 2010). As discussed in 1.2.9.2 Ca content of the SR is directly related to systolic Ca (Trafford *et al.*, 1997) and a small change in SR Ca content can result in a significant change to Ca amplitude given the steep relationship.

The reduction in SR Ca content may be attributed to a reduction in SERCA activity due to ROS (Greensmith *et al.*, 2010; Kuster *et al.*, 2010; Morris & Sulakhe, 1997; Reeves *et al.*, 1986; Scherer & Deamer, 1986) which would mean less Ca taken back up into the SR. Furthermore, ROS have been shown to increase NCX activity (Eigel *et al.*, 2004; Goldhaber, 1996; Kuster *et al.*, 2010; Reeves *et al.*, 1986) and as discussed in 1.2.9.4 recent evidence suggests NCX is a far more important regulator than originally thought with this contributing to SR Ca content to a greater extent than SERCA (Bode *et al.*, 2011). In addition to this ROS may also impact the LTCC and have been shown to reduce the voltage gated Ca current therefore Ca influx (Anzai *et al.*, 1998; Boraso & Williams, 1994; Kawakami & Okabe, 1998). The combined effect on both these transporters may account for the reduction in SR Ca content due to ROS which in turn can explain the decrease in Ca transient amplitude and reduced contractility. To identify the presence of oxidative stress in disease states various methods exist.

1.4.4 Measuring oxidative stress

Given the implications of ROS on ECC as discussed in 1.4.3 it is important to ascertain if ROS are increased in cells and if this increase in ROS exceeds the cells coping mechanisms. The impact on a cellular basis in turn effects whole organ function.

In *vivo*, one method that may be used on a clinical sample is the OXY-adsorbent test. This test measures the total antioxidant capacity by determining the ability of the sample to inactivate the oxidant solution which is added in excess, the results are then compared to the standard calibration curve (Vassalle *et al.*, 2012). This means that by measuring the excess oxidant solution you can indirectly measure the antioxidant capacity of the sample, in principle the higher the antioxidant capacity the lower the OS (Bonanni *et al.*, 2007). However, it can be argued that measuring the antioxidant capacity of a sample is not sufficient to make an indirect conclusion about OS. Various factors such as the consideration on non-enzymatic antioxidants and species variation suggest this method alone does not suffice and a method to determine oxidative damage would also be required (Costantini & Verhulst, 2009).

Methods to detect OS in *vitro* include detecting antioxidant enzyme levels as well as ROS using various techniques. For example, detection of lactate dehydrogenase may be used as an indicator of oxidative stress as leakage of LDH indicates damage to the cell membrane by free radicals (Zhou *et al.*, 2010). The detection of ROS may be achieved through fluorescent probes along with the flow cytometry and fluorescence microscopy (Shen *et al.*, 2013). For example, hydroethidine and 1,3-diphenylisobenzfuran can be used to detect superoxide radicals while 2,7-dichlordihydrofluorescein (DCFDA) can be used for detection of hydrogen peroxide (Gomes *et al.*, 2005). While these probes are commonly used due to their high sensitivity the detection of specific ROS may not be useful in the context of measuring overall OS.

While various methods exist for detecting OS and ROS, measuring these in cardiac myocytes poses new challenges. Isolation of primary cardiac myocytes yield varies, and identification of viable cells must be made based on visual characteristics meaning many methods may include data from unviable cells which will impact findings. To remove the non-viable cells is not always feasible as cardiac myocytes remain in suspension and would require the use of a micromanipulator which would be extremely time consuming (Kosloski *et al.*, 2009). While flow cytometry (FC) may seem a useful tool to sort and detect OS in cardiac myocytes it's use is limited in viable ventricular cardiac myocytes, and many experiments using FC with this cell type require them to be fixed (Diez & Simm, 1998). The major limitation in using FC with live primary ventricular cardiac myocytes is the variety in the cells shapes and sizes as this can impact validity and reproducibility (López *et al.*, 2017). There is the option to wash and filter the cardiac myocytes to select smaller cells as demonstrated by López *et al* (2017) as this would allow the cells to fit through the filter without causing equipment issues but this then presents its own problems with bias. Therefore, for the purposes of this project an alternative method was used as described in section 2.9.

1.5 Anthracyclines

As discussed in 1.1.4 anthracyclines are highly effective anti-cancer drugs with a main caveat of their use being cardiotoxicity.

1.5.1 Anthracycline chemical structure

In 1957 anthracyclines were first derived from the bacteria *Streptomyces*, in south-eastern Italy, with daunorubicin being the first antibiotic discovered (Weiss *et al.*, 1986). Doxorubicin (adriamycin) was isolated in 1967 and has the same anthraquinone and glycoside structure as daunorubicin (Arcamone *et al.*, 1969). The only chemical difference between these two anthracyclines is the hydroxyl group in doxorubicin compared to the methyl group in daunorubicin (DAUN) as highlighted in figure 1.14.

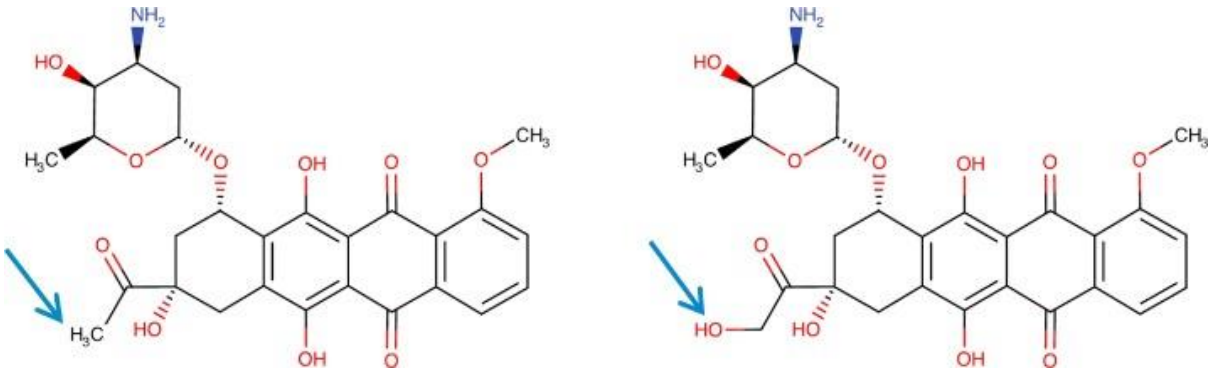


Figure 1.14: Chemical structure of daunorubicin (left) and doxorubicin (right) highlighting the hydroxyl group in doxorubicin compared to the methyl group in daunorubicin from Alves *et al* (2017).

1.5.2 Mechanism of action of anthracyclines

Research into anthracyclines suggest various mechanisms of action for their anti-cancer effects including cellular damage via free radical production, altered intracellular signalling and inhibiting topoisomerase type 2 (Fanous & Dillon, 2016).

1.5.2.1 Topoisomerase type 2 poisoning/inhibition

Topoisomerase II cleaves and relegates double stranded DNA to solve issues such as supercoiling and knotting (Hangas *et al.*, 2018). Studies have shown anthracyclines cause DNA cleavage via activation of topoisomerase II. Different anthracyclines have different DNA binding capabilities, DOX and DAUN are two anthracyclines that are known to bind strongly which is linked to inducing topoisomerase II cleavage (Capranico *et al.*, 1990). It is also important to note that anthracyclines have a strong binding capacity to topoisomerase 2 cleavage complex this is the intermediate that is present when the DNA is broken and can lead to apoptosis of the cell if the cuts are irreversible (Marinello *et al.*, 2018). It has been found that at low concentrations anthracyclines elicit poisoning activity on topoisomerase 2 compared to high concentrations which cause inhibition of activity. This means anthracyclines can intercalate with DNA base pairs as well as stabilising the topoisomerase 2 cleavage complex after DNA cleavage. To summarise, anthracyclines can increase the breaks in the DNA but also prevent ligation and therefore DNA synthesis.

1.5.2.2 Free radical production

Another proposed mechanism of action for anthracyclines is the generation of free radicals. The quinone structure in anthracyclines acts as an electron acceptor in reactions such as those mediated by NADH dehydrogenase, the free electrons then convert quinones in to a semiquinone and subsequently the formation of oxygen free radicals (Goodman & Hochstein, 1977). Anthracyclines can also produce ROS through increasing intracellular free iron (Fe^{2+} and Fe^{3+}) which leads to an increase in iron-mediated ROS production (Gammella *et al.*, 2014).

It has been shown that high levels of exogenous hydrogen peroxide cause DNA damage and severe cytotoxicity while low levels of exogenous H_2O_2 lead to a reduction in ATP, a delay in toxicity and the cell is seen to arrest in mitosis and can lead the cell to stop dividing known as senescence (Panieri *et al.*, 2013). This shows that by increasing the production of ROS, anthracyclines can stop division of cancer cells as well as induce DNA damage.

1.5.2.3 Altered intracellular signalling

Anthracyclines may also alter intracellular signalling either as a direct result of their ability to inhibit topoisomerase II or via another mechanism.

Formation of neutrophil extracellular traps are part of the innate immunity in response to factors such as foreign bodies and an increase in pH (Khan *et al.*, 2018). Neutrophil extracellular traps (NETs) are DNA-protein complexes that as well as responding to foreign bodies such as infection also combat conditions such as sepsis and cancer (Khan *et al.*, 2019). However, studies also show that NETs have an advantageous effect on cancer cells as they have been found in close proximity to tumour cells such as Lewis lung carcinoma and entrap these cells to aid survival as well as inhibiting immune cells (Demers & Wagner, 2014). Research is still ongoing into the effect of NETs on cancerous cells. Anthracyclines can suppress pathways that release NETs such as NADPH oxidase (NOX)-dependent and independent pathways (Khan *et al.*, 2019). If the cancer aiding properties of NETs outweigh the protection they provide from cancer, the ability of anthracyclines to suppress NETs would prove beneficial in preventing the survival of cancer cells.

Hypoxia-inducible factor 1 (HIF-1) overexpression is linked to the increased growth of tumours as well as vascularisation which aids metastasis (Semenza, 2007). Research has shown anthracyclines such as DOX and DAUN inhibit HIF-1 (Lee *et al.*, 2009). Anthracycline achieves this is by preventing HIF-1 from binding to DNA as well as inhibiting the transcriptional activity of HIF-1, this reduction in HIF-1 transcription can lead to a downstream reduction in other proteins that promote vessel formation such as vascular endothelial growth factor (Lee *et al.*, 2009). By reducing the expression of HIF-1, anthracyclines may prevent the growth of blood vessels around cancerous tumours and thereby reduce tumour growth and spread.

1.6 Anthracycline-induced cardiotoxicity

As discussed in 1.5, anthracyclines are effective anti-cancer agents and are commonly used in treatment regimes, however the off-target effect, particularly cardiotoxicity needs to be addressed. Cardiac complications are the most common cause of severe health issues in childhood cancer survivors (Trachtenberg *et al.*, 2011).

1.6.1 Clinical manifestation of anthracycline-induced cardiotoxicity

As discussed in section 1.5.2 anthracyclines have multiple mechanisms of action. This makes them effective anti-cancer drugs however, the effects are not exclusive to cancer cells. A major limitation of these drugs is the negative effect on the heart. Childhood cancer survivors treated with anthracyclines have a 15-fold increased risk of heart failure (Oeffinger *et al.*, 2006). The identification of cardiotoxicity is subcategorised. Occurrence of clinical heart failure in those treated with a low cumulative dose of anthracyclines is between 2 -4 % while cardiac injury has been found in 30 – 35 % of patients (McGowan *et al.*, 2017).

Clinically, left ventricular ejection fraction (LVEF) is used to quantify ventricular function in the heart with a poorer prognosis associated with a lower percentage (Wehner *et al.*, 2019). Cardiotoxicity is recognised by a decrease of 10 % or more in LVEF (Nakayama *et al.*, 2020). In childhood cancer survivors with subclinical cardiac dysfunction a decrease in left ventricular mass and wall thickness was detected as well as decreased contractility (Lipshultz *et al.*, 1991). This study also found 57 % of patients had an increase in afterload due to reduced wall thickness. The afterload is the pressure in the vasculature that the heart must exceed to successfully eject blood from the ventricles and is determined by the systolic pressure; an increase in afterload results in a decrease in stroke volume (LaCombe *et al.*, 2022). As well as left ventricular systolic dysfunction patients may suffer from myocardial ischaemia and hypertension (Galán-Arriola *et al.*, 2021). Myocardial ischemia may occur due to microvascular dysfunction, stenosis and mitochondrial dysfunction (Marzilli *et al.*, 2020). The

clinical manifestations though similar vary depending on the cumulative dose and age at treatment.

1.6.2 Acute and long-term anthracycline-induced cardiotoxicity

Anthracyclines are known to elicit acute and long term cardiotoxicity, with acute symptoms being present during or less than a year after treatment has ended and this increases the risk of the patient suffering from late cardiotoxicity (Grenier & Lipshultz, 1998). Chemotherapy such as DOX and DAUN are linked to adverse cardiac events including arrhythmias, myocarditis and heart failure (Lipshultz *et al.*, 2013). A longitudinal study recorded various cardiac parameters following DOX treatment in 115 childhood survivors of acute lymphoblastic leukaemia and some of the findings are illustrated in figure 1.15 (Lipshultz *et al.*, 2005). Lipshultz *et al* (2005) identified an initial reduction in left ventricular contractility which recovered over the next 6 years only to reduce again (fig 1.15A), this pattern of improvement followed by a reduction was evident regardless of cumulative dose of anthracycline. Following completion of DOX treatment, the left ventricular wall thickness was also reduced and became thinner over the course of the 15 years of the study with measurements after 6 years being significantly less than normal (Lipshultz *et al.*, 2005).

It stands to reason that although the cardiotoxic effects seen acutely during and shortly after anthracycline treatment improve over time they then decline again and can be a good indicator of future heart problems as a result of the treatment.

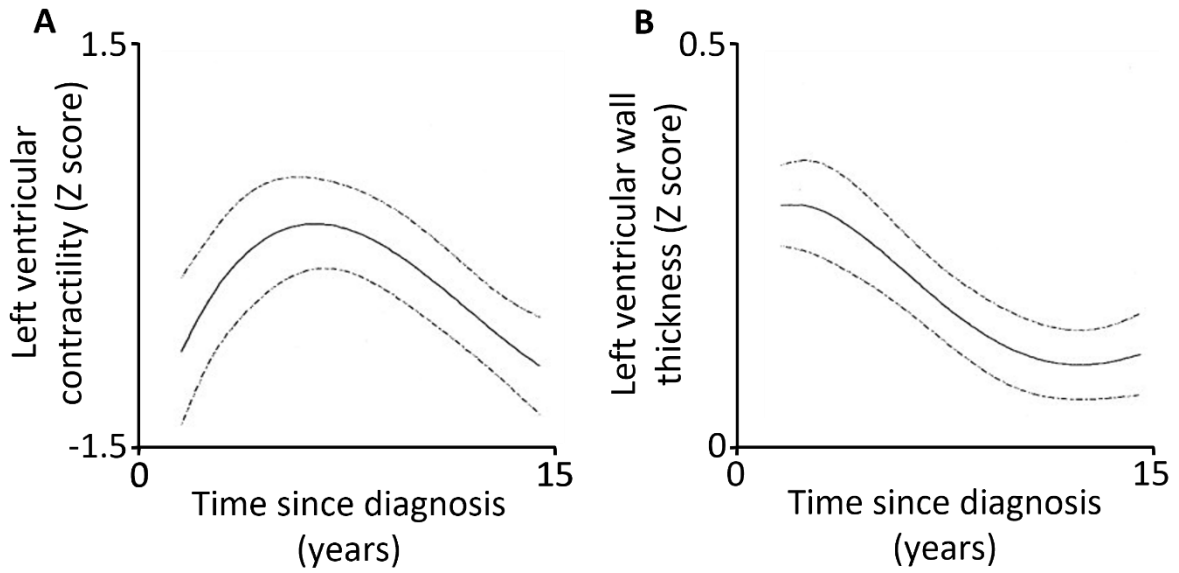


Figure 1.15: The effect of doxorubicin on left ventricular contractility and wall thickness. (A) mean left ventricular cardiac measurements from 115 survivors of ALL treated with doxorubicin with the upper and lower 95 % confidence interval. (B) mean left ventricular wall thickness for the same cohort. Adapted from Lipshultz *et al* (2005).

1.6.3 Adult versus childhood anthracycline-induced cardiotoxicity

The clinical features of cardiotoxicity following anthracycline treatment may present differently in children compared to adults. Adults are more likely to present with dilated cardiomyopathy whereas children may present with dilated cardiomyopathy which then develops into restrictive cardiomyopathy, however both are known to suffer with reduction of contractility (Giantris *et al.*, 1998). Dilated cardiomyopathy is characterised by dilated left ventricle with normal or thinning of the wall whereas restrictive cardiomyopathy is due to failure of the myocardium to relax (Davies, 2000). The difference in how the toxicity presents is likely due to the survival time following treatment as well as continuing growth. The proliferation of cardiomyocytes slows down as humans age, however these myocytes will increase in size meaning that in adults this growth will have already occurred whereas in children anthracycline treatment may impact this growth and proliferation (Mollova *et al.*, 2013). Not only may anthracyclines reduce cardiomyocyte number, but they may alter their function via various mechanisms.

1.6.4 The mechanisms of anthracycline-induced cardiotoxicity

The alteration to calcium handling in cardiomyocytes during and following anthracycline treatment may be due to a variety of mechanisms. As previously discussed, these drugs lead to an increase in ROS which have been theorised to activate Ca/calmodulin dependent protein kinase II (CaMKII) which then leads to SR Ca leakage (Sag *et al.*, 2011). CaMKII is expressed abundantly in the heart and phosphorylates various proteins involved in ECC (Bers & Grandi, 2009). If CaMKII is inhibited a leak of SR Ca has been found to be reduced indicating the importance of this protein in this process (Curran *et al.*, 2007). A calcium leak from the SR can contribute to a decrease in systolic Ca transient and lead to an abnormal heartbeat, this is termed an arrhythmia (Curran *et al.*, 2007).

Alternatively, the anthracycline may bind directly to a channel within the cardiac myocyte. Doxorubicin and its metabolite, doxorubicinol can bind to the RyR and at low concentrations (0.5 and 2.5 μM) can activate the channels as well as causing secondary inhibition of the receptor, the activation response has been found to be reversible however the inhibition has not (Hanna *et al.*, 2014). Hanna *et al* (2014) found when treated with 2.5 μM DOX the maximal RyR activation was seen after 2.5 minutes, then after 16 minutes inhibition was observed, they believed the inhibition to be due to RyR thiol modification. Alternatively, this inhibition could be due to the RyR becoming insensitive to the changes in luminal [Ca] as doxorubicinol disrupts calsequestrin 2 (CSQ2) which is a binding protein in the SR capable of altering the activity of the RyR (Hanna *et al.*, 2017).

Hanna *et al* (2014) also found low concentrations of doxorubicinol reduced SERCA2a activity but not DOX suggesting that the metabolite acts with greater efficacy than the original compound. Autoregulation may compensate for an increase in open probability in the RyR however this is assuming only one component has been affected, if SERCA is also affected the cell may not be able to compensate and this can lead to major consequences.

While one of the mechanisms of action for anthracyclines may be the production of ROS this may also be linked to how they cause cardiotoxicity. When rat cardiomyocytes were treated with DOX, ROS were produced immediately and increased over time and through the use of various channel blockers it was found that ROS production due to DOX opens RyR and causes an increase in systolic [Ca] which in turn generates more ROS (Kim *et al.*, 2006a). As discussed in 1.3.1 these free radicals have been linked to heart failure. This highlights some of the information research has discovered regarding anthracycline induced heart failure however these studies are often conducted on individual channels rather than the intact cardiac myocyte.

1.6.5 Prevention of anthracycline induced heart failure

Current research to prevent cardiac complication due to anthracycline treatment involves modification of anthracycline doses, use/production of less cardiotoxic analogues of anthracyclines and the use of cardioprotective agents alongside anthracyclines (Nathan *et al.*, 2016).

There is ongoing research into altering the chemical structure of certain anthracyclines via the addition of polyethylene glycol (PEG) or encapsulation to make delivery targeted and reduce concentrations of circulating anthracyclines such as DOX (Volkova & Russell, 2011). PEG is a polymer that can be added to the surface of liposomes which are used as nanocarriers to decrease the drugs interactions with blood components (Chen *et al.*, 2020). Pegylated-liposomal DOX is associated with a lower risk of DOX induced HF when looking at a retrospective group of patients who have received large cumulative doses (Skubitz *et al.*, 2017). However, issues may arise with PEGylated liposomal DOX if the patient has anti-PEG antibodies as this can lead to activation of the complement immune system and cause rapid release of DOX which will alter the safety for use in patients (Chen *et al.*, 2020). By creating a

targeted drug delivery, it is thought the toxic effect of anthracyclines on the heart will be minimised.

Dexrazoxane (DEX) is a drug used clinically in combination with certain anthracyclines to try and combat the cardiotoxic effects. DEX is an iron chelator which may work by preventing iron-dependent oxidative stress and/or via degrading topoisomerase II in heart tissues which prevents DNA damage (Deng *et al.*, 2014). In the presence of iron DOX may undergo redox cycling resulting in ROS production, as an iron chelator DEX reduces mitochondrial iron accumulation and therefore prevent iron mediated ROS production (Ichikawa *et al.*, 2014a). Use of DEX with DOX in children with acute lymphoblastic leukaemia showed their left ventricular function to be closer to normal than those treated with DOX alone indicating that dexrazoxane provides some form of long-term protection from the cardiotoxic effects of DOX (Lipshultz *et al.*, 2010). Impaired left ventricular function is often used as an indicator of heart failure (Harbo *et al.*, 2020). Another study conducted on children with solid tumours who received DEX along with DOX showed fewer cardiac events related to cardiotoxicity than those treated with DOX alone (Choi *et al.*, 2010). Choi *et al* (2010) found that 13 cardiotoxicities were found in the DEX treated group compared to 22 cardiotoxicities in patients treated with DOX alone, however the follow-ups were at 54 months and 86 months respectively meaning there may have been more cardiotoxicities reported after 86 months following DEX treatment. Furthermore, a risk of secondary malignancies (acute myeloid leukaemia and myelodysplastic syndrome) was identified in children who received DEX (Tebbi *et al.*, 2007). The co-treatment of DEX with anthracyclines appears to be beneficial though the exact mechanism of action is unclear. The effects in children are of greater concern due to the risk of secondary malignancies.

Various theories exist and studies have expanded knowledge on how the cardiotoxic effects of anthracyclines can be limited. However, few (if any) look at the effects of these combination or altered treatments on a cardiac cellular basis. Given the cardiac implications of anthracycline treatment the use of positive inotropic drugs may be of interest. These drugs

increase the force of contraction by increasing free intracellular Ca (Scholz, 1984) and provide an avenue of combating anthracycline cardiotoxicity.

1.7 Study main aims

The role of anthracycline-induced cardiotoxicity is not a new concept and various studies have sought to investigate how this occurs. However, many of these studies use small animal models or isolated ion channels which have limitations as previously mentioned. It therefore stands to reason that the impact on the components of excitation-contraction coupling may be altered to a different extent in an intact large animal model. Furthermore, the majority of these studies focus solely on doxorubicin with the assumption other anthracyclines will behave in the same way. This study will address the effects of another commonly used anthracycline that is believed to be less cardiotoxic; daunorubicin.

While previous research has highlighted the increase in ROS due to anthracyclines and implicated the role of ROS in altered cardiac cellular function there are limited studies on the impact of anthracycline-induced ROS on excitation-contraction coupling components in intact cardiac myocytes and the pathways involved.

This project aimed to:

1. Characterise the effects of doxorubicin and daunorubicin on excitation-contraction coupling and DAUN on ECC in the integrated model of the intact sheep ventricular myocytes.
2. Determine whether DOX and DAUN increase oxidative stress in sheep ventricular myocytes.
3. Determine to what extent changes to excitation-contraction coupling are dependent on oxidative stress.
4. Determine to what extent elevations in oxidative stress are dependent on NADPH oxidase and xanthine oxidase.

5. Measure the effects of DOX and DAUN on excitation-contraction coupling in myocytes isolated from a sheep with heart failure.

Chapter 2
General Methodology

2.1 The use of animal models to study excitation-contraction coupling

One of the main issues in studying heart disease states or the effect of drugs on cardiac function is the limitation of research methods. Due to ethical and practical constraints experiments on the human cardiac system are only possible in the rare cases of donated organs which poses its own limitations in terms of control data. Animal models offer a useful alternative.

Heart rates vary between species and generally the larger the mammal the slower the heart rate (Zhang & Zhang, 2009). Humans have a heart rate of around 80 beats per minute (bpm) compared to rats which are approximately 250 bpm and sheep which are 103 bpm (Noujaim *et al.*, 2004). There is variation in calcium handling between species and so there is a difference in the time of contraction and relaxation (Janssen & Periasamy, 2007).
While small rodents such as rats are easier to handle and house than larger mammals such as sheep, they have very high heart rates which means their hearts are different on a cellular level to facilitate the rapid contraction. For example, the action potentials of ventricular myocytes differ in rodents compared to humans as the outward potassium current and density of potassium channels differs meaning rodents such as rats have a large initial repolarisation phase and short plateau phase at lower membrane potentials compared to humans (Joukar, 2021).

Large animal models such as sheep share cardiac similarities to humans. For example, the removal of Ca via SERCA activity in sheep is similar to that of humans at 81.5 % (Dibb *et al.*, 2004) which as discussed in section 1.2.8.2 is responsible for 70 – 90 % of calcium removal. This means sheep serve as a good animal model for cardiovascular research particularly in terms of investigating excitation-contraction coupling.

2.2 Primary myocyte isolation

2.2.1 Ethics

All procedures were in accordance with the Animals (Scientific Procedures) Act, UK, 1986 and Directive 2010/63/EU of the European Parliament. All procedures carried out at the University of Manchester have ethical approval from the University of Manchester Welfare and Ethical Review board and the experiments have ethical approval from the University of Salford ethical review board.

2.2.2 Tissue preparation and cell isolation

Cells were isolated from the left ventricle of young (approx. 18 month), healthy sheep or sheep with tachypacing induced heart failure by collaborators at the University of Manchester. The sheep received heparin intravenously (20 ml) to prevent blood clots and then received a lethal injection of sodium pentobarbitone (200 mg/kg). Incisions were made to access and remove the heart to allow for cell isolation.

Langendorff apparatus was prepared as shown in figure 2.1, and all solutions were incubated in a water bath at 37^{o}C. Ca free solution (see table 2.1) was used to rinse the heart and remove excess blood prior to dissection of the ventricle and atria. The left ventricle was cannulated via the coronary artery onto the langendorff apparatus (see figure 2.1). Ca free solution was perfused through the left ventricle using a peristaltic pump for at least 10 minutes to clear intravenous blood and prevent blood clots forming in the tissue. Collagenase type 1 (Worthington, 0.096 mg/ml) and protease (Sigma, 0.018 mg/ml) were then added to the perfusing solution to commence enzymatic digestion of the tissue. Collagenase digests the extracellular matrix and collagen, allowing the dissociation of cells. Protease facilitates this while digesting the membrane. Digestion ranged from 5-15 minutes and was judged by visual observation of pericardium viscosity to achieve optimal cell quality.

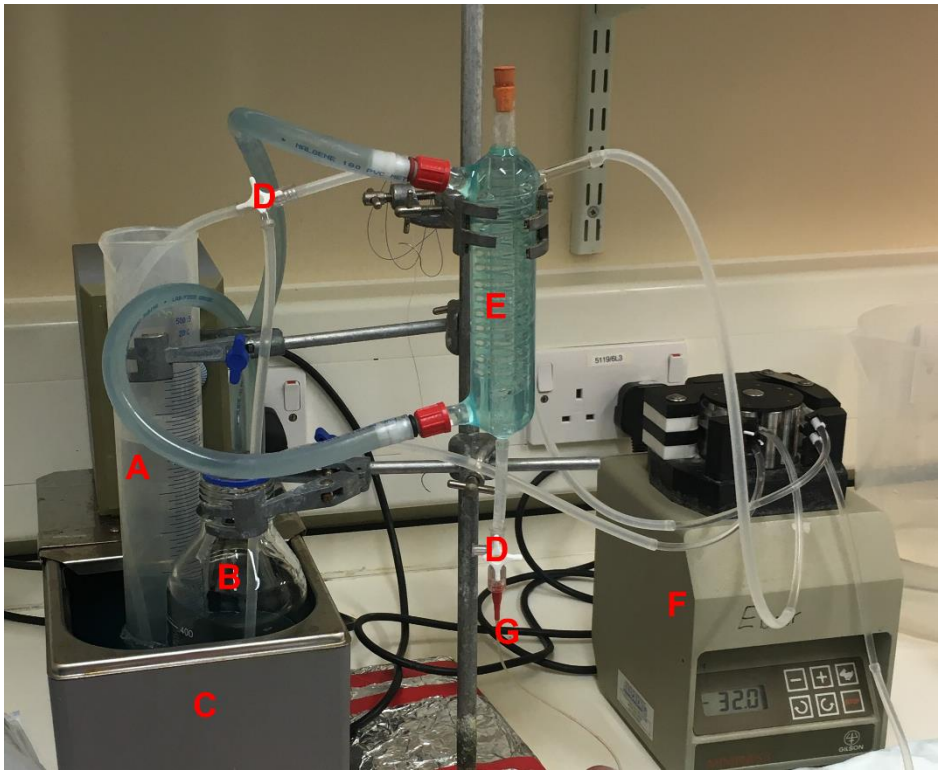


Figure 2.1: Langendorff apparatus used for cardiac myocyte isolation. (A) taurine solution. (B) Ca free solution. (C) water bath. (D) multi-way taps. (E) heat exchanger. (F) peristaltic pump. (G) cannula leading to sheep heart into the coronary artery.

Table 2.1: Calcium free/ Isolation solution	
Constituent (Fisher or Sigma Aldrich)	**Concentration (mM)**
Bovine Serum Albumin (BSA)	1 %
2,3- Butanedione Monoxime (BDM)	10
Sodium Chloride (NaCl)	134
HEPES	10
Glucose	11
Potassium chloride (KCl)	4
Magnesium sulfate ($MgSO_4$)	1.2
Monosodium Phosphate (NaH_2PO_4)	1.2
pH adjusted to 7.34 using 1M NaOH or 1M HCl prior to volumizing.	

After digestion, taurine solution (see table 2.2) was perfused through the tissue for 10 minutes to reintroduce Ca and wash out any enzyme from the tissue. The endocardium and epicardium were then removed, and the tissue was cut into small pieces and transferred to fresh taurine solution. The tissue was agitated using a pasteur pipette and filtered through 200 µM pored mesh. The cell suspension was then inspected under an inverted microscope to determine experimental suitability using visual characteristics such as distinctive rod shape and striations as shown in figure 2.2.

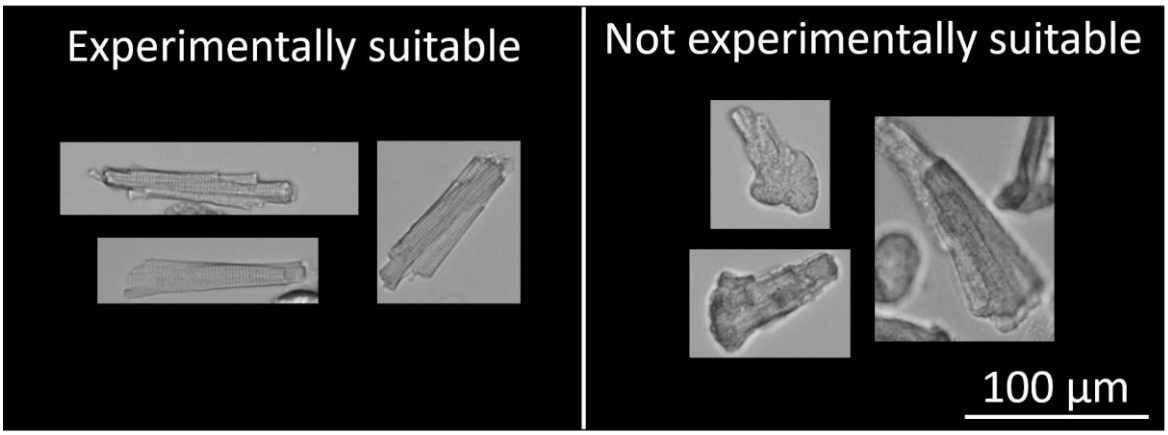


Figure 2.2: Images of experimentally suitable and non-experimentally suitable cells. Suitable cells are distinguished by the rod shape and striations.

Table 2.2: Taurine solution	
Constituent (Fisher or Sigma Aldrich)	**Concentration (mM)**
BSA	1 %
BDM	10
NaCl	108
HEPES	10
Glucose	11
KCl	4
$MgSO_4$	1.2
NaH_2PO_4	1.2
Taurine	50
Calcium chloride ($CaCl_2$)	0.1
pH adjusted to 7.34 using 1M NaOH or 1M HCl prior to volumizing.	

2.3 Experimental solutions

Sydney Ringer developed the first form of solution used in cardiac experiments and noted that when a frog heart was perfused with water contraction became more rapid and died quickly. From this observation Ringer added sodium chloride (NaCl), potassium chloride (KCl) and calcium chloride ($CaCl_2$) to produce physiologically normal contractions (Ringer, 1885). These contractions lasted for ~1.5 hours however the addition of sodium bicarbonate strengthened the contractions due to altering the pH of the solution (Ringer, 1885). The solution was further developed by Locke who observed adding glucose as an energy source for metabolism extended the survival of cardiac myocytes (Locke, 1895). The current solution used experimentally is called Normal Tyrode's (NT) solution and has a similar composition to the Ringer-Locke solution with the addition of magnesium chloride ($MgCl_2$) and sodium phosphates (Lee, 1981). Probenecid was added to reduce fluorophore leaking out of the cell. Addition of taurine has been found to retain Ca (Dolara *et al.*, 1973). Taurine containing solution is used during cell isolation and provides an intermediate calcium concentration.

All solutions were kept for a maximum of 4 days prior to experiments at 4^{o}C. Glucose and calcium were added on the day of use to limit contaminant growth.

Table 2.3: Normal Tyrodes solution	
Constituent (Fisher or Sigma Aldrich)	**Concentration (mM)**
NaCl	140
HEPES	10
Glucose	10
Probenecid	2
KCl	4
$MgCl_2$	1
$CaCl_2$	1.8
Probenecid dissolved in 1M NaOH before addition to solution. pH adjusted to 7.34 using 1M NaOH or 1M HCl prior to volumizing.	

2.4 Preparation of anthracyclines

Doxorubicin hydrochloride (Fisher) was dissolved in DMSO (Fisher) to create a stock concentration of 2 mM and aliquots were stored at -20^{o}C. All further dilutions of DOX were achieved using NT and ensured the concentration of DMSO present was less than 0.5 %

initially, most experiments were carried out at 0.001 µM which contained < 0.001% DMSO. The percentage of DMSO would not alter cell osmolality, pH, viability, action potential or field potential (Hyun *et al.*, 2017). Daunorubicin hydrochloride (Fisher) was prepared in the same manner.

2.4.1 Control of experimental cell environment / perfusion

Preparation of the experimental apparatus involved flushing the tubing with experimental solution to ensure no bubbles were present, as these can impact cell function and solution flow.

Solutions were preheated to 37^{o}C using a BubbleStop syringe heater. ValveLink was used to control which solution perfused on to the bath and allowed a seamless switch between solutions. Flow rate of solutions was controlled by solution level present in the syringe and aperture of the tip (250 µm). Experiments were conducted under perfusion conditions.

After cells were loaded with Fura-2, a small volume of cells (~0.5 ml) was pipetted on to the cell bath and allowed to settle for 5-10 minutes. Cells were brought into focus using the epi-fluorescent inverted microscope at x 40 magnification with the use of immersion oil. Cells that appeared rod shaped, striated and non-granulated were classified as viable. NT was perfused into the bath and field stimulation was used to stimulate cell contraction.

2.5 Field stimulation of cardiomyocytes

A circuit was formed between the electrodes on the cell bath, a DS2A isolated voltage stimulator and a DG2A train delay generator. The DS2A isolated voltage stimulator was set to the lowest voltage required to stimulate cells, typically between 55 – 75 V. The train delay generator controlled the frequency at which the cells were paced, at the voltage set on the isolated voltage stimulator. The pacing frequency used was 0.5 Hz with a duration of 100 ms.

2.6 Measurement of intracellular calcium

2.6.1 Equipment configuration

Various pieces of equipment are required to measure intracellular Ca and contractility simultaneously, the roles of each piece of equipment are described in table 2.4.

Table 2.4: Equipment to measure intracellular Ca and contractility

Equipment	**Purpose**
DG2A train delay generator	Controls the frequency of electrical stimulation
DS2A isolated voltage stimulator	Outputs electrical stimulation to the cell bath
Valvelink system	Allows seamless switch between solutions perfusing into the cell bath
ThermoClamp™-1	Enables heated perfusion of solutions into the cell bath
Photomultiplier tube	Receives and amplifies fluorescence signal from the cell
Multistream pro	Switches LED excitation between 340 nm and 380 nm at a set frequency
Data system interface	Communicates between the hardware and computer
Dual OptoLED power supply	Allows two LEDs independently
Myocam-S power supply	Powers the Myocam-S
Epi-fluorescent microscope	A series of filters and mirrors that enables excitation/emission of Fura-2 as illustrated in figure 2.4
BubbleStop syringe heater	Heats the solutions for perfusion and prevents bubbles in the tubing

Myocam-S high speed camera	Video detection of sarcomere which allows measurement of sarcomere length in combination with SarcLen Sarcomere Length Acquisition Module (IonOptix)
Anti-vibration table	Reduces vibrations and disturbance
Blackout case	Limits other light sources

2.6.2 Loading of cardiomyocytes with Ca fluorophore

Primary sheep cardiomyocytes were stored at room temperature. Cells were acclimatised to experimental concentrations of calcium gradually by storing in a solution of half NT (see table 2.3) and half taurine solution (see table 2.2) to produce an intermediary Ca concentration of 0.95 mM. Experiments were carried out in NT at a Ca concentration of 1.8 mM (see table 2.2). The acetoxymethyl ester (AM) form of the fluorophore Fura-2 (Invitrogen) was used to measure intracellular Ca. The AM group allows the otherwise negatively charged compound to enter through the membrane of the cell, where the cell esterases then cleave the AM groups leading to strong fluorescent indicators to be retained within the cell (Grynkiewicz *et al.*, 1985). Cells were incubated with Fura-2-AM at 0.1 µM for 10 minutes at room temperature. Loading of cells ceased after 10 minutes when an excess of solution was added. The cells were then kept in the dark at room temperature for a further 30 minutes to allow for de-esterification of Fura-2. After 30 minutes cells were ready for experimental use.

2.6.3 Excitation and emission protocol

Fura-2 is a dual excitation (340/380 nm) indicator. At 340 nm the fluorescence of Fura-2 increases with an increasing Ca concentration. At 380 nm, the fluorescence of Fura-2 decreases with an increasing Ca concentration (Grynkiewicz *et al.*, 1985). This is illustrated in figure 2.2. Fura-2 emits at 510 nm in response to excitation at both wavelengths. The ratio between these signals is not affected by loss of signal or photobleaching unlike non-

ratiometric fluorophores. Therefore, it provides a reflection of real changes in intracellular Ca.

Fura-2 was excited sequentially at 200 Hz with the multistream pro at 340 and 380 nm using OptoLED light sources. Emitted fluorescence was measured via a 515 nm long-pass filter using a photomultiplier tube coupled to a photometry system.

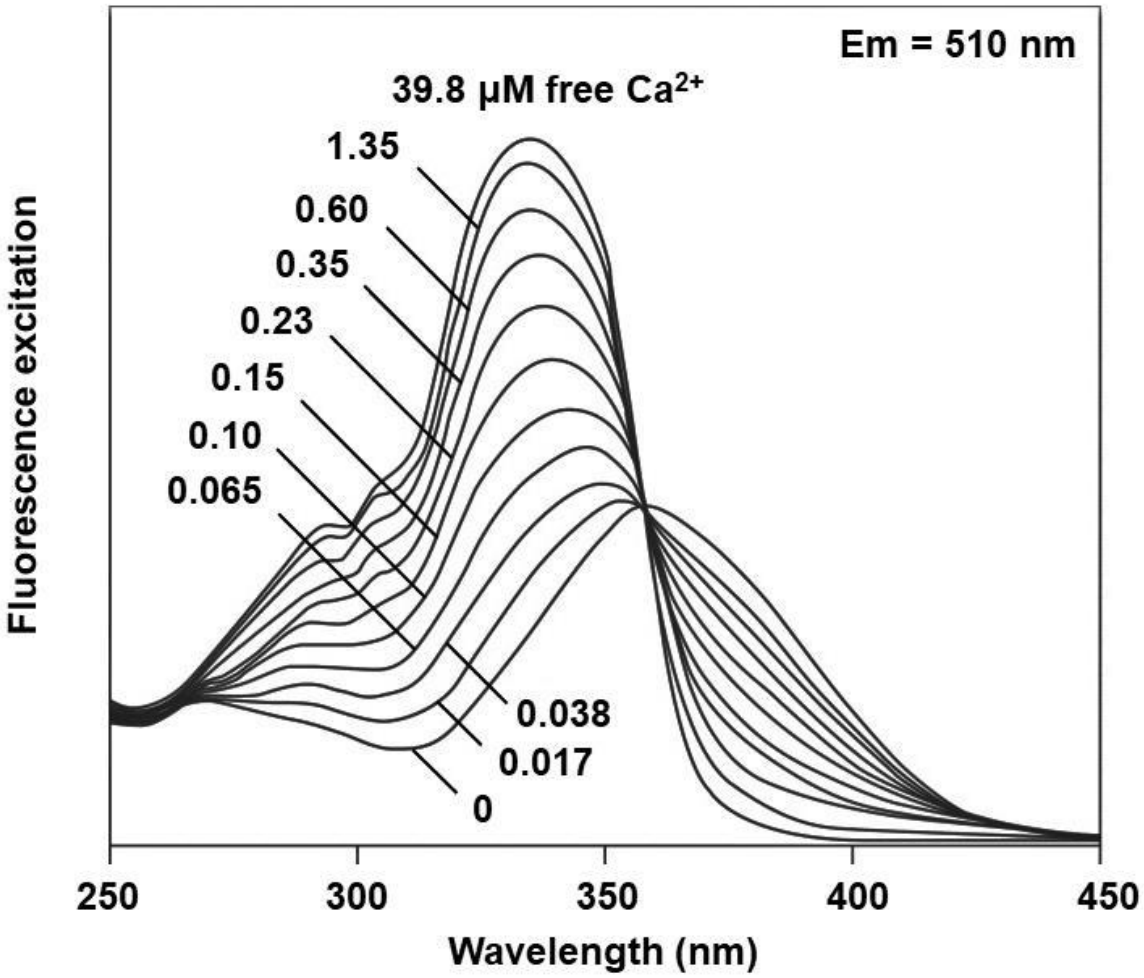


Figure 2.3: Excitation spectra of Fura-2 from Trabalza *et al* (2021).

2.6.4 Epi-fluorescence microscope configuration for Ca measurement

The configuration of the epi-fluorescence microscope is illustrated in figure 2.4. The use of Fura-2 to detect relative changes in intracellular [Ca] means it is important to limit exposure

to light from outside sources. A Faraday cage surrounds the microscope, and a black curtain covers the front to prevent outside light. As an extra precaution during experiments the lights in the room are switched off and to protect the photomultiplier tube.

As mentioned in section 2.6.3 Fura-2 excites at 340/380 nm and this is achieved by placing band pass filters in front of the LED light sources and alternating at a frequency of 200 Hz by the Multistream Pro (Cairn). The 370 nm long pass dichroic mirror allows the 380 nm light beam to pass, and this then converges with the 340 nm light beam before being directed to the cell bath by the 425 nm and 500 nm long pass dichroic mirrors. After excitation, the light emitted due to Fura-2 in the cells is deflected by standard mirrors and the 600 nm long pass dichroic mirror into the PMT. The PMT amplifies this fluorescent signal from the cell before it is integrated in the IonWizard (IonOptix) software for analysis as described in section 2.4. The amplification from the PMT was kept consistent throughout experiments while the intensity of LED excitation of Fura-2 was altered on a cell-by-cell basis due to variation in fluorophore uptake.

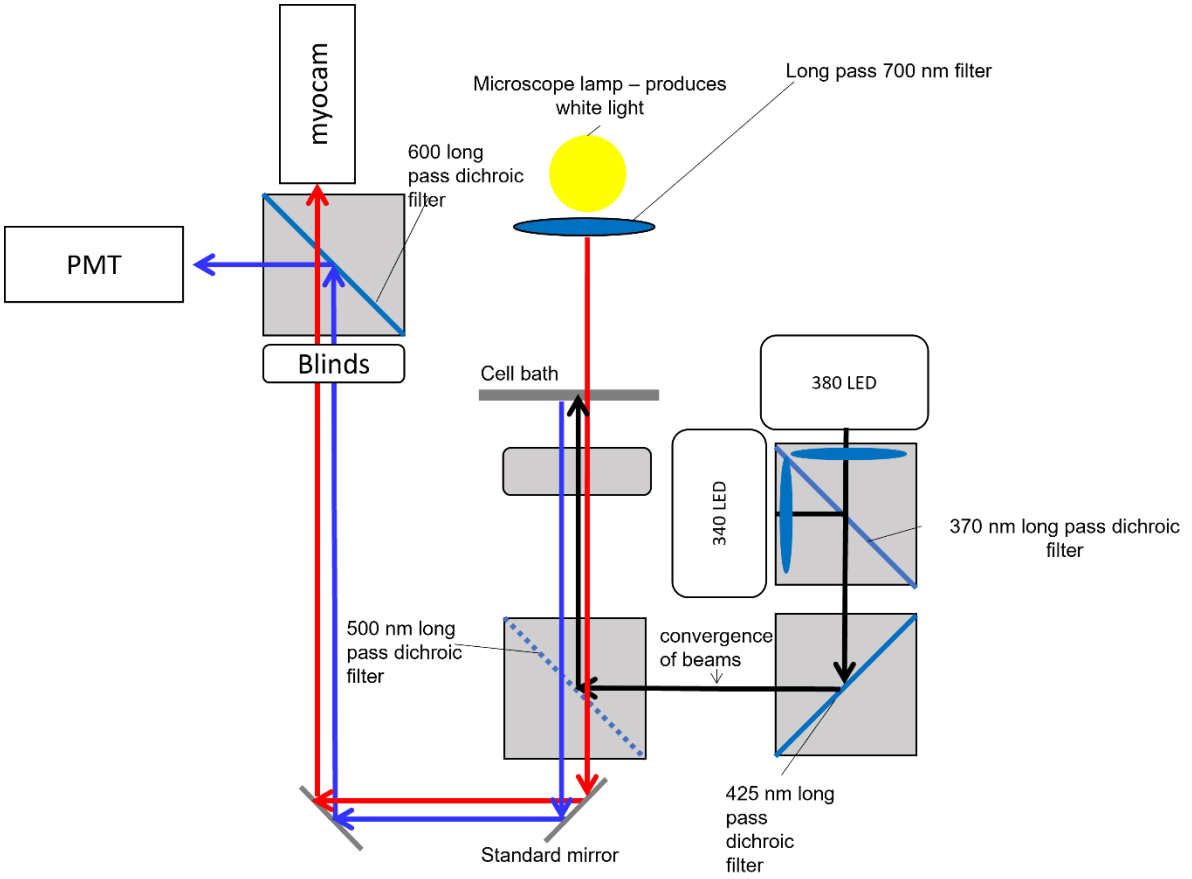


Figure 2.4: Configuration of epi-fluorescence microscope illustrating filters and light pathways for use with Fura-2.

2.7 Measurement of sarcomere length

An Ion Optix MyoCam-S high speed camera was utilised to detect changes in sarcomere length. Data regarding sarcomere length was acquired by the SarcLen Sarcomere Length Acquisition Module along in combination with the IonWizard 6.5 system. Once a cardiac myocyte was positioned in the field of view with the striations aligned vertically a box of appropriate size was positioned around the striations (sarcomeres). This determined the sarcomere length that was measured. A density trace was produced, and the peak produced converted in the software to pixels per sarcomere.

2.8 Data Analysis for calcium and sarcomere length

2.8.1 Diastolic and systolic Ca

Analysis of diastolic and systolic Ca was conducted by exporting 5 averaged Ca transients from IonWizard software (Ion Optix) into Ca Multi which is an excel program to analyse transients obtained using fluorescent indicators (Greensmith, 2014a). This was done for each steady state condition (e.g. steady state control, steady state drug etc). The program allows the identification of parameters such as peak Ca and diastolic Ca using cursors, the program then uses this information to determine Ca amplitude. The background (no cell) for each cell was also taken after each experiment by moving the cell out of field of view. Both signals obtained for the no cell from the 340 nm and 380 nm were averaged and a ratio was determined by dividing 380 by 340. The program subtracts the background fluorescence (no cell) from the raw fluorescence, so fluorescence analysed is that from the cell.

2.8.2 Sarcomere length

The average of 5 sarcomere lengths at each steady state were exported from IonWizard software to the Ca Multi program described in section 2.8.1 and diastolic and systolic length was determined. These values also allow the program to calculate the peak amplitude of contraction.

The rate of sarcomere relaxation and shortening was analysed using the excel program APD single 2010 v2. The relaxation of the sarcomere was determined by calculating the time taken for 90 % rate of decay minus 10 % rate of decay.

2.8.3 Statistical Analysis for Ca handling and contractility

The raw data for each cell was extracted from the Ca Multi program and input into a new excel spreadsheet. This data was then normalised against each cells own control state. Statistical analysis was carried out on IBM SPSS. Unless otherwise stated a one-way ANOVA

with Tukey post-hoc test was carried out and a statistically significant result was identified if $P < 0.05$.

2.9 Measurement of intracellular oxidative stress

2.9.1 Loading and preparation of cardiomyocytes for oxidative stress experiments

Throughout the oxidative stress experiments cells were stored at room temperature in half NT (see table 2.3) and half taurine solution (see table 2.2) at Ca concentration of 0.95 mM. Cardiac myocytes were split into tubes corresponding to treatment conditions and treated with anthracyclines of varying concentrations for a set period. After incubation the drug solution was removed from the cells. Cells were then loaded with 5 µM CellROX Deep Red (Invitrogen) (Ex 640/Em 665) for 30 minutes. CellROX deep red is a cell permeable fluorophore that becomes fluorescent upon oxidation and is localised to the cytoplasm (Manoil & Bouillaguet, 2018). CellROX Deep Red has the advantage of being used with viable cells and detects increases in oxidative stress (Habibalahi *et al.*, 2020). Cells pelleted during this period allowing the solution containing fluorophore to be removed before being resuspended in fresh solution.

Following resuspension in fresh solution 100 µl of cell suspension was pipetted into wells on a 96 well plate and allowed to settle before images were captured using the Biotek cytation imaging system.

2.9.2 Image capturing and analysis

The Biotek cytation imaging system 3 was set to 25^{o}C (room temperature) and focused to capture images of cells using both the brightfield and Texas red filter cube at x 10 magnification. Gain and intensity were kept consistent for all experiments.

Analysis was conducted using imageJ software. Brightfield images were overlayed on the corresponding Texas red image and viable cells were identified as previously mentioned by shape, striation, and granularity. The integrated density and area of the cells were measured and areas surrounding the cells selected to subtract the background. The total corrected cellular fluorescence (TCCF) was calculated by subtracting the background fluorescence reading ((integrated density – (area of cell x mean fluorescence of background reading)) (McCloy *et al.*, 2014).

2.9.3 Statistical analysis for oxidative stress data

For these experiments each plate (animal) determined the n. The average fluorescence for each treatment condition was calculated and normalised against the untreated average for each plate. Statistical analysis was carried out on IBM SPSS. Unless otherwise stated a one-way ANOVA with Tukey post-hoc test was carried out and a statistically significant result was identified if $P < 0.05$.

Chapter 3
The effects of doxorubicin and daunorubicin on Ca handling and contractility in sheep ventricular myocytes

3.1 Introduction

As discussed in detail in 1.1.4 and 1.5, anthracyclines are highly effective against many cancer types, so frequently form part of treatment strategies. The most commonly used anthracyclines are doxorubicin (DOX), daunorubicin (DAUN), idarubicin and epirubicin (Armenian & Bhatia, 2018). Of these, epirubicin is predominantly used for the treatment of breast cancer (Levine, 2000). While doxorubicin, daunorubicin and idarubicin are used for the treatment of childhood cancer – the focus of this project – the use of idarubicin is relatively less common (Feijen *et al.*, 2019). For this reason, doxorubicin and daunorubicin were selected for this investigation. As per 1.1.4, the anti-cancer effectiveness of these drugs is not disputed however, early and late cardiotoxic effects remain a considerable limitation. The extent to which doxorubicin and daunorubicin-mediated *cell* dysfunction contributes remains unclear.

3.1.1 Anthracyclines and excitation-contraction coupling

As discussed in 1.2.1 whole heart function is dependent on that of the single cardiac myocyte. As such, if we are to fully understand how anthracyclines cause cardiotoxicity, it is essential to elucidate their effects on ECC. Limited, previous studies provide an insight into the effects of anthracyclines on the many components of ECC.

In terms of global cell function, several studies agree that doxorubicin reduces the Ca transient amplitude and contractility in a variety of models (Maeda *et al.*, 1997; Sag *et al.*, 2011; Temma *et al.*, 1996; Zheng *et al.*, 2011). However, the *direct* effects of daunorubicin on these inotropic indicators have yet to be investigated. For doxorubicin, the reduction in Ca transient amplitude and contractility may be explained by alterations to the components of ECC, though again, previous studies are remarkably limited.

As discussed in section 1.6.4 calcium-calmodulin protein kinase (CaMKII) regulates phosphorylation of various proteins of ECC. DOX increases phosphorylation of CaMKII which

is implicated in cardiomyocyte apoptosis. However, this is suppressed by blocking LTCC (Ikeda *et al.*, 2019a). CaMKII activity is regulated by intracellular Ca, these findings suggest that DOX increases intracellular Ca via increased LTCC activity thereby increasing activation of CaMKII pathways. Others show DOX directly increases L-type Ca channel activity leading to an increase in intracellular Ca (Keung *et al.*, 1991). This is surprising given negatively inotropic effects of DOX, observed by others, at cell level. This inconsistency may be explained by effects of anthracyclines on SR Ca content and the RyR. Evidence shows DOX causes a reduction in SR Ca content (Ondrias *et al.*, 1990; Sag *et al.*, 2011). This is also the case for DAUN (Shadle *et al.*, 2000).

Studies in planar membranes reveal a concentration-dependent biphasic effect on the RyR (Hanna *et al.*, 2014). Here, low concentrations of DOX (<2.5 μM) increase the open probability (activate) the RyR whereas higher concentrations produced an irreversible inhibition. *In vivo* studies show DOX to increase (Llach *et al.*, 2019) and decrease (Gambliel *et al.*, 2002; Olson *et al.*, 2005) RyR expression. DAUN produced a time-dependent biphasic effect on RyR activity (Hanna *et al.*, 2011b). Here, short-term exposure (10 minutes) to concentrations between 0.5 and 10 μM increased RyR activity. However, at higher concentrations (≥ 2.5 μM), an inhibitory effect developed after 20 minutes. These studies highlight a controversy surrounding the effects of anthracyclines on RyR.

The effects on the SR Ca content and RyR may be explained by alterations to the Ca removal pathways (see 1.2.9 for review). The expression of SERCA has been found to be reduced following DOX treatment (Arai *et al.*, 1998). Furthermore, a modulatory effect was observed by Hanna *et al* (2014) who show low concentrations of doxorubicinol (a metabolite of DOX) reduce SERCA2a activity. However, this was not the case for DOX itself suggesting an important role for DOX metabolic products.

A shift in the relationship between systolic Ca and force of cell contraction may also explain the apparent negatively inotropic effects of DOX and DAUN. This may occur due to a change in myofilament sensitivity. While the impact of DOX on myofilament sensitivity has been

studied in small animal models, whether this sensitivity is increased, decreased or unaltered remains controversial. In one study, DOX did not alter myofilament sensitivity (Boucek *et al.*, 1993) while in others, a decrease was observed (Chakouri *et al.*, 2020). Whether DAUN alters myofilament remains unknown.

While these previous studies provide certain insight into anthracycline cardiotoxicity there are many limitations. For instance, most studies use doxorubicin on the assumption that other anthracyclines will produce similar effects. Clearly, this isn't the case yet few studies have investigated the impact of daunorubicin on calcium handling. Furthermore, many of these studies use artificial planar membranes or small animal models and look at components individually. This makes integration of discrete findings into a unified model problematic. Our experiments overcome this by using cardiomyocytes from a large animal model. This integrative approach allows all aspects of ECC to be studied in the dynamic setting of a physiologically intact cell.

3.1.2 An experimentally relevant anthracycline concentration

In vitro studies are conducted on individual intact cardiac myocytes so the experimental concentrations of anthracyclines must not alter cell viability during experiments designed to elucidate mechanistic effects. Clinically, plasma concentrations of DOX following treatment in female patients reached levels of 630.4 ± 22.1 ng/ml to 39.8 ± 15.3 ng/ml after 0.66 and 24.66 hours respectively (Barpe *et al.*, 2010). We do not know if the cells of the hearts in patients are exposed to these concentrations, nonetheless, this information provides a useful indication.

In cultured cardiac myocytes (both primary and iPS-derived) a considerable range of concentrations have been used (0.01 to 10 µM) (Barpe *et al.*, 2010; Burridge *et al.*, 2016; Chaudhari *et al.*, 2016; Hanna *et al.*, 2014; Hanna *et al.*, 2017; Kim *et al.*, 2006a; Vavrova *et*

al., 2013; Zhao & Zhang, 2017). In iPS derived cardiomyocytes, DOX-dependent cytotoxicity became evident at 0.5 µM with an IC_{50} of 3.5 µM (Zhao & Zhang, 2017).

While the cellular effects of anthracyclines may differ, it remains useful to consider DOX as a reference compound to develop working concentrations for experimental methodology (Chaudhari *et al.*, 2016).

3.1.3 Summary

Given that anthracyclines are unquestionably linked to cardiac toxicity, there remains significant gaps in the knowledge surrounding their effects on cardiac myocytes. This is critically important if we are to (1) fully understand anthracycline mediated cardiotoxicity and (2) design new therapeutic strategies that allow the continued use of anthracyclines with mitigation of cardiotoxicity.

3.1.4 Aims

- Establish a suitable working AC concentration.
- Use integrative analysis to measure the effects of DOX and DAUN on ECC in the dynamic setting of intact sheep ventricular myocyte.
- Investigate the effect of doxorubicin and daunorubicin on myofilament sensitivity.

3.2 Methods

3.2.1 Cell preparation

Primary left ventricular cardiomyocytes were isolated from healthy, ~18-month-old sheep (see section 2.2.2). Cells were loaded with fura-2 as described in section 2.6.2 then stored in a solution produced by mixing NT and taurine (see 2.6.2) solution in equal parts to produce an intermediate Ca concentration of 0.95 mM.

3.2.2 Assessment of cell suitability

Cell suitability was assessed using visual characteristics of cardiac myocytes as described in 2.2.2. Cells were viewed in NT solution prior to acute exposure to DOX (0.001 µM – 10 µM) followed by a wash out period. Cell experimental suitability was assessed in each condition. Overall experiments lasted 3-10 minutes. Cells that remained striated, rod shaped and non-granulated throughout the experiment were classed as suitable.

Percentage suitability of cells for each animal at each concentration was determined and an average calculated. Statistical significance between treatment groups was established using a one-way ANOVA.

3.2.3 Excitation-contraction coupling measurements

Loaded cells were paced at 0.5 Hz using field stimulation (see section 2.5) in NT. Once Ca and sarcomere length transients reached steady state, the perfusing solution was switched to DOX or DAUN at concentrations of 0.001, 0.01, 0.1, 1 and 10 µM. Once a new steady state was reached, the reversibility of any effect was determined with a washout (NT). Original recordings were calibrated and analysed offline using custom software (see 2.8).

3.2.4 Measurement of myofilament sensitivity

Alterations to myofilament sensitivity were investigated by plotting phase-plane loops of cytoplasmic Ca versus sarcomere length of each experiment. The phase-plane loops proceed counter-clockwise with cell shortening denoted by the ascending limb (see fig 3.1). The relaxation phase of the cardiac myocyte defines a quasi-equilibrium of systolic Ca, myofilament Ca binding and force, hence cell length (Spurgeon *et al.*, 1992). Therefore, in each plot the cytoplasmic Ca at 50 % relaxation (EC_{50}) was used to quantify the change in myofilament sensitivity and statistical significance was calculated using a paired T-test.

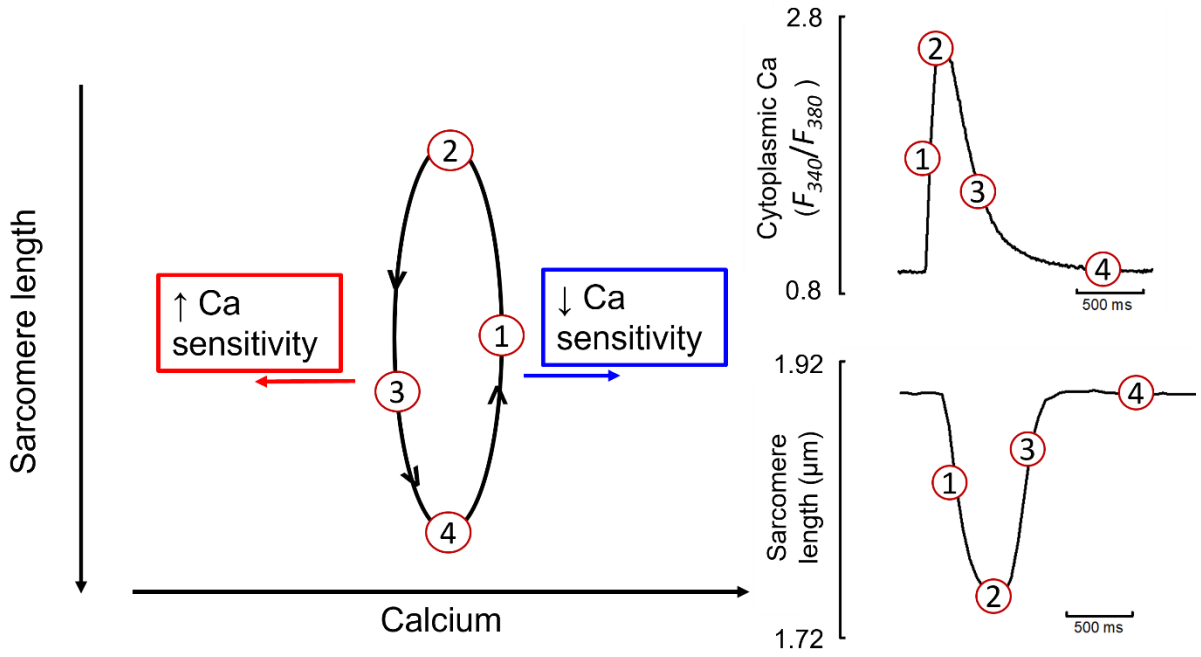


Figure 3.1: Phase plane loops highlighting indications of change in myofilament sensitivity.

Further investigation of myofilament sensitivity was carried out by permeabilising cells using saponin (25 µg/ml) in a Ca free version of the NT solution outlined in table 2.3. Cardiac myocytes were exposed to saponin for 1 – 5 minutes depending on the visual integrity of the cell. Once permeabilised, resting sarcomere length was measured in the absence of Ca then incremental Ca concentrations (0.35, 3.5 and 35 µM). This manoeuvre was then repeated in the presence of DOX and DAUN. Mean sarcomere lengths were measured at each Ca concentration and normalised to the length in the absence of Ca. The gradient of the relationship between [Ca] and sarcomere length was compared to determine if DOX or DAUN altered myofilament sensitivity. Statistical analysis was carried out using a 2-way ANOVA and a Holm-Sidak post-hoc test was used to allow comparison between groups.

3.2.5 Measurement of sarcoplasmic reticulum Ca content

Changes in SR Ca were estimated by comparison of Ca transients evoked by rapid application of caffeine (10 mM). This works on the principle that caffeine increases the open probability of the RyR to the extent that mass SR Ca release occurs (Eisner *et al.*, 2017).

3.2.6 Measurement of the activity of the Ca removal mechanisms

As noted in section 1.2.9.1 the main mechanisms of Ca removal in mammalian cardiomyocytes are SERCA and NCX. The rate constant (RC) of decay of systolic Ca transient (k_{sys}) therefore represents the combined activity of SERCA and NCX. Caffeine increases RyR open probability (see 3.2.5) to the extent that SERCA activity is rendered futile, thus the RC of decay of the caffeine-evoked Ca transient (k_{caff}) represents the activity of NCX alone. Subtraction of k_{caff} from k_{sys} provides k_{SERCA} and so the activity of SERCA alone.

3.3 Results

3.3.1 The effects of doxorubicin on cell suitability

To establish a suitable anthracycline experimental concentration, the effects of DOX on cell suitability were assessed. A concentration that produced a graded effect on ECC without reducing cell suitability was required.

Exposure to 10 µM DOX (fig 3.2), reduced cell suitability to 54.17 % ± 17.58 %. Cell suitability was reduced to 80.56 % ± 10 %, 90 % ± 10 and 96.67 ± 3.33 % by 1, 0.1 and 0.01 µM DOX respectively. Cell suitability was unaltered during the time course of our experiments at 0.001 µM DOX. While no statistically significant difference was detected between the suitability of cells from each animal ($n = 3$, $p < 0.066$) there is a clear correlation between an increase in concentration and a reduction in cell suitability as shown in figure 3.2.

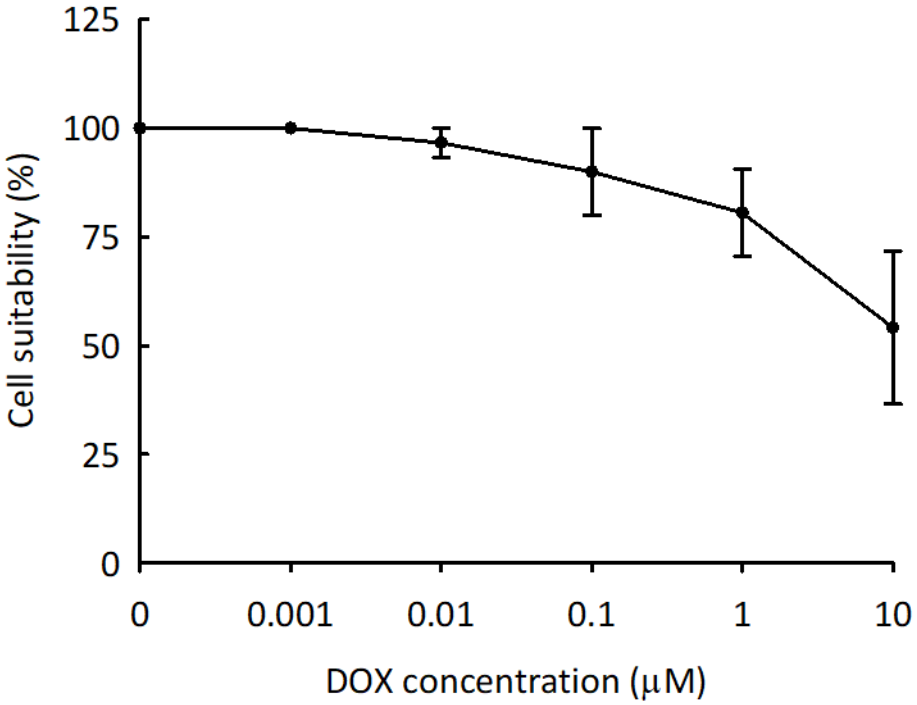


Figure 3.2: Cell suitability of left ventricular cardiac myocytes isolated from control sheep when treated with 0.001 µM- 10 µM doxorubicin and washout (n = 3, p = 0.066).

3.3.2 The dose-dependent effect of doxorubicin on intracellular Ca and contractility

At the concentrations used, no dose dependent effect of DOX was observed (fig 3.3A-F). This was the case for diastolic Ca (n = 13, p = 0.38; fig 3.3A) and diastolic sarcomere length (n = 12, p = 0.599; fig 3.3B). The effect on Ca transient amplitude was not dose dependent (n = 13, p = 0.086; fig 3.3C) and was accompanied by no dose dependent effect on sarcomere shortening (n =10, p = 0.156; fig 3.3D). This was also the case for peak intracellular Ca (n = 13, p = 0.637; fig 3.3E) and rate of sarcomere relaxation (n = 10, p = 0.156; fig 3.3F).

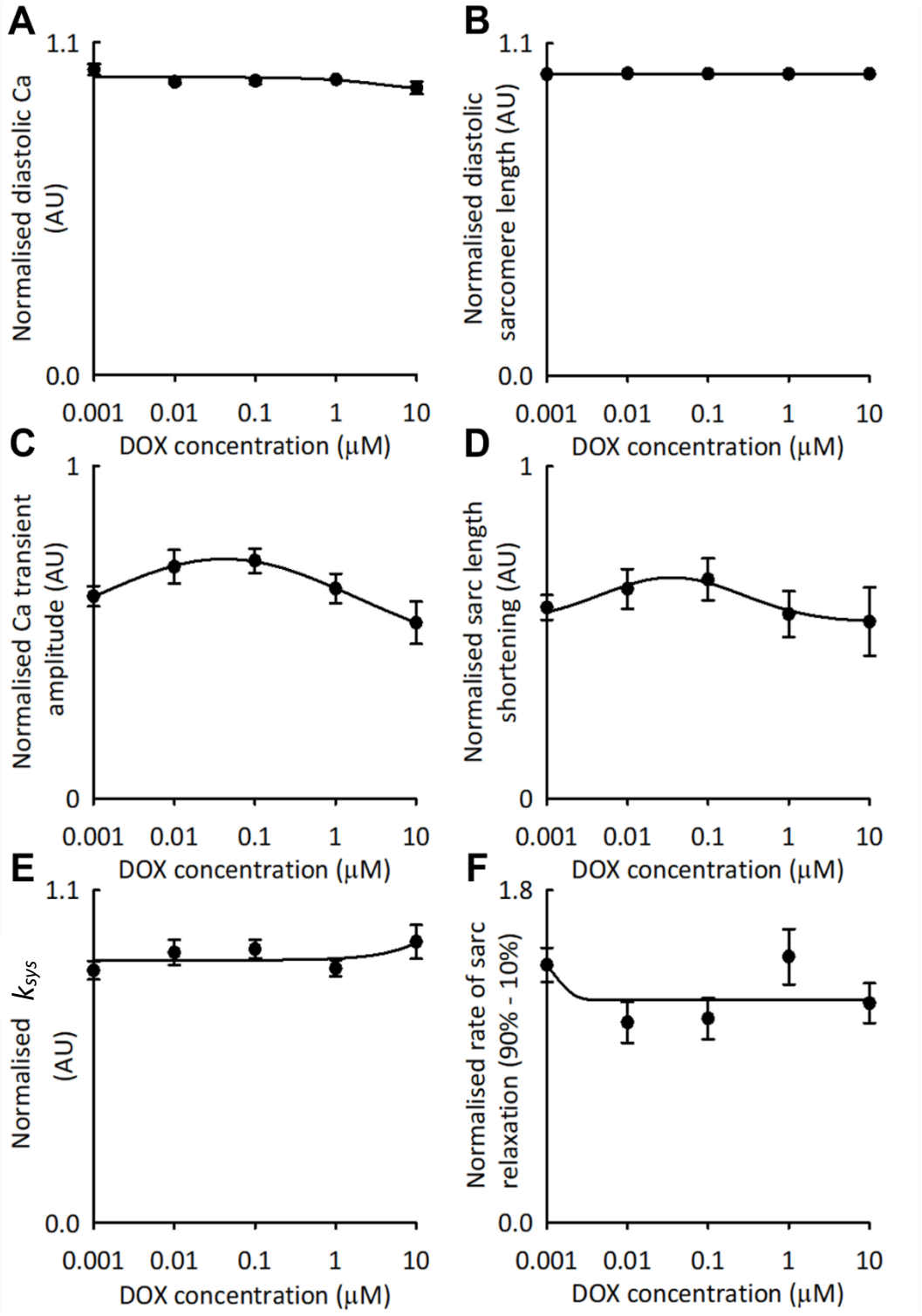


Figure 3.3: The effects of DOX (0.001 – 10 μM) on intracellular Ca and contractility in sheep ventricular myocytes. (A) mean normalised diastolic Ca. (B) mean normalised diastolic sarcomere length. (E) mean normalised peak sarcomere length. (C) mean normalised Ca

transient amplitude. (D) mean normalised sarcomere length shortening. (E) mean normalised peak Ca. (F) mean normalised 90 % - 10 % decay time of sarcomere length. n = 10 – 21 (from 3 animals).

3.3.3 A detailed investigation of the effects of DOX on Ca and contractility

A working concentration of 1 nM was selected for subsequent detailed investigations given (1) no concentration-dependence of effect was observed in 3.3.2 and (2) this concentration produced no significant effect on cell suitability (3.3.1).

The specimen traces in figure 3.4 show the effect of 1 nM DOX on intracellular Ca and contractility and are representative of the means shown in fig 3.5. On average, no significant effect on diastolic Ca ($n = 42$, $p = 0.213$; fig 3.5A) or diastolic sarcomere length ($n = 40$, $p = 0.06$; fig 3.5B) was observed. However, DOX reduced the Ca transient amplitude by 39.13 % ± 3 % ($n = 43$, $p < 0.001$; fig 3.5C) which partially reversed upon washout ($n = 43$, $p < 0.001$; control vs wash and $p = 0.004$; drug vs wash). This effect on Ca was accompanied by a 42.46 % ± 3.79 % ($n = 36$, $p < 0.001$) decrease in systolic sarcomere shortening (fig 3.5D) which reversed fully on washout ($n = 38$, $p = 0.159$; control vs wash and $p < 0.001$; drug vs wash). On average, peak Ca decreased by 19.11 % ± 2.13 % ($n = 43$, $p < 0.001$) which partially reversed upon washout ($n = 43$, $p < 0.001$; control vs wash and $p = 0.011$; drug vs wash; fig 3.5E). The rate of sarcomere relaxation was increased on average by 39.46 ± 9.21 % ($n = 43$, $p < 0.001$) which recovered in washout ($n = 36$, $p = 0.895$; control vs wash and $p = 0.002$; drug vs wash; fig 3.5F).

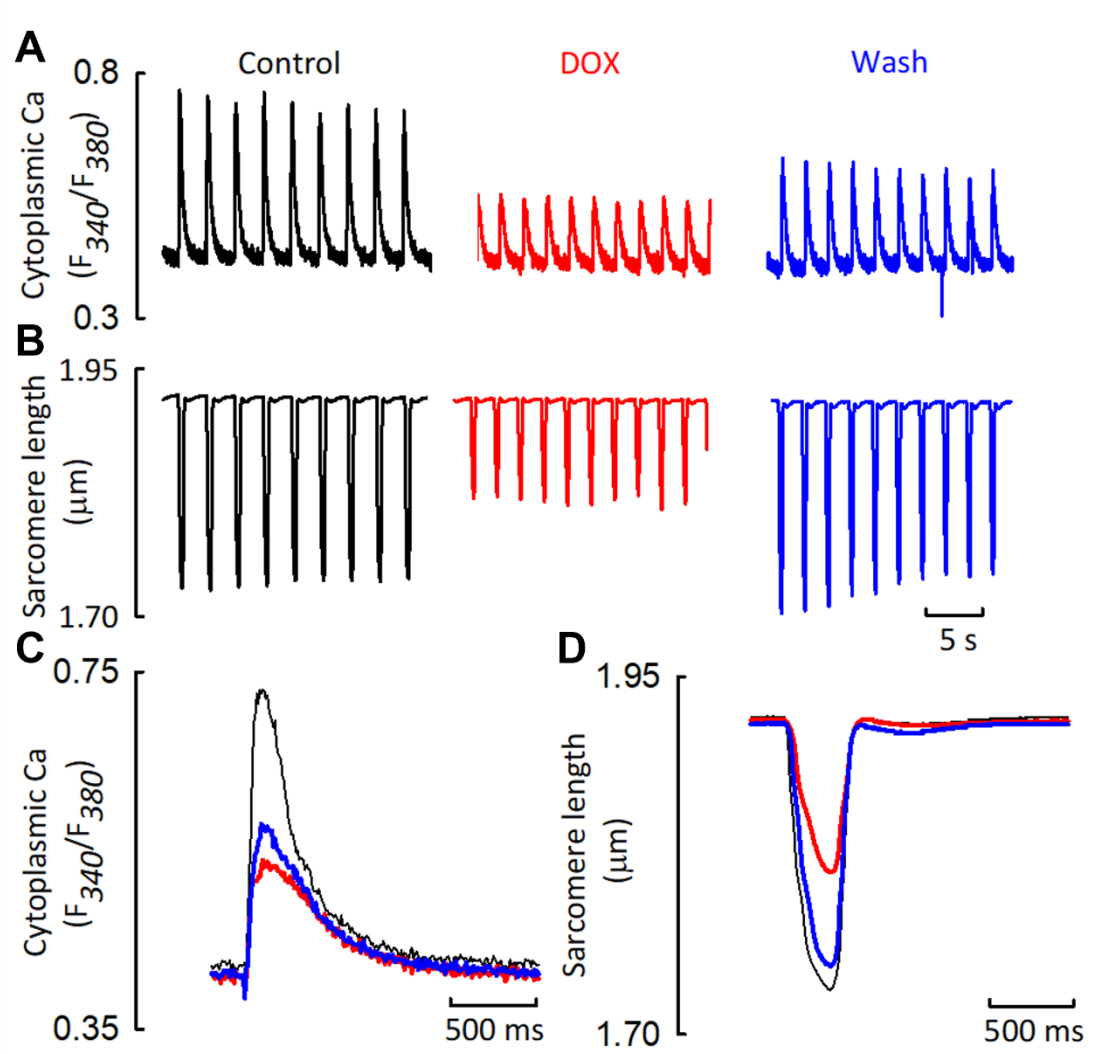


Figure 3.4: The effect of 1 nM DOX on cytoplasmic Ca and sarcomere shortening. (A) long time base record that shows specimen Ca transients. (B) specimen contractility transients. (C) Ca transient overlays. (D) contractility transient overlays. Long time base records represent steady state from which five Ca transients were averaged to produce the overlays. Data from this single cell is representative of the mean data shown in fig 3.5.

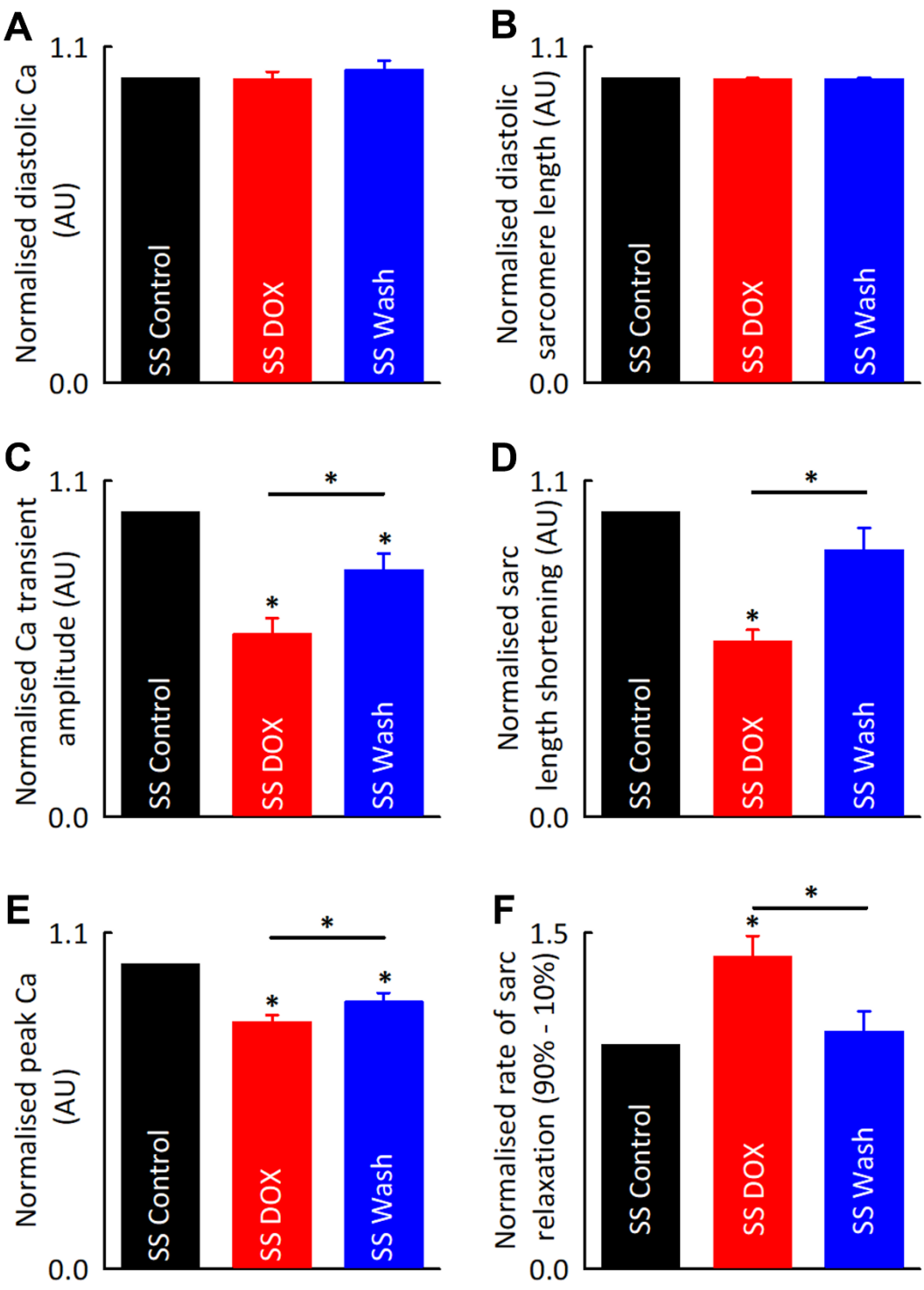


Figure 3.5: The effect of 1 nM DOX on intracellular Ca and contractility in sheep ventricular myocytes. (A) mean normalised diastolic Ca. (B) mean normalised diastolic sarcomere length. (C) mean normalised Ca transient amplitude. (D) mean normalised sarcomere shortening. (E)

mean normalised peak Ca. (F) mean normalised 90-10 % decay time of sarcomere length. n = 36 - 43 (from 9 animals), * denotes statistical significance.

3.3.4 The effect of doxorubicin on SR Ca content and rate of Ca removal

As described in section 3.2 caffeine allows the investigation of alterations to SR Ca content. Specimen shown in figure 3.6A shows the effect of DOX on the amplitude of the caffeine evoked Ca transient. On average this was reduced by 33 % ± 5.16 % (n = 24, $p < 0.001$) and did not recover in washout (n = 20, $p < 0.001$; fig 3.7A). Specimen figure 3.6B illustrates the effect of DOX on the Ca transient rate of constant decay (k_{sys}). On average, k_{sys} is reduced by 16.56 % ± 3.02 % (n = 46, $p < 0.001$) which then recovers in wash (n = 40, p = 0.059; fig 3.7B). The caffeine-evoked Ca transient rate constant of decay (K_{caff}) shown in figure 3.6C increased on average by 64 % ± 25.52 % (n = 20, p = 0.017) and recovered in wash (n = 20, p = 0.107; fig 3.7C). On average, k_{SERCA} was significantly reduced by DOX by 34.07 % ± 8.49 % (n = 20, p = 0.002) and recovered in wash (n = 19, p = 0.588; fig 3.7D).

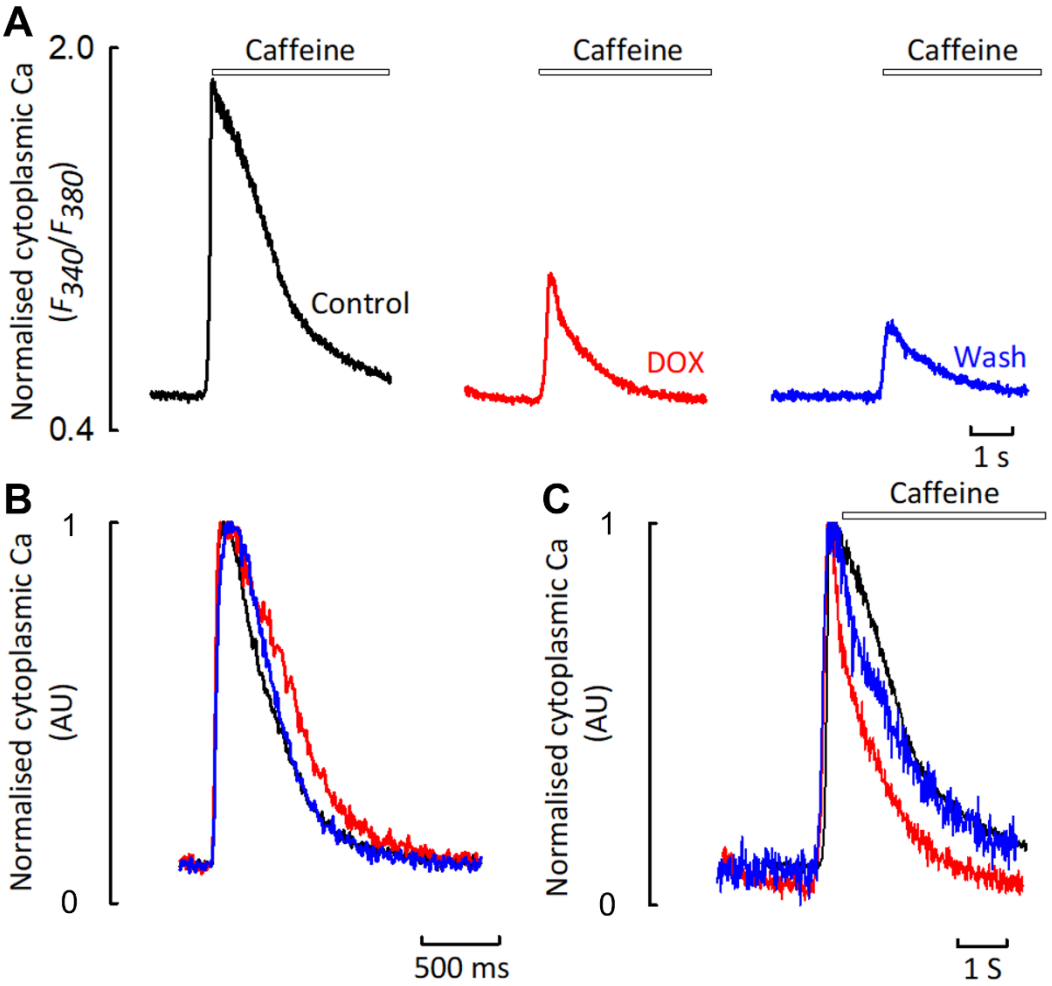


Figure 3.6: The effect of 1 nM DOX on SR Ca content. (A) specimen Ca transients in the presence of caffeine. (B) specimen normalised Ca transients to permit direct comparison of the rate of Ca decay. (C) specimen normalised caffeine-evoked Ca transients to permit direct comparison of the rate of Ca decay in the presence of caffeine.

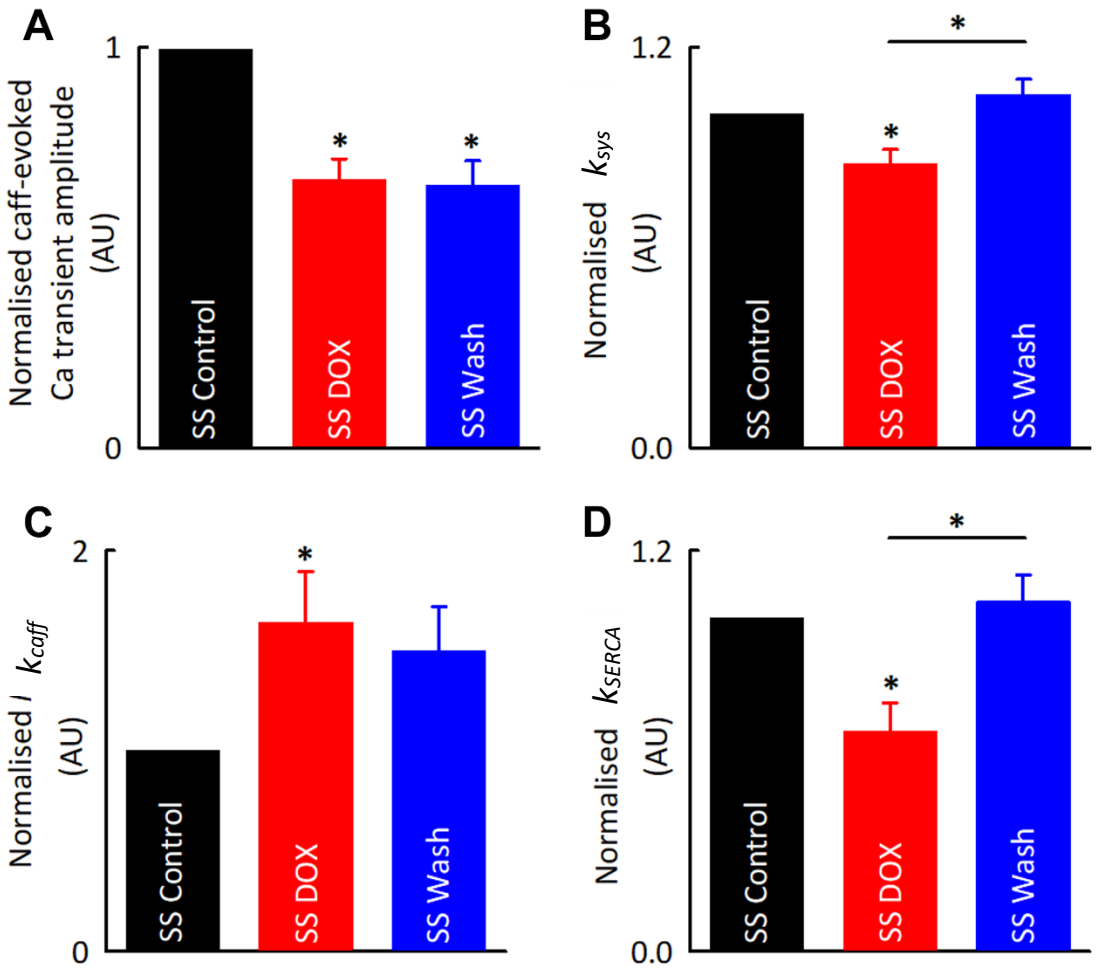


Figure 3.7: The effect of 1 nM DOX on Ca removal in sheep ventricular myocytes. (A) mean normalised amplitude of caffeine evoked Ca transient. (B) mean normalised k_{sys}. (C) mean normalised k_{caff}. (D) mean normalised k_{SERCA}. n = 19 - 24 (from 5 animals), * denotes statistical significance.

3.3.5 A detailed investigation of the effects of daunorubicin on Ca and contractility

The specimen traces in figure 3.8 show the effect of 1 nM DAUN on intracellular Ca and contractility. On average, no significant effect was seen on diastolic Ca (n = 20, p = 0.776; fig 3.9A) however the change to diastolic sarcomere length although minor at an increase of

0.32 % ± 0.078 was significant (n = 18, p = 0.003; fig 3.9B). DAUN decreased Ca transient amplitude by 45.31 % ± 4.51 % (n = 20, p < 0.001) which reversed upon washout (n = 20, p = 0.138; fig 3.9C). This was accompanied by a decrease in sarcomere shortening of 64.38 % ± 4.51 % (n = 19, p < 0.001) that recovered upon washout (n = 18, p = 0.313; fig 3.9D). On average, peak Ca was decreased by 18.42 % ± 2.19 % (n = 20, p < 0.001) which partially reversed upon washout (n = 20, p = 0.045; control vs wash and p < 0.001; drug vs wash; fig 3.9E). Sarcomere relaxation was increased on average by 88.5 % ± 26.21 % (n = 18, p < 0.001) which recovered upon wash out (n = 16, p = 0.509; fig 3.9F).

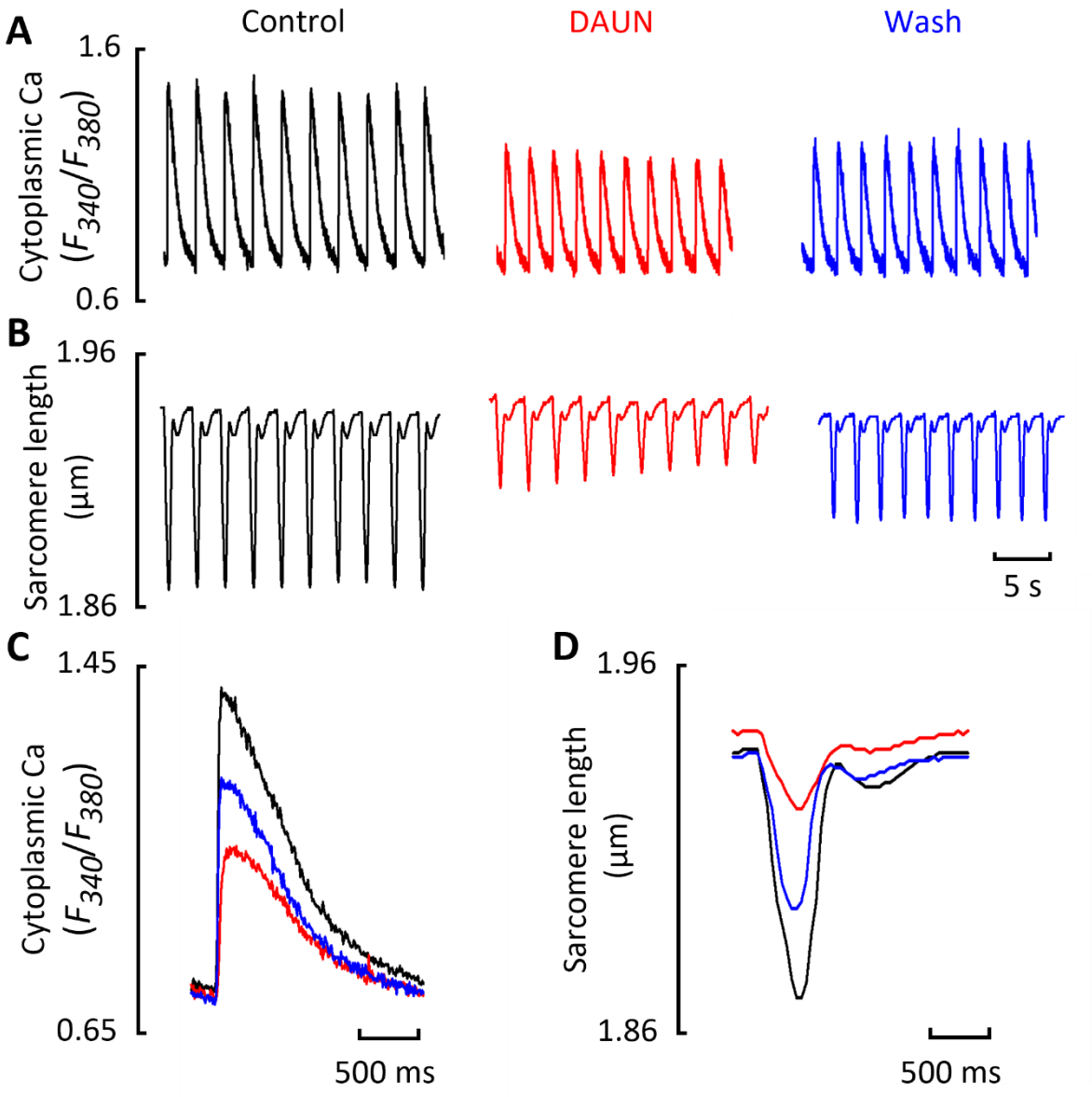


Figure 3.8: The effect of 1 nM DAUN on cytoplasmic Ca and sarcomere shortening. (A) long time base record that shows specimen Ca transients. (B) specimen contractility transients. (C) Ca transient overlays. (D) contractility transient overlays. Long time base records represent steady state from which five Ca transients were averaged to produce the overlays. Data from this single cell is representative of the mean data shown in fig 3.9.

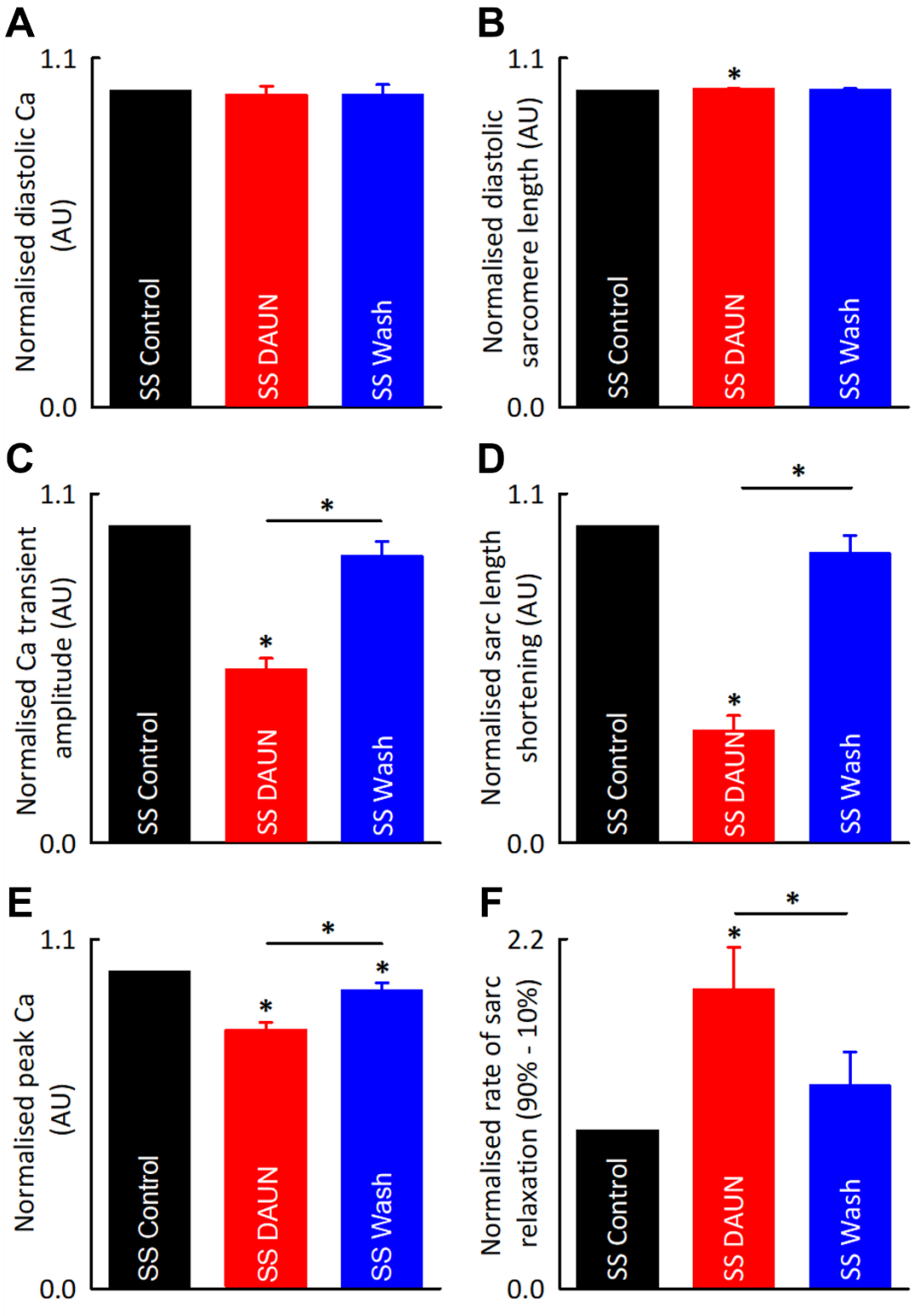


Figure 3.9: The effect of 1 nM DAUN on intracellular Ca and contractility in sheep ventricular myocytes. (A) mean normalised diastolic Ca. (B) mean normalised diastolic sarcomere length.

(C) mean normalised Ca transient amplitude. (D) mean normalised sarcomere shortening. (E) mean normalised peak Ca. (F) mean normalised 90-10 % decay time of sarcomere length. n = 16 - 20 (from 6 animals), * denotes statistical significance.

3.3.6 The effect of daunorubicin on SR Ca content

As described in section 3.2 caffeine allows the investigation of alterations to SR Ca content. Specimen shown in figure 3.10A shows the effect of DAUN on the amplitude of the caffeine evoked Ca transient. On average this was reduced by 14 % ± 6.29 % (n = 23, p = 0.035) and did not recover in washout (n = 20, p < 0.001; fig 3.10B).

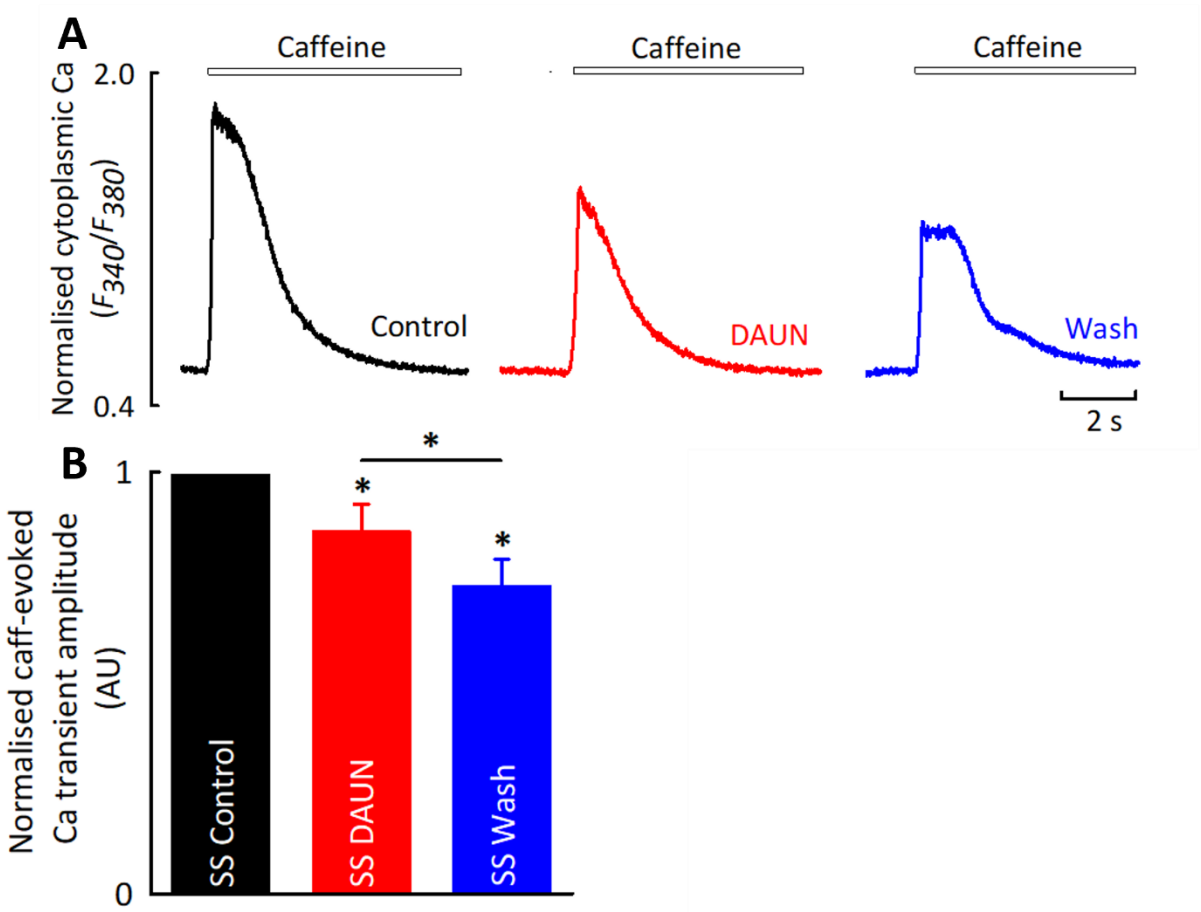


Figure 3.10: The effect of 1 nM DAUN on SR Ca content. (A) specimen Ca transients in the presence of caffeine. (B) mean normalised caffeine evoked Ca transient amplitude. n = 20 – 23 (from 5 animals), * denotes statistical significance.

3.3.7 The apparent interaction of daunorubicin and caffeine

In the presence of caffeine, the rate constant of decay was not significantly altered following exposure to DAUN treatment (n = 19, p = 0.06) as shown in figure 3.11. Without caffeine DAUN caused a decrease in k_{sys} of 23.12 % ± 4.51 % (n = 18, p < 0.001) whereas this change was not detected in the k_{sys} after cells had been exposed to caffeine. Due to this apparent interaction of DAUN and caffeine the removal of Ca could not be measured in the presence of DAUN.

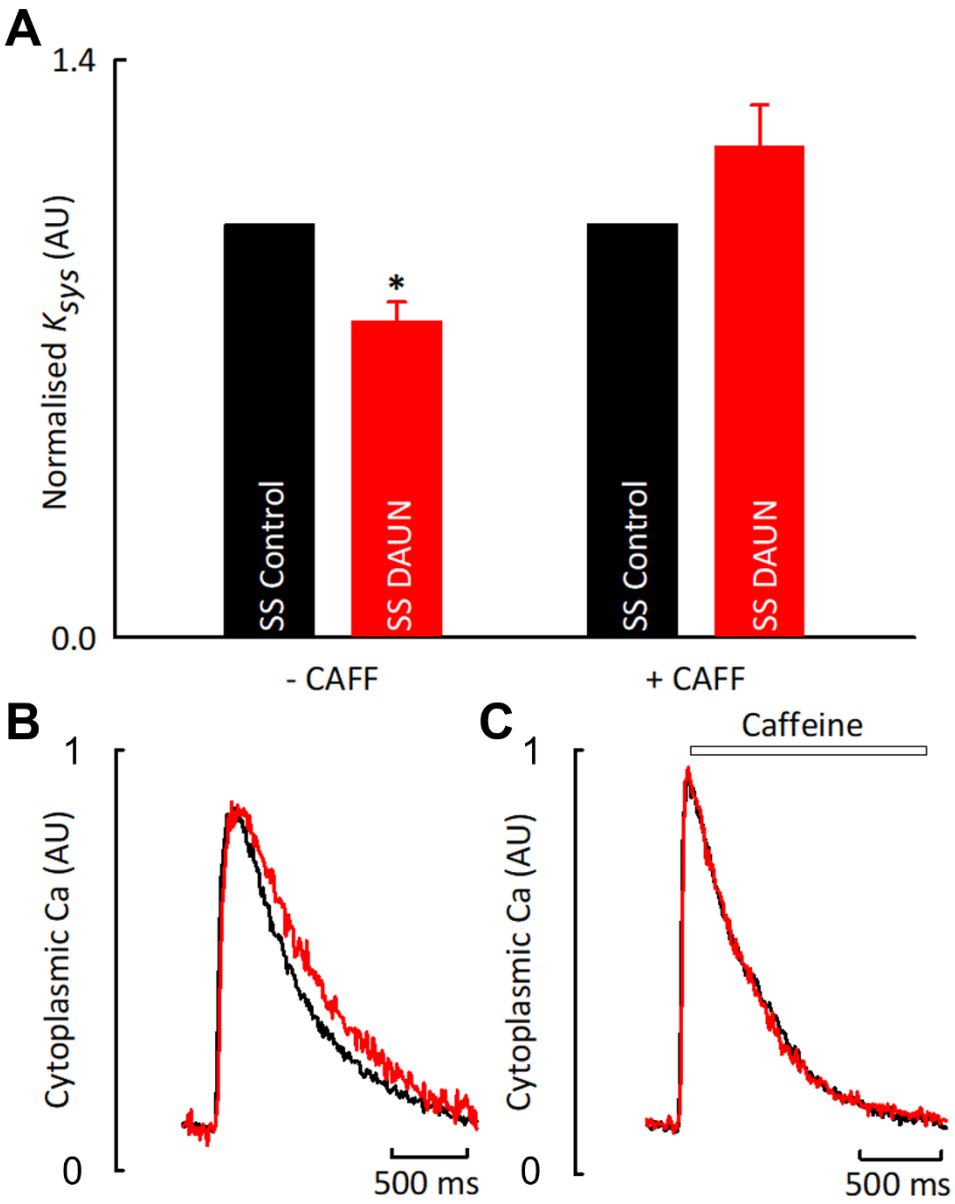


Figure 3.11: The effects of 1 nM DAUN on Ca removal in sheep ventricular myocytes. (A) mean normalised rate constant of decay in the presence and absence of caffeine. (B) specimen normalised Ca transients to permit direct comparison of the rate of Ca decay. (C) specimen normalised Ca transients to permit direct comparison of the rate of Ca decay in the presence of caffeine. n = 18 – 19 (from 6 animals), * denotes statistical significance.

3.3.8 The effects of doxorubicin and daunorubicin on myofilament sensitivity

Certain factors may reduce myofilament Ca sensitivity such as; acidosis, an increase in the concentration of free magnesium ([Mg]) and β-adrenergic stimulation (Bers, 2001b). Drugs have also been found to impact myofilament Ca sensitivity and alter the force-Ca relationship and so it is important to establish if anthracyclines alter the force-Ca relationship.

Figure 3.12A and C show representative phase-plane loops for cells treated with 1 nM DOX and DAUN. Phase-plane loops proceed counter-clockwise and plot the change of cytoplasmic Ca vs the change of sarcomere length in the absence and presence of DOX and DAUN. Both DOX and DAUN caused the loops to shift leftwards. This shift was quantified by measuring the intracellular Ca concentration required for half relaxation (EC_{50}). On average, DOX reduced EC_{50} by 6.12 % ± 1.9 % (n = 20, p = 0.026). DAUN reduced EC_{50} on average by 8.5 % ± 2.4 % (n = 20, p < 0.001). Furthermore, due to systolic effects reported in 3.3.2 and 3.3.4 both loops are compressed (fig 3.12 A&C). These observations suggest increased myofilament sensitivity due to anthracyclines.

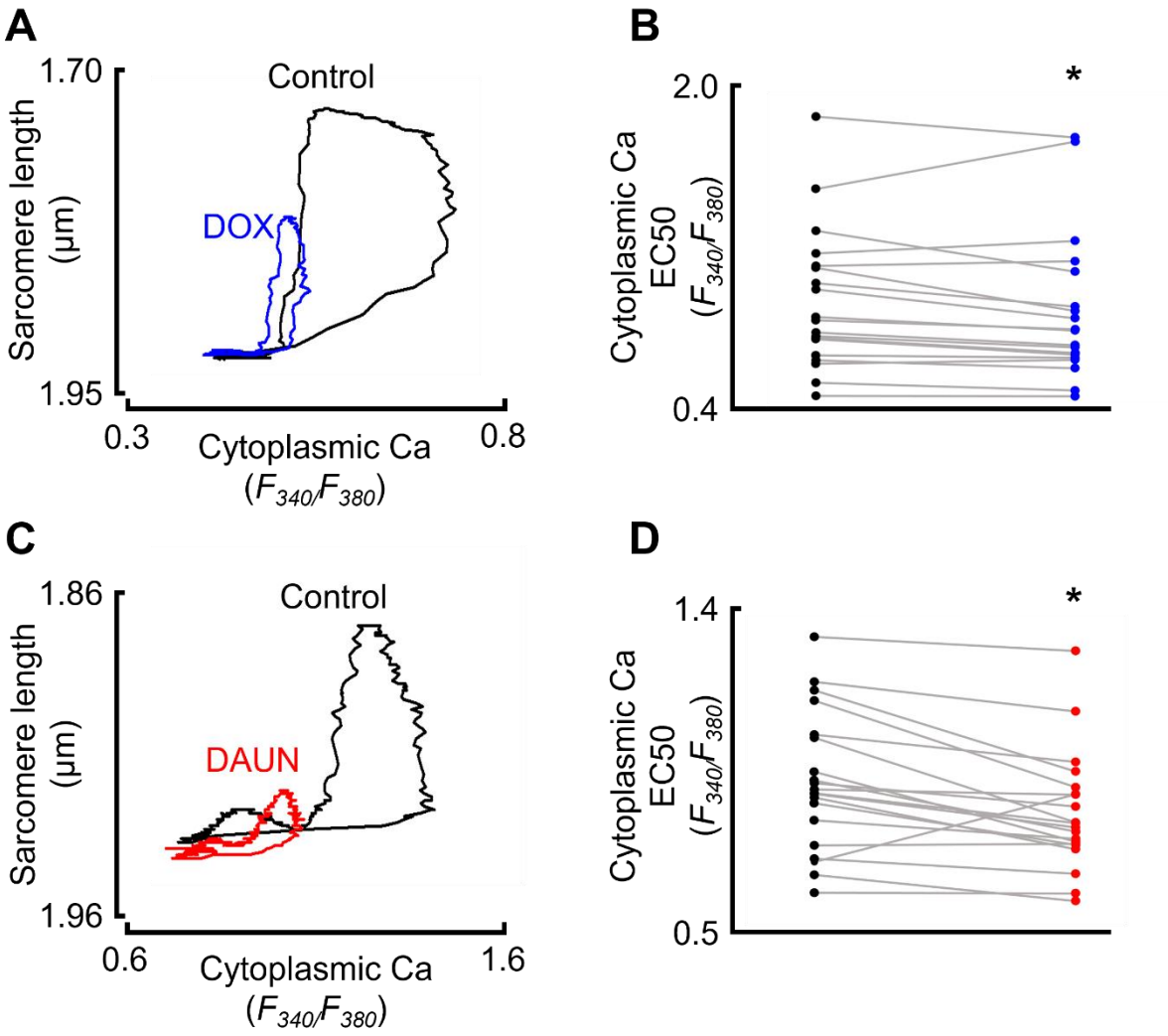


Figure 3.12: **The effect of DOX and DAUN on myofilament sensitivity in sheep left ventricular myocytes.** (A) phase-plane loop of sarcomere length versus cytoplasmic Ca when treated with 1nM DOX. (B) EC_{50} (cytoplasmic Ca at 50 % relaxation) of cells in NT compared to 1nM DOX treatment. (C) phase-plane loop of sarcomere length versus cytoplasmic Ca when treated with 1nM DAUN. (D) EC_{50} of cells in NT compared to 1 nM DAUN treatment. n = 20 (from 3 – 6 animals), * denotes statistical significance.

Further experiments were conducted to verify changes to myofilament sensitivity due to anthracycline treatment. As Ca concentration increases the resting sarcomere length reduces as shown in figure 3.13, with a significant shortening at 35 µM (n = 16, p = 0.036). 1 nM DAUN

resulted a significant reduction in sarcomere shortening at 3.5 and 35 µM (n = 18, p < 0.001) and was significantly shorter than the control at these concentrations (n = 16, p < 0.001) indicating an increase in myofilament sensitivity. 1 nM DOX did not cause a significant change.

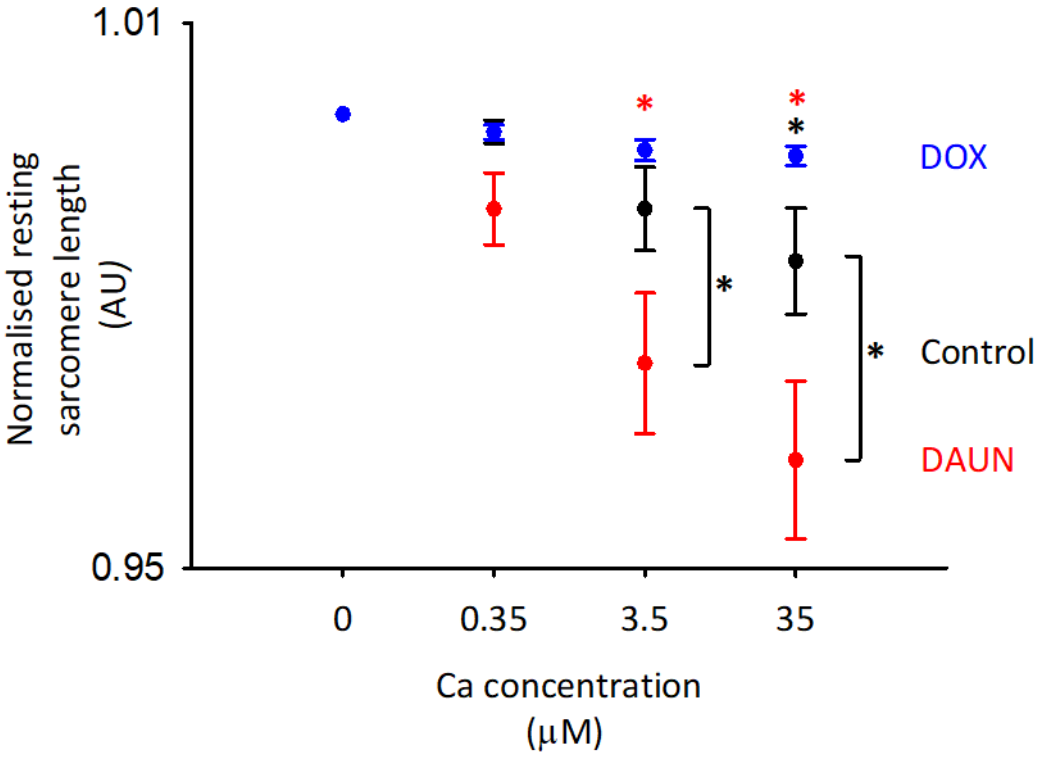


Figure 3.13: The effect of 1 nM DOX and DAUN on myofilament sensitivity. n = 15 (from 5 animals), * denotes statistical significance.

3.4 Discussion

3.4.1 What is an experimentally relevant anthracycline concentration?

Using clinically relevant concentrations for reductionist experiments like these is not useful. The concentrations administered clinically will not be the concentration present in the heart or in each cardiac myocyte. These experiments aim to elucidate mechanistic effects on anthracyclines at a cellular level. Therefore, a concentration that does not impact cell experimental suitability while allowing mechanisms to be clarified is required.

As shown in figure 3.2 as the concentration of DOX increases (0.001 µM to 10 µM), suitability reduces in primary cardiac myocytes. DOX elicits death in human cardiac myocytes within hours of administration (Unverferth *et al.*, 1983) supporting the rapid response seen in our experiments. In cultured human pluripotent stem cell derived cardiomyocytes cell viability was unaltered after a single 3-hour treatment with 150 nM and 300 nM DOX, however a reduction in viability was seen if this treatment was followed by washout (Louisse *et al.*, 2017). Our calcium handling experiments are short term (3 – 10 minutes) and include a wash out period. Maintaining viability and therefore suitability for the length of the experiment is important. A concentration that allows the identification of subtle mechanisms and alterations to Ca handling and contractility while maintaining viability is ideal.

To assist in choosing an appropriate concentration the dose-response of Ca handling and contractility was established. All aspects of calcium dynamics measured (diastolic, peak, transient amplitude, and rate constant of decay) were not altered when cells were treated with the different concentrations of DOX (0.001 – 10 µM; as shown in figure 3.3).

From this information we can deduce that since no dose-dependent effect was evident the lowest concentration that did not alter cell experimental suitability is suitable for experimental use (0.001 µM). While we cannot claim this concentration is clinically appropriate, given the reductionist nature of the experiments we can be confident that this concentration will elucidate mechanistic effects of anthracyclines that will aid in the knowledge of how these drugs impact the heart on a cellular level. Thereby providing valuable insight into the potential implications of these drugs on global Ca handling and contractility.

3.4.2 Does 1 nM doxorubicin alter intracellular Ca handling and contractility in sheep cardiomyocytes?

1 nM DOX did not alter diastolic Ca or sarcomere length in sheep ventricular myocytes (fig 3.4). In rat cardiomyocytes following a 30 minute treatment with 10 µM DOX an increase in

diastolic Ca was detected (Sag *et al.*, 2011) which was also found to be the case in acute exposure (1 minute) (Mijares & López, 2001). However, this acute study also found no change in diastolic Ca at 1 μM. In a mouse atrial cardiac myocyte cell line (HL-1) diastolic Ca was not altered by 5 μM DOX after 30 minutes, however after 60 minutes an increase was evident (Asensio-López *et al.*, 2016). These findings suggest that at higher concentrations of DOX or longer treatment times than those used in our studies an increase diastolic Ca occurs.

DOX resulted in a reduction in Ca amplitude which was accompanied by a decrease in sarcomere shortening, these findings are supported by other experiments conducted on alternative models using various methods. Sag *et al* (2011) saw a reduction in Ca amplitude due to DOX in rat cardiac myocytes which was also found to be the case by Zheng *et al* (2011). Zheng *et al* (2011) measured cell shortening separately and found this was also reduced. It is important to note that these experiments incubated the cardiomyocytes with DOX for a longer period; 30 minutes and 12 hours respectively and they did not investigate recovery after removal of the anthracycline. The time taken for Ca amplitude to reduce in sheep ventricular myocytes was rapid whereas experiments conducted on rats showed variation in the time taken to reduce Ca amplitude with some studies taking up to 3 hours (Timolati *et al.*, 2006). However, once reduced this observation was maintained for the period of exposure (18 hours) along with sarcomere shortening. In our experiments Ca amplitude partially recovered in wash and sarcomere shortening fully recovered. From these data it is evident that DOX reduces Ca amplitude and sarcomere shortening.

Peak Ca was also found to be reduced and partially recover in wash (fig 3.4). While Ca amplitude represents the relative change in Ca it is not an absolute level. A decrease in peak Ca confirms absolute systolic Ca levels – that which activates the myofilaments - are lower.

The rate of sarcomere relaxation (90 % - 10 %) was increased in the presence of DOX as shown in figure 3.5F, indicating a decrease in the rate of removal of Ca. This was investigated by quantifying the rate of Ca removal illustrated in figure 3.7B which was found to be reduced. These findings suggest that DOX reduces the rate of removal of Ca which in turn increases the time taken for the sarcomere to relax (Bers, 2001a).

As discussed in 1.2.8.2, a major regulator of systolic Ca Is SR Ca content (Trafford *et al.*, 2000). Therefore, it is logical that SR Ca content may be impacted by DOX. The reduction in the rate of Ca removal and increase in rate of sarcomere relaxation indicates an alteration in the function of the main removal mechanisms, SERCA and NCX (see 1.2.9.4). Therefore, it is important to investigate whether SR Ca content and ion transporter activity are altered due to DOX.

3.4.3 Is SR Ca altered by 1 nM doxorubicin?

In our experiments, DOX decreased SR Ca content (fig 3.6A). The Ca transient amplitude is proportional to the third power of SR Ca (Dibb *et al.*, 2007). DOX reduced SR Ca content to 67 % of control. This would be expected to decrease the Ca transient amplitude to $(0.67)^3$ = 30 %. The Ca transient amplitude only decreased to 61 %, indicating that the decreased SR Ca can account for the change in Ca transient amplitude. However, we do not know if DOX alters Ca buffering and so we cannot quantify the absolute change in SR Ca content (Trafford *et al.*, 1999b). DOX was found to decrease SR Ca content in canine SR vesicle and primary rat cardiac myocytes (Ondrias *et al.*, 1990; Sag *et al.*, 2011), supporting the findings in sheep ventricular myocytes.

3.4.4 Does doxorubicin alter the activity of Ca removal mechanisms?

We next investigated the cause of reduction in SR Ca content. As shown in figure 3.7 DOX increased NCX activity (k_{caff}) and decreased SERCA activity (k_{SERCA}) which can account for the decrease in SR Ca content (Eisner, 2014). While SERCA activity is restored upon washout, NCX recovery is partial. This, and that recent work highlights a relatively small dependence of SR Ca on SERCA activity (Bode *et al.*, 2011) may account for the lack of recovery of SR Ca content during washout. It also supports the suggestion that NCX plays a key role in the regulation of SR Ca content, which is apparently the case here. SERCA and NCX activity are integral to SR regulation but have not been directly quantified.

Long term studies found a downregulation in SERCA expression as well as SR leak leading to a decrease in SR Ca content (Altomare *et al.*, 2021; Timolati *et al.*, 2006). Our findings show the effect of DOX on SERCA and NCX to be rapid (3- 10 minutes) indicating a direct effect of DOX on these channels as protein expression would not be altered. Studies report that DOX activates Ca/calmodulin protein kinase II (CaMKII) (Ikeda *et al.*, 2019b; Tscheschner *et al.*, 2019) which phosphorylates phospholamban and promotes reuptake of SR Ca via SERCA, while also phosphorylating RyR and promoting SR Ca leakage (Yuchi *et al.*, 2012). The roles of CaMKII are vast but its activation would suggest an increase in SERCA activity which is not the case in our study.

With regards to direct interaction with SERCA Hanna *et al* (2014) found SERCA to be inhibited by the metabolite of DOX but not DOX itself. However, DOX was able to bind directly to SERCA. As these findings were not carried out in intact cardiomyocytes it stands to reason that this direct interaction may alter SERCA activity, and result in a decrease in Ca uptake into the SR via SERCA. It is also possible that anthracyclines do not impact SERCA through direct binding but by the production of reactive oxygen species. As mentioned in section 1.5.2.2 anthracyclines have been shown to increase OS. Greensmith *et al* (2010) have shown hydrogen peroxide which is a ROS results in a reduction in SERCA activity which supports this theory.

DOX treatment results in an increase in NCX activity (fig 3.7C). While most studies focus on the impact of DOX on SERCA and RyR few investigate the impact on NCX. The expression of NCX is dependent on CaMKII via stimulation of β-adrenergic receptor (Mani *et al.*, 2010). A study in mice found correlation between increased activity of CaMKII and NCX current in heart failure (Xu *et al.*, 2012). In turn if CaMKII is activated via DOX, this may then result in an increase in NCX current as found by Xu *et al* (2012). This however would only occur after long term exposure. It may be the effect on NCX is due to ROS production as ROS has been shown to increase NCX activity (Goldhaber, 1996; Reeves *et al.*, 1986).

In summary, we are the first to provide mechanistic explanation for decreased SR Ca content by showing that DOX decreased SERCA activity while increasing NCX activity.

3.4.5 Does 1 nM daunorubicin alter intracellular Ca handling and contractility in sheep cardiomyocytes?

There is very little literature regarding the overall impact of daunorubicin on Ca handling and contractility in cardiac myocytes. As mentioned in 3.4.5 there is information regarding the anthracyclines impact on individual channels and the effect on SR Ca, but we are the first to explore the effect of DAUN on global intracellular Ca and contractility.

DAUN did not alter diastolic Ca (fig 3.9A) however the change to diastolic sarcomere length although minor at an increase of 0.32 % was significant. This increase may not be physiologically important as it is < 1 %. A reversible decrease in Ca amplitude was accompanied by a reversible decrease in sarcomere shortening. This is as expected as explained by the force-calcium relationship in section 1.2.9.1. DOX caused an increase in sarcomere relaxation indicating a decrease Ca removal.

3.4.6 Is SR Ca altered by 1 nM daunorubicin?

The caffeine-evoked Ca transient amplitude was decreased by DAUN (fig 3.10). Ca transient amplitude is proportional to the third power of SR Ca content (Dibb *et al.*, 2007). DAUN reduced SR Ca content to 86 % of control. This would be expected to decrease Ca transient to $(0.86)^3$ = 64 %. The Ca transient decreased to 55 % indicating that a decrease in SR Ca content can account for this reduction.

However, it was noted that k_{sys} was altered in the presence of caffeine and DAUN suggesting overall Ca removal is impacted by this interaction therefore impacting the caffeine-evoked Ca amplitude detected. Given the magnitude of the change in the caffeine-evoked Ca transient amplitude it is highly likely that SR Ca content is in fact reduced however this is a relative

change. In canine cardiac SR vesicle nanomolar ranges of DAUN inhibited SR Ca release and prevented caffeine-induced SR Ca release (Olson *et al.*, 2000). This suggests DAUN has an inhibitory effect on RyR2. Reduction in the open probability of RyR2 (activity) was identified by treatment with DAUN at 2.5 µM for 10 minutes (Hanna *et al.*, 2011a). Within the same study a concentration < 2.5 µM for less than 10 minutes increased the open probability of RyR (activity).

3.4.7 Does daunorubicin alter the activity of Ca removal mechanisms?

It was noted that caffeine exposure in combination with DAUN treatment resulted in no change in the rate constant of as shown in figure 3.11. However, in experiments conducted without caffeine DAUN resulted in a decrease in k_{sys}. Due to this apparent interaction the removal of Ca was unable to be investigated for DAUN.

Interestingly, a study conducted using rabbit skeletal SR vesicles identified that anthraquinones can bind to ryanodine receptors at a different site to caffeine and showed non-competitive interaction (Abramson *et al.*, 1988). This study investigated daunorubicin-ryanodine binding and found binding to be reduced in the presence of caffeine. Caffeine was also shown to inhibit daunorubicin-stimulated SR Ca release but resolves upon washout. Given that our results only found an apparent interaction between DAUN and caffeine and not DOX and caffeine it may be that a different interaction occurs with DOX. More chemical research is required to understand this.

The activity of SERCA needs to be considered when investigating SR Ca content. If the mechanism of action is similar to that of DOX, we would expect a decrease in SERCA activity. This theory is supported by a study in cardiac myocytes isolated from rabbits that had been treated with DAUN for 2 weeks as an inhibition in Ca uptake into the SR was observed (Cusack *et al.*, 1993) indicating a reduction in SERCA activity. While a decrease in SERCA may contribute to a reduction in SR Ca content, the effect of DAUN on NCX is unknown. Given both

SERCA and NCX massively contribute to Ca removal in mammalians both would need to be considered to account for a decrease in SR Ca content.

3.4.8 Do doxorubicin and daunorubicin alter myofilament sensitivity?

By plotting phase-plane loops it was determined that the EC_{50} for cytoplasmic Ca at 50 % relaxation for DOX and DAUN was significantly decreased. This infers that less calcium is required to cause the same change in sarcomere length indicating an increase in myofilament sensitivity highlighted by a shift of the force versus Ca amplitude relationship shown in figure 1.8 to the left.

Further experiments shown in figure 3.11 confirmed that DAUN increased myofilament sensitivity ($n = 16$, $p < 0.001$) whereas the results for DOX were inconclusive given no change in sarcomere length at different Ca concentrations was detected. This increase in myofilament sensitivity would (for all things being equal) likely offsetting the effect of the decrease in Ca amplitude on sarcomere shortening. It would be expected that sarcomere shortening would be altered to a greater degree for any given change in Ca. An increase in myofilament sensitivity leads to a rise in force of contraction due to an increase in sensitivity to Ca. This may be due to an increased affinity for troponin C to Ca or increase in the effectiveness of this attachment (Lee & Allen, 1997). For example, the Ca sensitiser levosimendan increases myofilament sensitivity by binding to both the N-terminal and C-terminal of cardiac troponin C, therefore stabilising the complex and increasing the force of contraction (Haikala *et al.*, 1995).

Permeabilised rabbit cardiac myocytes treated with DOX did not alter the Ca concentration-tension relationship (Boucek *et al.*, 1993), which suggests DOX does not alter myofilament sensitivity. Whereas, in male rats exposed to DOX over 6 weeks a decrease in myofilament sensitivity was detected (Chakouri *et al.*, 2020). Interestingly, there may be a gender difference in the effect of DOX on myofilament sensitivity. An increase in myofilament

sensitivity was detected in female rats treated with DOX over 10 weeks however no change was found in male rats (Rattanasopa *et al.*, 2019). The same study showed that in the absence of testosterone myofilament sensitivity was increased.

To the best of our knowledge there is no literature identifying the impact of DAUN on myofilament sensitivity.

As discussed in 1.5.2.2 anthracyclines increase ROS. This production of ROS may impact myofilament sensitivity. Hydroxyl radicals lead to a leftward shift in the force-calcium relationship indicating an increase in myofilament sensitivity in a rabbit model (Haizlip *et al.*, 2012). These data along with the data shown in figure 3.13 suggest that DAUN and DOX increase myofilament sensitivity.

3.5 Concluding remarks

In conclusion, this study has elucidated the mechanistic effects of DOX and DAUN on intracellular Ca and contractility in cardiac myocytes. The autoregulation of the cardiomyocyte appears to be altered as a reduction in SR Ca content would normally result in an increase in SERCA activity and reduction in NCX, however the opposite is shown in our findings with DOX. Our findings also highlight differences between DOX and DAUN with DAUN showing a potential caffeine interaction suggesting changes in protein interactions between the two anthracyclines. Furthermore, myofilament sensitivity is increased by both DOX and DAUN.

It is unknown if the impact of DOX and DAUN on Ca handling and contractility is a direct interaction or due to the production of reactive oxygen species (potentially both). This requires further investigation.

Chapter 4
Measurement of anthracycline-induced oxidative stress in sheep ventricular myocytes

4.1 Introduction

As discussed in 1.4 oxidative stress is a state in which the production of ROS outweighs the cells antioxidant mechanisms and is implicated in various cardiovascular diseases (Burton & Jauniaux, 2011). Anthracyclines are known to induce ROS in cancer cells as a mechanism of cytotoxicity as discussed in 1.5.2.2 (Attanasio *et al.*, 2021; Doroshow, 2019; Sheibani *et al.*, 2022). The increase in OS in the heart may contribute to cardiotoxicity.

4.1.1 ROS production in cardiac myocytes

ROS play a role in normal signalling within the heart and are involved in normal excitation-contraction coupling, however they are also implicated in cardiovascular disease (Burgoyne *et al.*, 2012). There are several sources of ROS within cardiomyocytes and examples include; the mitochondrial electron transport chain, NADPH oxidases (NOX) (specifically NOX2 and NOX4) and xanthine oxidoreductase (XO) (Akki *et al.*, 2009).

The mitochondrial electron transport chain consists of four enzymatic complexes and is a main source of physiological ROS (Peoples *et al.*, 2019). This is due to a leakage of electrons (2 -5 %) into the cytoplasm resulting into the formation of superoxides (Kagan *et al.*, 2009). ROS may then leak out of the mitochondria and lead to ROS-induced ROS release from neighbouring mitochondria (Zorov *et al.*, 2006).

Activation of enzymes may induce ROS production. NADPH oxidases are one of the main sources of ROS in the heart (Gray *et al.*, 2019) with NOX2 and NOX4 being the main isoforms expressed (Nabeebaccus *et al.*, 2011). NOX4 is primarily expressed in the mitochondria in cardiac myocytes (Kuroda *et al.*, 2010) whereas NOX2 is localised in the plasma membrane and cytosol (Krijnen *et al.*, 2003). Another enzyme involved in the production of super oxide species and hydrogen peroxide are xanthine oxidases. ROS are produced through a series of reactions in the process of converting xanthine to uric acid (Berry & Hare, 2004a). The

production of ROS via xanthine oxidase is particularly implicated in heart failure (Hajjar & Leopold, 2006).

All these processes contribute to ROS production in cardiac myocytes and this production may be altered in disease states or by drugs.

4.1.2 Anthracyclines and ROS

As discussed in 1.5.2.2 anthracyclines increase ROS in cancer cells and this contributes to their mechanism of cytotoxicity therefore it is plausible that this also occurs in cardiac cells. Anthracyclines such as DOX may lead to semi-quinone production by reduction via xanthine oxidase (Bates & Winterbourn, 1982). This redox cycling of anthracyclines produces various ROS such as hydrogen peroxide (Menna *et al.*, 2010) which may then create an imbalance resulting in oxidative stress in cardiac myocytes. This reaction may also occur due to NOX and by the mitochondrial electron transport chain (Huang *et al.*, 2022). In isolated cardiomyocytes DOX was shown to accumulate in the mitochondria (Ichikawa *et al.*, 2014b) suggesting that NOX4 and/or the mitochondrial transport chain may be involved in the reduction reaction of DOX that produces ROS given the localisation of both the drug and enzyme.

An imbalance in ROS and antioxidant properties of a cell results in oxidative stress and may impact calcium handling within cardiac cells.

4.1.3 Oxidative stress and calcium handling

The modulation of ECC by OS underpins many cardiovascular diseases (Dubois-Deruy *et al.*, 2020; Moris *et al.*, 2017b; Peoples *et al.*, 2019). ROS are known to perturb Ca handling and contractility (D'Oria *et al.*, 2020). This is due a wide range of effects on ECC components.

ROS have been shown to reduce Ca transient amplitude and contractility (Goldhaber & Liu, 1994; Greensmith *et al.*, 2010; Kuster *et al.*, 2010). This decrease in amplitude can be explained by a reduction in SR Ca content (Boraso & Williams, 1994; Greensmith *et al.*, 2010;

Kuster *et al.*, 2010). ROS leads to a decrease in SERCA activity (Greensmith *et al.*, 2010; Kuster *et al.*, 2010; Morris & Sulakhe, 1997; Reeves *et al.*, 1986; Scherer & Deamer, 1986) and an increase in NCX activity (Eigel *et al.*, 2004; Goldhaber, 1996; Kuster *et al.*, 2010; Reeves *et al.*, 1986) which can account for the reduction in SR Ca content. Furthermore, the open probability of RyR has been shown to be increased by ROS (Anzai *et al.*, 1998; Boraso & Williams, 1994; Kawakami & Okabe, 1998) and the voltage gated Ca current reduced (Anzai *et al.*, 1998; Boraso & Williams, 1994; Kawakami & Okabe, 1998). Combined these findings identify the impacts of ROS on ECC components in a wide range of models and provide vital insight into the mechanisms by which Ca amplitude and contractility are reduced.

ROS clearly has implications on calcium handling and contractility. Therefore, it is logical to suggest that the anthracycline-induced ROS may directly lead to Ca handling and contractility dysfunction.

4.1.4 Antioxidants

Cardiac myocytes are highly metabolically active cells and so contain many mitochondria biogenesis (Bartz *et al.*, 2015). As discussed in 4.1.1 the mitochondrial electron transport chain produces superoxides and NOX4 is expressed in the mitochondria and contribute to mitochondrial ROS. Intracellular antioxidant defence mechanisms exist to cope with ROS production such as glutathione peroxidases and catalase as discussed in 1.4.1.2.

The heart has lower levels of antioxidant enzymes compared to other organs (Costa *et al.*, 2013) with catalase activity being particularly lower in the heart (Thayer, 1986). Under normal physiological conditions the coping mechanisms within the heart are adequate to manage ROS production, however in states of pathology such as increased ROS production the antioxidant defences may not be sufficient.

The susceptibility of cardiac myocytes to pathological ROS production may account for anthracycline-induced cardiotoxicity if ROS is increased. This increase in ROS is more likely to result in oxidative stress in the heart and result in dysregulation of Ca handling.
The imbalance in ROS to coping mechanisms of a cell may be altered by introducing antioxidants. This is also a useful tool to highlight the role of ROS on cellular dysfunction such as that seen in Ca handling and contractility.

N-acetyl cysteine (NAC) has long been considered a broad antioxidant as it maintains total antioxidant capacity (ROS scavenging) and increases glutathione levels (Mahmoudinezhad *et al.*, 2023). Furthermore, emerging evidence suggests that NAC may work by increasing sulfane sulphur inside cells leading to an increase in the oxidant scavenging capacity of the cell, as well as providing protection against irreversible oxidative damage (Pedre *et al.*, 2021). NAC has been used in various studies as a tool to reduce ROS in primary and cultured cardiac myocytes at a range of concentrations and incubation times (0.1 - 4mM) (Chen *et al.*, 2013; Chen *et al.*, 2012b; Liu *et al.*, 2019). It is a well-established tool and is therefore useful as a generic scavenger of ROS.

4.1.5 Aims

- Validate the novel methodology for measurement of oxidative stress in cardiac myocytes.
- Determine the effect of anthracyclines on levels of oxidative stress in cardiac myocytes.
- Establish to what extent anthracycline-induced oxidative stress are responsible for the alterations in Ca handling and contractility.

4.2 Methodology

4.2.2 Cell preparation

Primary left ventricular cardiac myocytes were isolated from young healthy sheep as described in section 2.1.2 and stored in a solution produced by mixing NT and taurine (see 2.1.1) solution in equal parts to produce an intermediate Ca concentration of 0.95 mM.

4.2.3 Oxidative stress measurements in cardiac myocytes

4.2.3.1 Preparation of cells to measure oxidative stress

As discussed in 1.4.4 existing methods used to measure oxidative stress may be unsuitable for use with cardiac myocytes. For example, the size of the cells and the inability to distinguish live from dead poses an issue. To overcome this a fluorophore compatible with live cells and a live imaging microscope was utilised (see section 2.9 for detail). Primary left ventricular myocytes were exposed to DOX or DAUN (0.001, 0.01, 0.1, 1 and 10 µM) for 30 minutes before loading with CellROX red (final concentration 5 µM) for 30 minutes. Cells were then washed and resuspended in NT solution (see table 2.3).

In some experiments, cells were pre-incubated with N-acetyl cysteine (NAC). NAC was prepared by dissolving NAC in sodium hydroxide and pH was readjusted to 7.34 before volumizing using NT. This was then further diluted to 1mM for use on the cells for 30 minutes. To investigate the time-dependent effects (fig 4.3) cells were exposed to DOX (with and without 1 mM NAC pre-treatment) for the period stated prior to loading with 5 µM CellROX deep red.

4.2.3.2 Measurement of oxidative stress in cardiac myocytes

As discussed in 2.9.2, using the Biotek cytation imaging system brightfield and fluorescent images of cells were captured. The Texas red filter cube was utilised at x 10 magnification.

Analysis was conducted using imageJ software. Brightfield images were overlayed on to the corresponding fluorescence images to identify viable cells. As discussed in 2.5.2 fluorescence was quantified and the background subtracted.

4.2.4 Excitation-contraction coupling measurements.

Cells were loaded with fura-2 (see section 2.6.2) after pre-treatment with 1mM NAC. Field stimulation at 0.5 Hz was used to pace cells as described in section 2.2.4. Cells were exposed to NT and a steady state obtained prior to exposure to 1 nM DOX. Once a new steady state was reached, the reversibility of any effect was determined with a washout (NT). Original recordings were calibrated and analysed offline using custom software (see 2.8).

4.2.5 Sarcoplasmic reticulum Ca content

Comparison of Ca transients evoked by rapid application of 10 mM caffeine allowed changes in SR Ca content to be estimated. Elements of ECC were measured as described in 3.2.5.
Statistical significance was determined by conducting a one-way ANOVA unless otherwise stated.

4.3 Results

4.3.1 Validation of method sensitivity

CellROX deep red reagent allows detection of OS in the cell cytoplasm (see 2.9.1). To be useful, the method must be sensitive enough to detect and discriminate pathophysiological levels of oxidative stress. Therefore, cells were exposed to sub-lethal concentrations of hydrogen peroxide. Hydrogen peroxide produced a significant, concentration-dependent increase in fluorescence (thus oxidative stress) reaching 83 % ± 17 % at 200 µM ($n = 6$, $p < 0.001$) (fig4.1B).

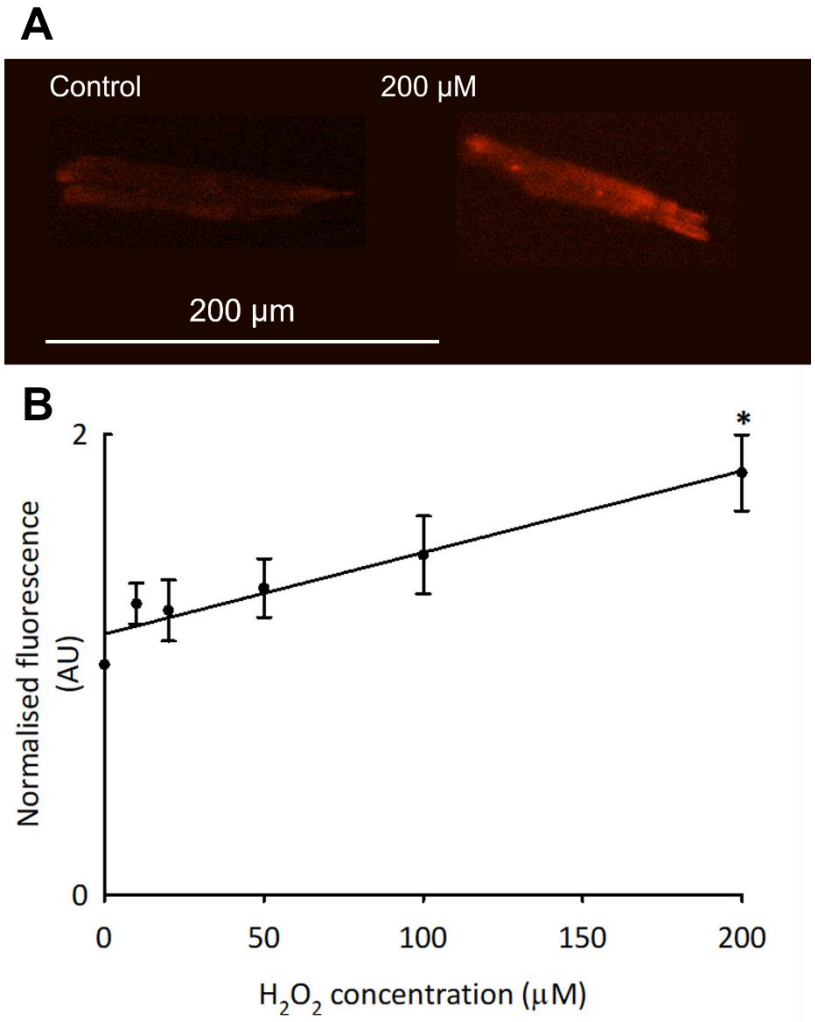


Figure 4.1: ROS detected from sheep ventricular cardiac myocytes treated with various concentrations of hydrogen peroxide (10, 20, 50, 100 and 200 µM). (A) specimen fluorescent image of cardiac myocytes in control and 200 µM hydrogen peroxide treatment. (B) mean

normalised fluorescence of cardiac myocytes treated with hydrogen peroxide. n = 12-32 cells from 4 animals, * denotes statistical significance.

4.3.2 The effect of DOX and DAUN on cytoplasmic oxidative stress

Initial experiments aimed to determine if anthracyclines increase oxidative stress in a concentration dependent manner. DAUN only produced a significant increase in fluorescence at the highest concentration of 10 µM; 86 ± 6 % (n = 4, p = 0.005; fig 4.2C). Incubation with DOX for 30 minutes produced a concentration-dependent increase in oxidative stress reaching with a significant increase of 52% ± 11 % (n = 6, p = 0.008; fig 4.2D) and 63.2 % ± 16.1 % (n = 5, p = 0.002; fig 4.2D) at 1 and 10 µM respectively.

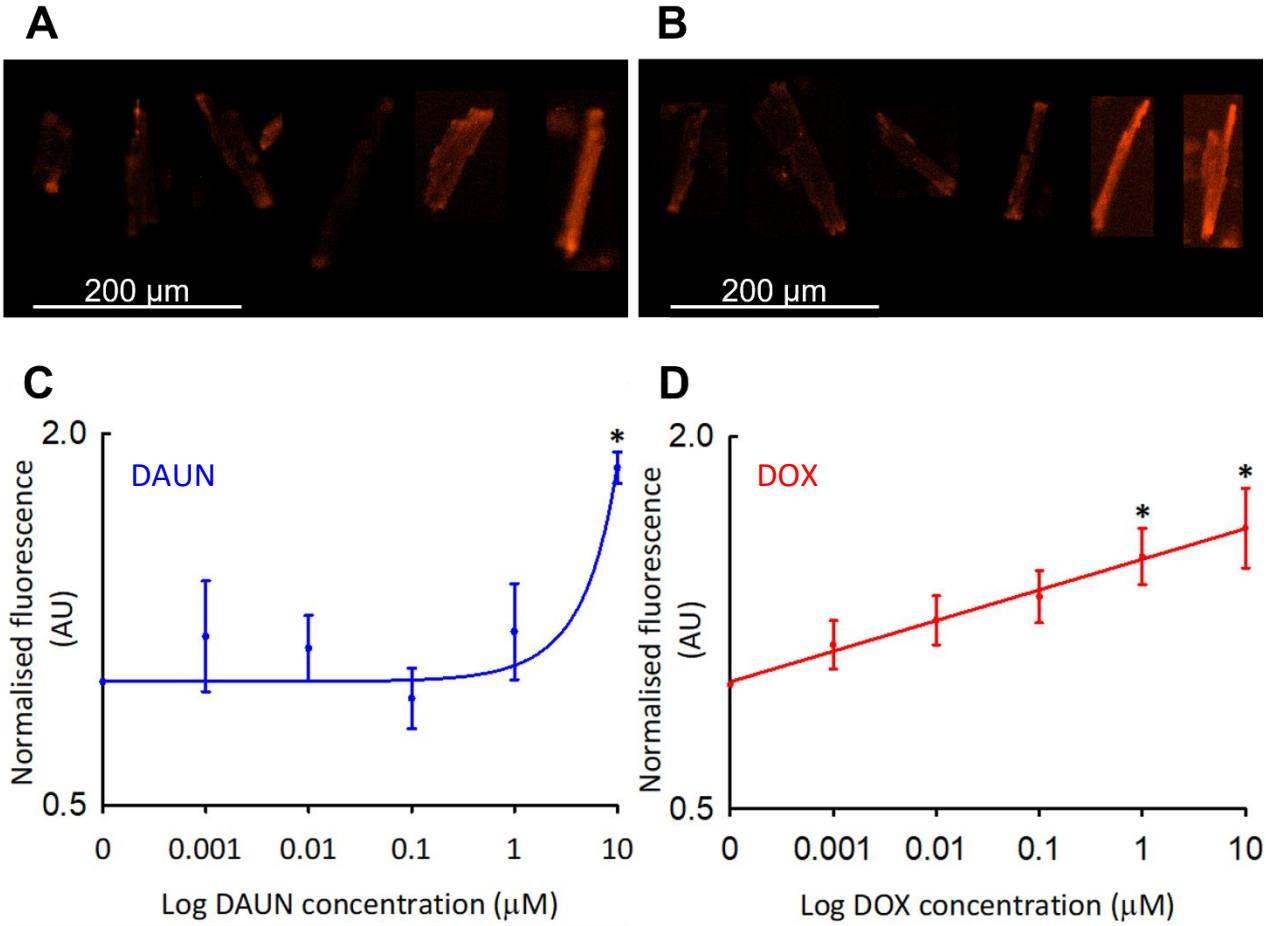


Figure 4.2: The effect of DOX and DAUN on OS production in sheep ventricular myocytes. (A) specimen fluorescent image of cardiac myocytes treated with various concentrations of DAUN (0, 0.001, 0.01, 0.1, 1 and 10 µM). (B) specimen fluorescent image of cardiac myocytes

treated with various concentrations of DOX (0, 0.001, 0.01, 0.1, 1 and 10 µM). (C) OS detected in cardiac myocytes when treated with varying concentrations of DAUN for 30 minutes (n = 29-39 cells from 4 animals). (D) OS detected in cardiac myocytes when treated with varying concentrations of DOX for 30 minutes (n = 41-53 cells from 5 animals), * denotes statistical significance.

4.3.3 The attenuation of DOX-induced OS by N-acetyl cysteine pre-treatment

The reduction of OS via 1 mM NAC and the time dependent effect of DOX was investigated as shown in figure 4.3. These experiments sought to (1) provide further validation that the increase in fluorescence due to DOX was indeed due to OS and (2) to ensure that NAC is suitable to reduce DOX-induced OS for future experiments (see 4.3.4 and 4.3.5).

No change in ROS was detected when cardiac myocytes were pre-treated with 1 mM NAC at any time point (n = 3, $p > 0.05$; fig 4.3). 0.1 µM DOX resulted in a significant increase in OS of 47.42 % ± 12.43 % at 120 minutes (n = 10, $p = 0.012$) and 49.97 % ± 12.54 % at 240 minutes (n = 10, $p = 0.007$). Furthermore, NAC pre-treatment resulted in significantly lower OS by 36.57 % at 240 minutes (n = 3, $p = 0.018$).

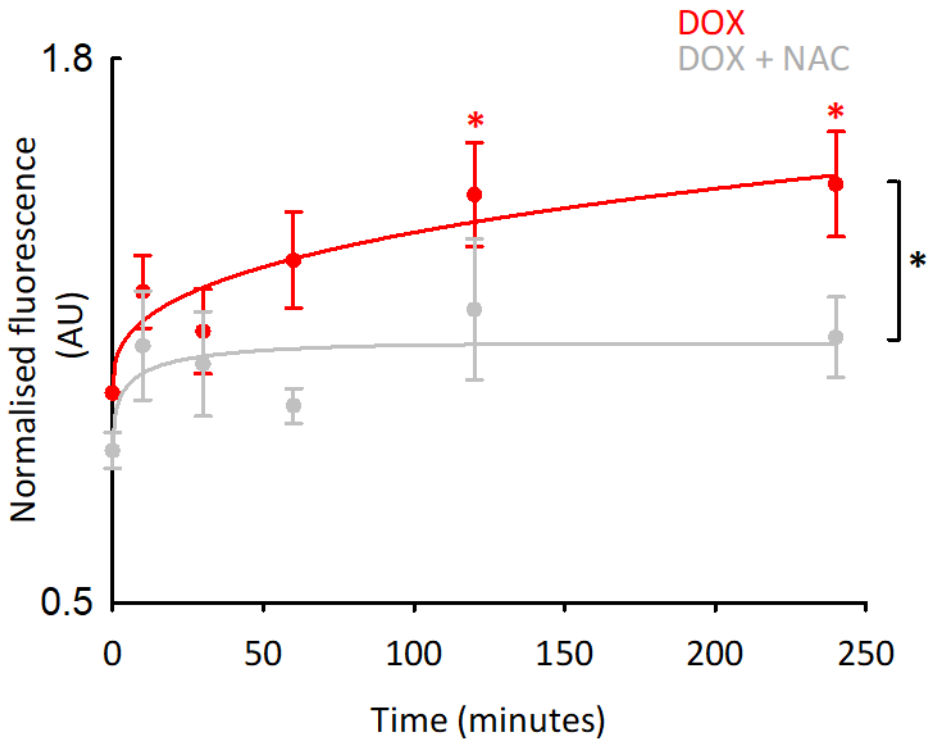


Figure 4.3: The time dependent effect of 0.1 µM DOX on ROS production in sheep ventricular myocytes with and without 1 mM NAC pre-treatment. Significant results were determined using a two-way ANOVA. n = 15-46 cells from 4-8 animals, * denotes statistical significance.

4.3.4 The effects of DOX on global Ca and contractility following pre-treatment with NAC

The results presented in chapter 3 show DOX alters aspects of ECC in cardiac myocytes. To determine the extent to which those changes are dependent on oxidative stress, the experiments were repeated in the presence of NAC.

The specimen traces in figure 4.4 show the effect of 1 nM DOX on intracellular Ca and contractility in cells pre-treated with 1 mM NAC. On average, no significant effect on diastolic Ca (n = 28, p = 0.181; fig 4.5A) or diastolic sarcomere length (n = 28, p = 0.256; fig 4.5B) was

observed. However, Ca transient amplitude reduced by 18.95 % ± 2.77 % (n = 29, p < 0.001) and did not recover in wash (n = 27, p < 0.001; fig 4.5C). This effect was accompanied with a decrease in systolic sarcomere shortening by 17.54 % ± 5.63 % (n = 29, p = 0.007; fig 4.5D) which reversed upon washout (n = 28, p = 0.267). On average, peak Ca decreased by 8.94 ± 1.63 % (n =29, p < 0.001) which did not recover upon washout (n = 28, p < 0.001; fig 4.5E). On average, no significant effect on sarcomere relaxation was observed (n = 27, p = 0.287).

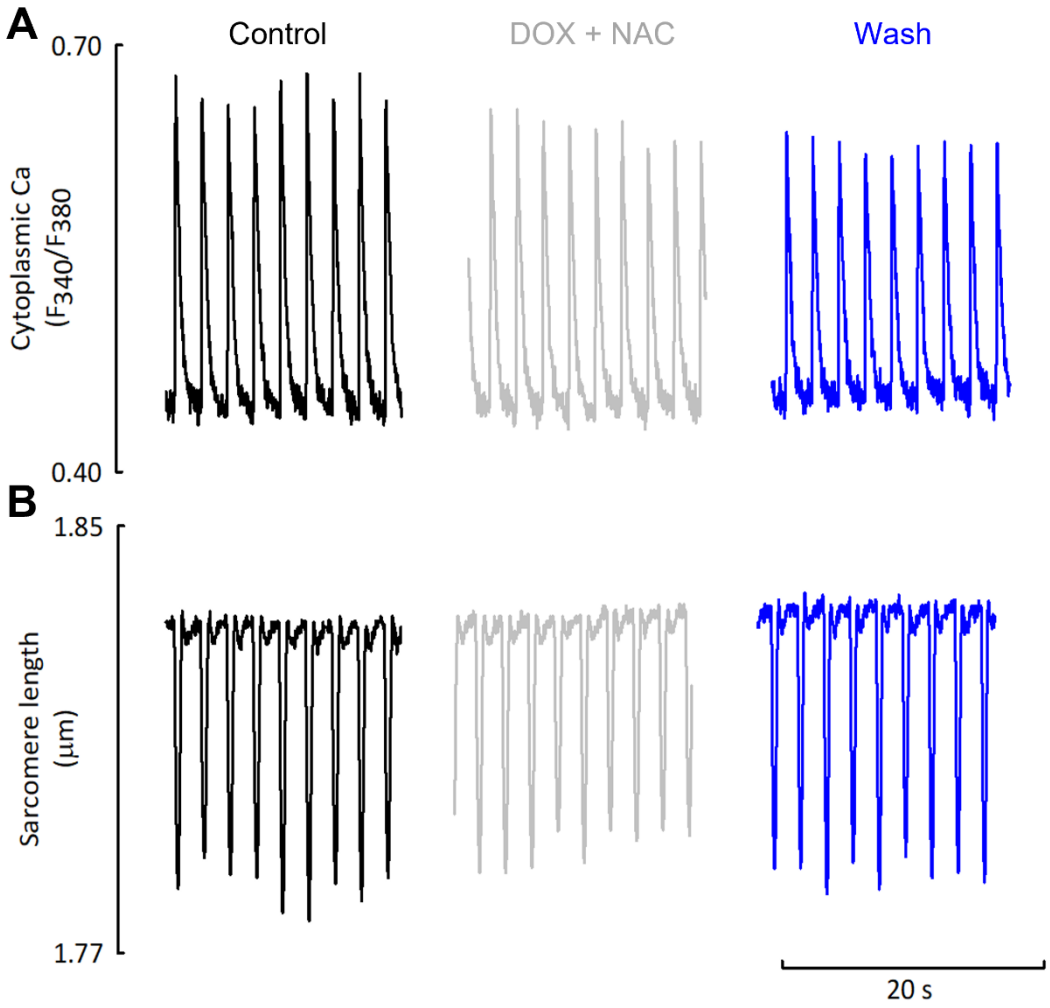


Figure 4.4: Specimen traces of the effect of 1 mM NAC pre-treatment along with 1 nM DOX treatment on cytoplasmic Ca and contractility in sheep ventricular myocytes. (A) specimen trace of Ca transients. (B) specimen trace of sarcomere length.

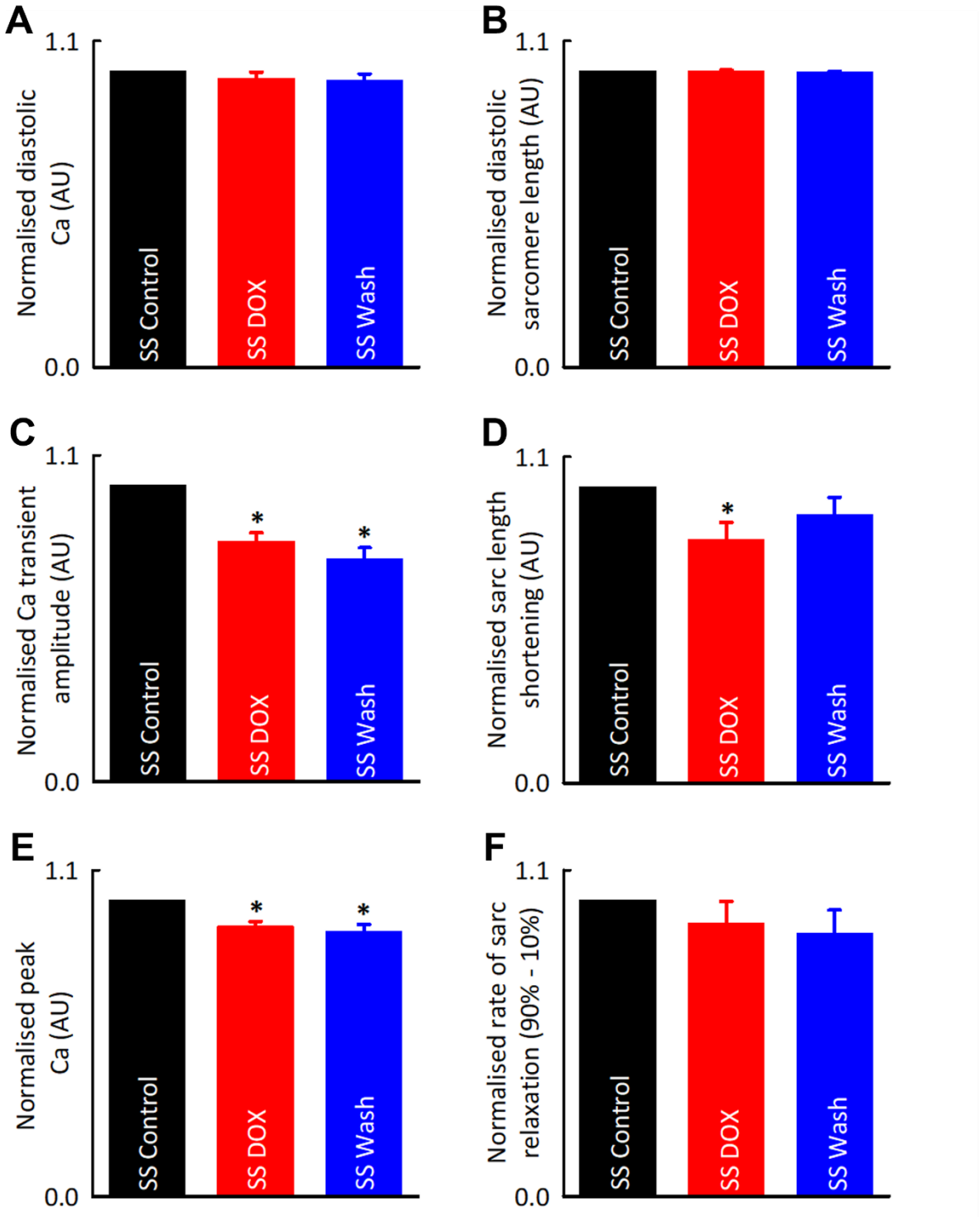


Figure 4.5: The effect of 1 mM NAC pre-treatment on Ca handling and contractility in sheep ventricular myocytes when treated with 1 nM DOX. (A) mean normalised diastolic Ca. (B) mean normalised diastolic sarcomere length. (C) mean normalised Ca transient amplitude.

(D) mean normalised sarcomere shortening. (E) mean normalised peak Ca. (F) mean normalised 90-10 % decay time of sarcomere length. n = 27 (from 5 animals), * denotes statistical significance.

4.3.5 The dependence of DOX-mediated changes to global Ca and contractility on oxidative stress

Fig 4.6 shows that pre-treatment with 1 mM NAC significantly attenuated the effects of DOX on both Ca transient amplitude by 52 % ($n = 29$, $p < 0.001$; fig 4.6A) and sarcomere length shortening by 58 % ($n = 29$, $p < 0.001$; fig 4.6B). NAC pre-treatment also significantly reduced the effect on peak Ca by 68 % ($n = 29$, $p < 0.001$; fig 4.6C) and rate of sarcomere relaxation by 120 % ($n = 28$, $p < 0.001$; fig 4.6D).

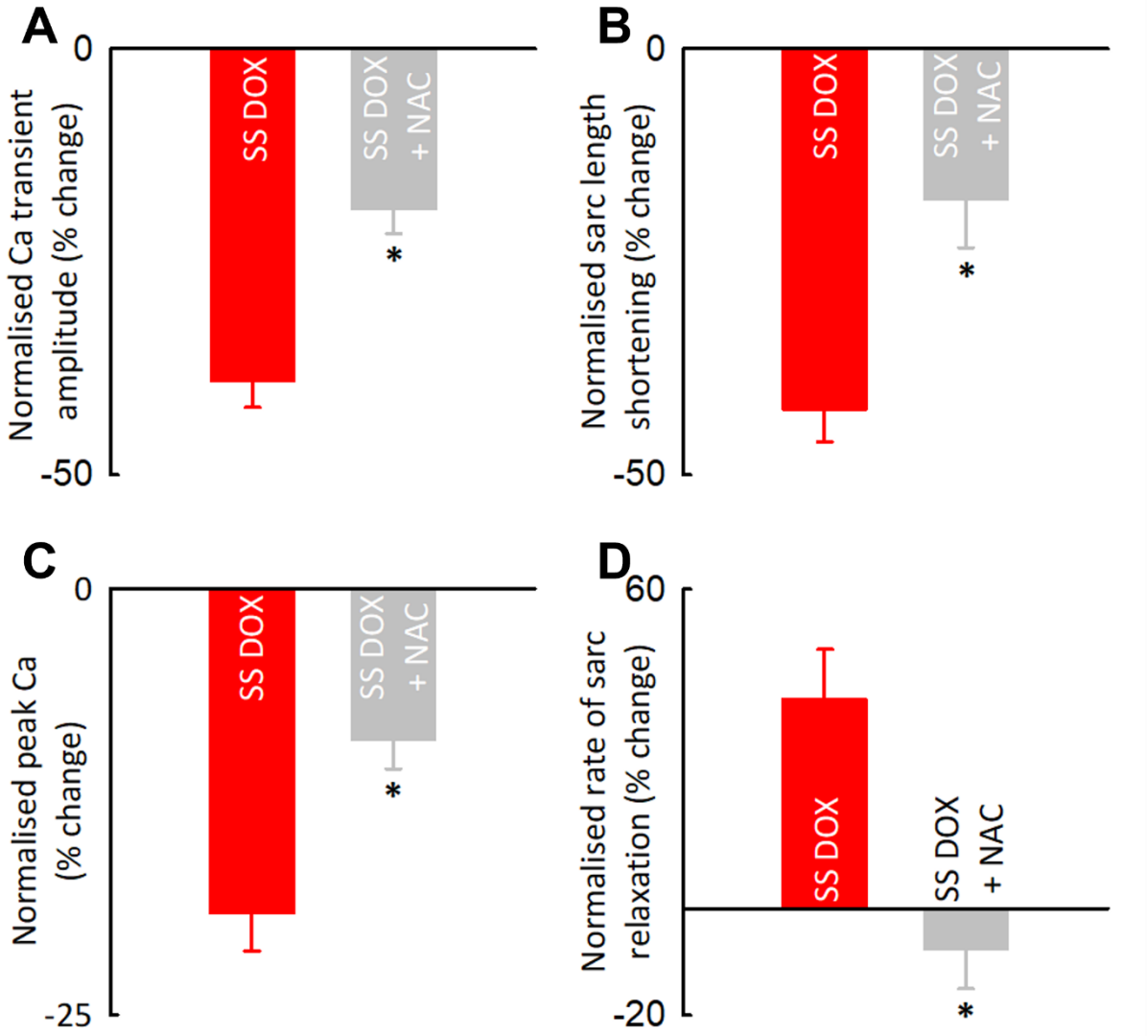


Figure 4.6: The percentage change compared to control in cardiac myocytes treated with 1 nM DOX only (n = 36 from 9 animals) and those treated with 1 mM NAC prior to DOX treatment (n = 27 from 5 animals). (A) mean percentage change compared to control of Ca transient amplitude. (B) mean percentage change compared to control of sarcomere length shortening. (C) mean percentage change compared to control of peak Ca. (D) mean percentage change compared to control of rate of sarcomere relaxation. Significance was determined using a T-test, * denotes statistical significance.

4.3.6 The effects of DOX on SR Ca content and cytoplasmic Ca removal following pre-treatment with NAC

DOX was found to reduce SR Ca content as shown in section 3.3.3. As described in section 3.2 caffeine allows the investigation of changes to SR Ca content. On average, following pre-treatment with NAC the amplitude of the caffeine evoked Ca transient was reduced by DOX by 18.34 % ± 3.56 ($n = 15$, $p < 0.001$) and did not recover upon washout ($n = 15$, $p < 0.001$; fig 4.7A). On average, the Ca transient rate of constant decay (k_{sys}) was unaltered ($n = 14$, $p = 0.119$; fig 4.7B). The caffeine-evoked Ca transient rate of decay (k_{caff}) increased by 13.52 % ± 7.87 % ($n = 16$, $p = 0.165$) in DOX and significantly increased by 18.86 % ± 7.15 % ($n = 15$, $p = 0.025$) in wash (fig 4.7C). The activity of SERCA (k_{SERCA}) was calculated by subtracting k_{caff} from k_{sys} and was not significantly altered ($n = 14$, $p = 0.362$; fig 4.7D).

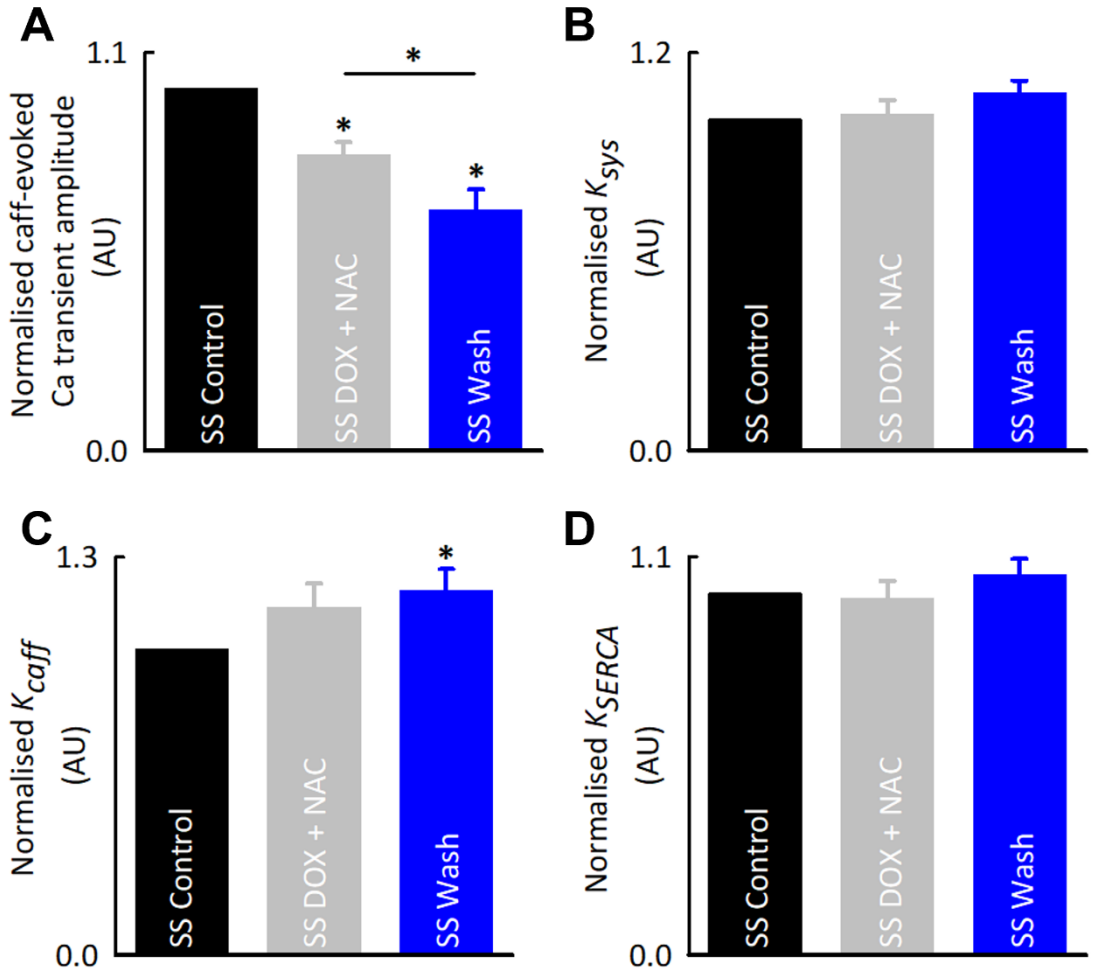


Figure 4.7: The effect of 1 nM DOX on Ca removal in sheep ventricular myocytes were pre-treated with 1 mM NAC. (A) mean normalised amplitude of caffeine evoked Ca transient. (B) mean normalised k_{sys}. (C) mean normalised k_{caff}. (D) mean normalised k_{SERCA}. n= 14 - 16 (from 3 animals, * denotes statistical significance.

4.3.7 The dependence of DOX-mediated changes to SR Ca and cytoplasmic Ca removal on oxidative stress

As can be seen in figure 4.8, NAC significantly reduced the effect of DOX on the caffeine-evoked Ca transient amplitude by 45 % (n = 15, p = 0.046; fig 4.8A). The effect on k_{sys} and k_{SERCA} was attenuated by 102 % (n = 28, p < 0.001; fig 4.8B) and 96 % (n = 14, p < 0.001; fig

4.8D) respectively. NAC pre-treatment caused a 79 % attenuation on k_{caff} however this was not significant (n = 16, p = 0.117; fig 4.8C).

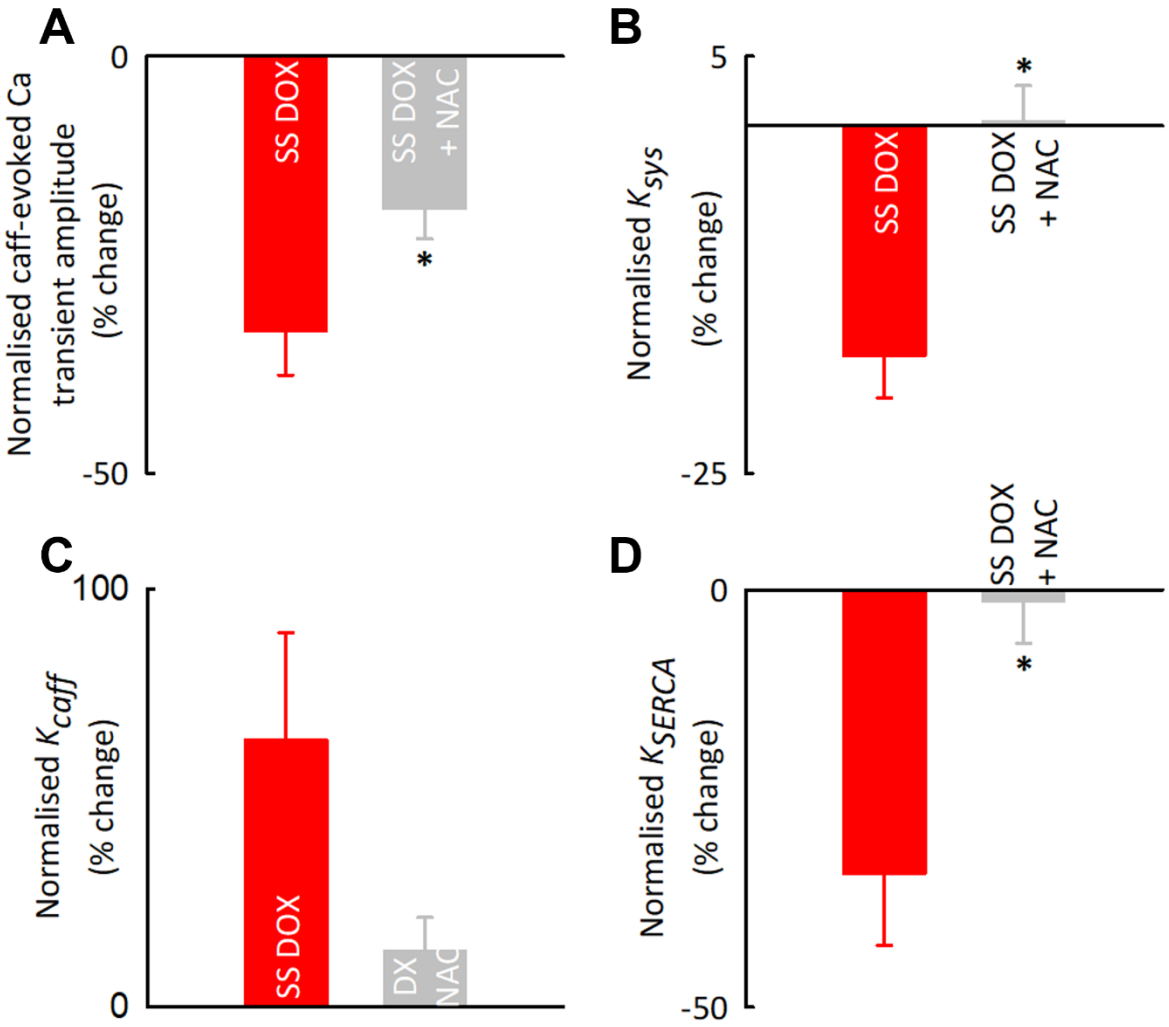


Figure 4.8: The percentage change compared to control in cardiac myocytes treated with 1 nM DOX (n = 19 from 5 animals) and those pre-treated with 1 mM NAC prior to DOX treatment (n = 14 from 3 animals). (A) mean percentage change of caffeine-evoked Ca transient amplitude. (B) mean percentage change of k_{sys}. (C) mean percentage change of k_{caff}. (D) mean percentage change of k_{SERCA}. Significance was determined using a T-test, * denotes statistical significance.

4.4 Discussion

4.4.1 Is this method sensitive enough to detect changes in oxidative stress?

Cells were exposed to hydrogen peroxide (10, 20, 50, 100 and 200 µM) as a positive control to check the validity of this method. A concentration-dependent increase in fluorescence was detected indicating an increase in OS (fig 4.1). This confirms that this method can detect an increase in OS and is sensitive enough to detect a concentration-dependent effect.

The validity of this method was further confirmed using NAC (see fig 4.3). NAC pre-treatment resulted in a decrease in fluorescence compared to DOX treatment alone confirming that the changes in fluorescence are due to OS.

4.4.2 Does DOX increase levels of oxidative stress in sheep ventricular myocytes?

DOX produced a concentration-dependent increase in cytoplasmic oxidative stress after 30 minutes of treatment, with a significant increase evident at 1 and 10 µM.

Other studies have shown that DOX increases ROS using the fluorophore DCF-DA however have not investigated a concentration dependent-effect (Kim *et al.*, 2006a; Ma *et al.*, 2013; Zhou *et al.*, 2001). While DCF-DA is a commonly used fluorophore it has lower photostability to CellROX reagents which may impact reproducibility (Souza *et al.*, 2020). Furthermore, CellROX reagents are selective to OS as evidenced by the inhibitor experiment (fig 4.3) and shown by the manufacturer (Invitrogen- Molecular probes). Invitrogen specify that CellROX reagents are used for generalised OS measurements, whereas DCF-DA detects hydrogen peroxide, with some versions of the fluorophore suitable for hydroxyl and peroxyl radical detection, however it does not detect singlet oxygen or superoxide anions. While other studies show an increase in ROS in cardiac myocytes due to DOX the use of DCF-DA means

not all ROS production is considered. Our experiments support the findings that DOX increases ROS and shows DOX increases OS in a concentration-dependent manner (fig 4.2).

Furthermore, our studies showed a time dependent effect in OS with an increase of 24 % being evident after 10 minutes (fig 4.3). This indicates a rapid response in DOX-induced OS which is supported by a study that found an increase in ROS production due to DOX close to the mitochondria after only 20 minutes of exposure (Sarvazyan, 1996). This suggests mitochondrial ROS are a large contributor and may then diffuse into the cytoplasm (Munro & Pamenter, 2019). As discussed in 4.1.4 cardiac myocytes are densely packed with mitochondria (Barth *et al.*, 1992) and under normal physiological conditions the cells are able to cope with ROS production. However, our findings show that DOX increases the levels of ROS beyond the coping mechanisms of the cell resulting in OS.

4.4.3 Does DAUN increase levels of oxidative stress in sheep ventricular myocytes?

In this study DAUN increased OS by 86.36 ± 6.3 % after 30 minutes at 10 µM (n = 4, p = 0.005), however did not result in a concentration-dependent increase. The lower concentrations of DAUN (0.001, 0.01, 0.1 and 1 µM) did not increase OS. This is supported by a study that found 1 µM DAUN did not increase ROS in cardiac myocytes after 3 hours of treatment (Sawyer *et al.*, 1999). Furthermore, in cancer cell lines 10 µM DAUN increases ROS after an incubation of 4 hours and remained high post-recovery (Al-Aamri *et al.*, 2019). These findings along with our own suggest that increased ROS production and ultimately OS requires high concentrations of DAUN (10 µM). The effects of DAUN on ROS production in cardiac cells is less studied than DOX due to the assumption that the compound will produce similar effects, however our findings contradict that. As shown in figure 1.10 DAUN has a quinone structure similar to DOX (Alves *et al.*, 2017) meaning it has the capability of being reduced to a semiquinone as discussed in 4.1.2 and producing ROS as a biproduct. The biochemical

properties are beyond the scope of this study but given the difference in OS production between DAUN and DOX may be an area of interest for further investigation.

4.4.4 To what extent are the effects of DOX on Ca handling and contractility dependent on oxidative stress?

DOX increases oxidative stress as shown in figures 4.2D and 4.3 however to what extent this causes alterations in Ca handling and contractility in cardiac myocytes remains unknown. The data discussed in 4.4.1 confirms NAC reduced OS in cardiac myocytes and is supported by others who show a reduction in ROS (Kuznetsov *et al.*, 2011), therefore confirming that NAC is a suitable tool to ascertain the extent to which DOX-induced OS alters Ca handling and contractility.

When pre-treated with NAC, DOX did not alter diastolic Ca but lead to a significant reduction in Ca transient amplitude, systolic shortening, and peak Ca (fig 4.5C). Despite a reduction in these Ca handling and contractility parameters, the NAC pre-treatment still attenuated the effect of DOX on Ca amplitude, systolic shortening, peak Ca and rate of sarcomere length shortening by 52, 58, 68 and 120 % respectively (fig 4.6).

These results suggest that OS is responsible for more than half of the decrease in Ca transient amplitude and systolic shortening due to DOX treatment. The Ca handling and contractility experiments are conducted over 3 -10 minutes meaning that any impact due to an increase in ROS production must be rapid. An increase of 24 ± 9 % in cytoplasmic OS was detected after 10 minutes of incubation with 0.1 µM DOX, which indicates a quick response in ROS production with OS increasing over the 4-hour period (fig 4.3). This is supported by a study that found under stress ROS production increases within 5 minutes (Kuznetsov *et al.*, 2011) and supports that alterations to Ca handling and contractility as a result of DOX treatment may be due to increased ROS production leading to OS.

NAC attenuated the effect of DOX on Ca amplitude and systolic shortening by 52 % and 58 % (fig 4.6) which indicates OS plays at least half a role in this alteration. As discussed in 4.1.3 studies have shown ROS reduces Ca amplitude and contractility (Goldhaber & Liu, 1994; Greensmith *et al.*, 2010; Kuster *et al.*, 2010). Our findings indicate that DOX-induced OS can account for at least half the reduction in Ca amplitude and contractility identified in chapter 3. Interestingly sarcomere length relaxation was fully attenuated by NAC pre-treatment indicating that DOX-induced OS is a major contributor to this alteration and is potentially related to altering the rate of Ca removal.

4.4.5 To what extent are the effects of DOX on SR Ca dependent on oxidative stress?

The caffeine-evoked Ca transient amplitude was reduced by DOX after NAC pre-treatment indicating a decrease in SR Ca content. The Ca transient amplitude is proportional to the third power of SR Ca (Dibb *et al.*, 2007). NAC-pre-treatment resulted in DOX reducing SR Ca content to 82 % of control. This would be expected to decrease the Ca transient amplitude to $(0.82)^3$ = 55 %. The Ca transient amplitude only decreased to 77 % indicating that the decreased SR Ca can account for the change in Ca transient amplitude.

When compared to DOX treatment alone NAC attenuated the effects to the SR Ca content by 45 % (fig 4.8A). ROS has been shown to reduce SR Ca content (Boraso & Williams, 1994; Greensmith *et al.*, 2010; Kuster *et al.*, 2010) and this along with our findings suggests DOX-induced OS is responsible for more than a third of the reduction in SR Ca content.

4.4.6 To what extent are the effects of DOX on Ca removal mechanisms dependent on oxidative stress?

NAC pre-treatment fully attenuated the effect of DOX on K_{sys} and K_{SERCA}. The effects on NCX were partially attenuated. This suggests that OS is the direct cause of a decrease in SERCA activity due to DOX which is supported by various studies that found ROS decreased SERCA

activity (Greensmith *et al.*, 2010; Kuster *et al.*, 2010; Morris & Sulakhe, 1997; Reeves *et al.*, 1986; Scherer & Deamer, 1986). ROS has been shown to increase NCX activity (Eigel *et al.*, 2004; Goldhaber, 1996; Kuster *et al.*, 2010; Reeves *et al.*, 1986) and the partial attenuation of 79 % shown in this study (fig 4.8C) indicates that OS has a substantial role in the alteration to NCX as a result of DOX treatment.

While the increase in NCX activity shown in figure 4.7 is not significant it may still lead to a reduction in SR Ca content. However, as discussed in 3.1.1 DOX may alter L-type Ca channel activity as well as the open probability of RyR which can impact SR Ca content and these alterations may not be dependent on DOX-induced OS. This may explain the decrease in SR Ca content identified in this study.

4.5 Concluding remarks

In conclusion, this study has highlighted the role of DOX-induced OS on Ca and contractility in ventricular myocytes. DOX resulted in an increase in intracellular OS in cardiac myocytes which may originate both from the cytoplasm and mitochondria. The oxidative stress response in cardiac myocytes due to DAUN and DOX differed. DAUN increased OS at high concentrations, whereas DOX resulted in a rapid response and showed a concentration-dependent increase. DAUN has been associated with less cardiotoxicity than DOX (Feijen *et al.*, 2015a) which based on this study may be due to higher concentrations being required to increase OS.

NAC was utilised to lower OS and resulted in attenuation on various Ca handling and contractility parameters that DOX impacted. Approximately half of the effect on Ca amplitude, systolic shortening and SR Ca content can be attributed to DOX-induced OS. Interestingly, the effect of DOX on the rate Ca removal was fully attenuated by the reduction of OS, suggesting that ROS directly impacts SERCA and NCX. The pathway by which DOX increases OS is still unknown.

Chapter 5
The role of xanthine oxidase and NADPH oxidase in anthracycline-induced oxidative stress

5.1 Introduction

5.1.1 The mechanisms of increased oxidative stress by anthracyclines

Anthracyclines have been shown in this study to increase ROS in cardiac myocytes. In chapter 4, removal of OS with NAC - a generic ROS scavenger - demonstrated that OS plays a role in the alterations in Ca handling and contractility caused by DOX. However, the sources -namely ROS production - of this OS remain unknown. As discussed in 4.1.2 anthracyclines may undergo redox reactions by various pathways in cardiac myocytes forming a semi-quinone leading to ROS production (Menna *et al.*, 2010).

Several studies on both primary and culture cardiac myocytes show DOX can increase intracellular ROS production (Kim *et al.*, 2006b; Ma *et al.*, 2013; Zhou *et al.*, 2001). Enzymatic pathways for ROS production such as xanthine oxidase and NADPH oxidase have been thought to play a role in anthracycline-induced ROS (Bates & Winterbourn, 1982; Huang *et al.*, 2022).

5.1.2 ROS inhibition

As discussed in 1.4 ROS may be produced through various pathways including via NADPH oxidases and xanthine oxidases with physiological removal pathways of ROS including catalase and glutathione peroxidase.

Experimentally and clinically, various inhibitors exist to reduce the production of ROS. As previously mentioned in 1.4.1.1, both NADPH oxidase and xanthine oxidase may be sources of ROS in cardiac myocytes (Akki *et al.*, 2009). Inhibitors of these enzymes can be used to establish the impact of different pathways on ROS production and whether this ROS production is leading to other cellular alterations. Inhibition of ROS production provides a preventative strategy rather than a reactionary approach to reducing ROS. While this inhibition may not be physiological it is (1) pharmacologically useful and (2) a useful tool to determine the sources of DOX-induced ROS shown in chapter 4.

5.1.3 Xanthine oxidase inhibition

Xanthine oxidoreductase is an enzyme that produces ROS as a biproduct of uric acid production from hypoxanthine metabolism (Kang & Ha, 2014) as shown in fig 5.1. It exists in two interconvertible forms in mammals; xanthine dehydrogenase and xanthine oxidase (XO), with the latter having an important role when cells are under stress (Chen & Meng, 2022) This enzyme is thought to have a role in the physiology of congestive heart failure (Berry & Hare, 2004b). Inhibition of xanthine oxidase in heart failure models increases contractility and improves left ventricular function (Ekelund *et al.*, 1999; Ukai *et al.*, 2001), highlighting the key role of this enzyme in cardiac dysfunction.

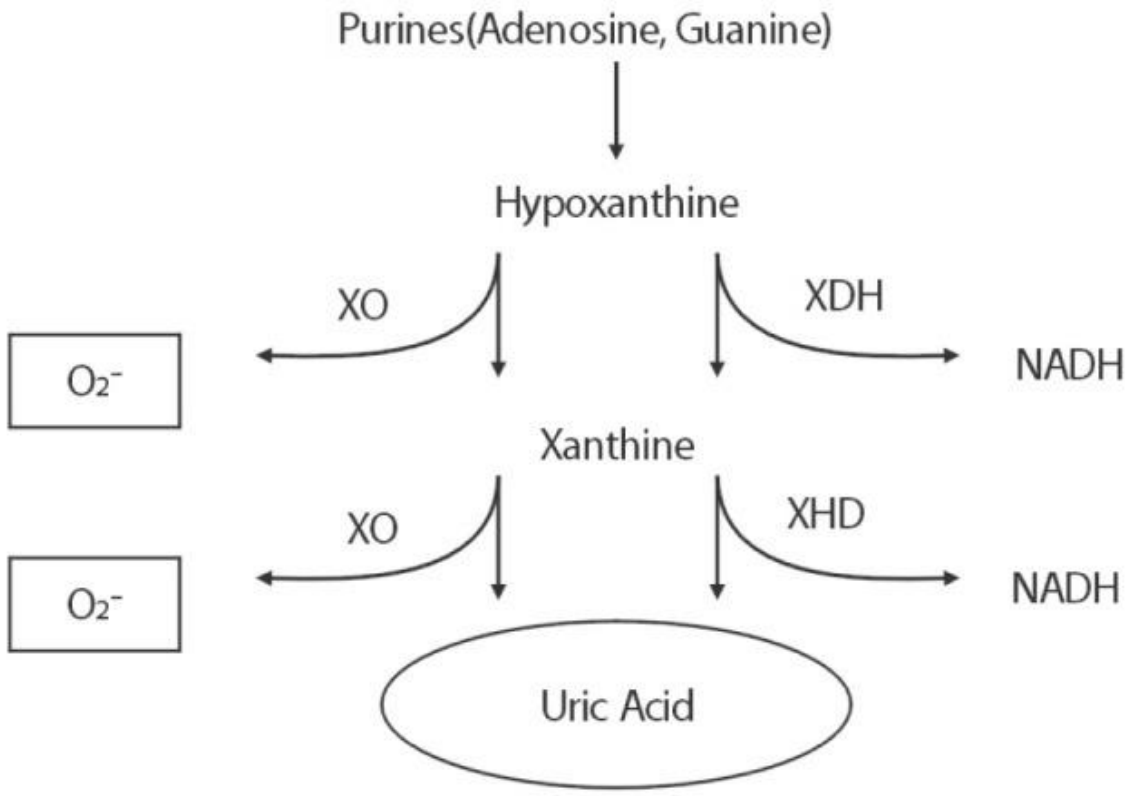


Figure 5.1: Production of ROS via xanthine oxidase. From (Kang & Ha, 2014).

Xanthine oxidase inhibitors are used therapeutically for the reduction of serum urate levels to treat gout, with febuxostat (FEB) approved for use medically in 2009 (White, 2018). The cardiovascular safety of febuxostat has been investigated and while one trial found patients

treated with FEB had higher cardiovascular mortality (White *et al.*, 2018), a more recent trial found that there was not an increased risk of serious adverse events or cardiovascular death (Mackenzie *et al.*, 2020).

5.1.4 NADPH oxidase inhibition

NADPH oxidase was first identified as having a role in immune defence by producing ROS (Kuroda & Sadoshima, 2010). Within the NADPH oxidase group, two isoforms (NOX2 and NOX4) are present in cardiac myocytes and are associated with cardiac dysfunction (Szekeres *et al.*, 2021a). This cardiac dysfunction may be due to pathological activation especially considering the relatively low antioxidant capacity of the heart (Costa *et al.*, 2013).

VAS3947 (VAS) is a specific inhibitor of NOX2 and NOX4 (Reis *et al.*, 2020) making it an ideal compound to investigate the production of ROS via NADPH oxidases in cardiac myocytes.

5.1.5 Summary

Chapter 3 showed that DOX and DAUN perturb Ca handling and contractility and chapter 4 highlighted the contributing role of oxidative stress. As reviewed above, inhibition of XO and NOX - the main enzymatic sources of ROS in the heart – is pharmacologically useful. Here, we use those inhibitors to determine the extent to which XO and NOX contribute to DOX-mediated oxidative stress.

5.1.6 Aims

- Determine if xanthine oxidase and NOX2 and NOX4 contribute to DOX-induced oxidative stress.
- Investigate if the effects of DOX on Ca handling and contractility can be attenuated by XO and/or NOX inhibition.
- Investigate whether the effects of DOX on myofilament sensitivity can be attenuated by XO and/or NOX inhibition.

5.2 Methods

5.2.1 Cell preparation and excitation-contraction coupling measurements

Primary left ventricular cardiac myocytes were isolated from 18-month-old sheep as described in section 2.2.2. After pre-treatment with inhibitors described in 5.2.4, they were loaded with fura-2 (see section 2.6.2) and stored in 0.95 mM Ca.

Cells were paced at 0.5 Hz using field stimulation as described in section 2.5. NT solution (see table 2.3) was perfused on to cells and allowed to reach steady state before being switched to 1 nM DOX. Following DOX treatment, a wash period was allowed to establish if the effects of the drug were reversible. The DOX treatment data shown in figure 3.4 are used as a comparison in figure 5.2 to highlight the impact of the antioxidants used.

5.2.2 Reactive oxygen species inhibitor preparation and treatment

Cells were pre-treated with one of two pathway specific antioxidants.

Xanthine oxidase inhibition was evident at 25 nM of FEB in vascular endothelial cells (Malik *et al.*, 2011), while in macrophages a concentration of 30 µM was shown to cause inhibition (Nomura *et al.*, 2013). A study in rat cardiomyocytes found 10 µM FEB for 1 hour treatment to be effective (Wang *et al.*, 2015) and therefore this concentration and treatment time were used in this study.

Studies have demonstrated the reduction in ROS due to NOX inhibition due to VAS with IC50 values ranging from 2 µM – 12 µM in cancer cell lines (El Dor *et al.*, 2020; Wind *et al.*, 2010b). A reduction in ROS in spontaneously hypertensive rat aortas was detected when treated with 10 µM VAS for 30 minutes (Wind *et al.*, 2010b). Furthermore, using purified human NOX4 Reis *et al* (2020) found an IC50 of 31.9 µM for VAS and almost 50 % inhibition after 10 minutes

with 100 % inhibition evident after 1 hour of treatment, while NOX2 inhibition was 50 % after 1 hour. Given this data and initial assessment of suitability a concentration of 30 µM for 1 hour was utilised.

Both FEB and VAS3947 were prepared to a stock solution of 50 mM in DMSO. FEB dilutions at 10 µM and VAS dilutions at 30 µM were prepared in NT solution. Cells were incubated with the NADPH oxidase inhibitor VAS3947 at 30 µM for 30 minutes prior to loading with fura-2. A separate group of cells were treated with febuxostat; a xanthine oxidase inhibitor at 10 µM for 1 hour before fura-2 loading.

These concentrations and incubation durations have been shown to reduce ROS as shown in figure 5.1 and have been used in other studies (Altenhöfer *et al.*, 2014; Nomura *et al.*, 2013; Wang *et al.*, 2015; Wind *et al.*, 2010a).

5.2.3 Measurement of sarcoplasmic reticulum Ca content and the activity of Ca removal mechanisms

Changes in SR Ca content were investigated by rapid caffeine application (10mM) as described in section 3.2.5. The rate constant of decay of systolic Ca transient (k_{sys}) represents the activity of NCX and SERCA. In the presence of caffeine SERCA activity is rendered futile due to the increase in open probability of RyR (Eisner *et al.*, 2017). Therefore, the RC of decay of the caffeine-evoked Ca transient (k_{caff}) represents the activity of NCX alone. By subtraction of k_{caff} from k_{sys} the relative activity of SERCA can be determined.

5.2.4 Measurement of myofilament sensitivity

Myofilament sensitivity was investigated by plotting phase-plane loops of cytoplasmic Ca versus sarcomere length of each cardiomyocyte and the EC_{50} (cytoplasmic Ca at 50 % relaxation) was used to quantify any change in myofilament sensitivity as described in detail in 3.2.4. Statistical significance was identified as $p < 0.05$ and calculated using a paired T-test.

5.3 Results

5.3.1 The effect of inhibition of XO and NOX2 and NOX4 on DOX-induced oxidative stress

Pre-treatment with 10 µM FEB reduced levels of 1nM DOX-mediated OS on average by 22 % and 29 % after treatment with DOX for 10 and 240 minutes. While this change in OS was not statistically significant (n = 12-20 cells from 3 animals, $p > 0.05$), it indicates that XO has a role (at least partially) in DOX-induced OS. No increase in OS was detected in cells pre-treated with FEB following DOX incubation (n =12-20 cells from 3 animals, $p > 0.05$; fig 5.2). Pre-treatment with 30 µM VAS3947 (NOX2 and NOX4 inhibitor) for 30 minutes reduced levels of oxidative stress by 65 %, 54 %, 70 % and 50 % when compared to DOX treatment alone at 10, 60, 120 and 240 minutes (n = 10-16 cells from 3 animals, $p < 0.05$; fig 5.2) and no change in OS over the 240 minutes was detected (n = 10-16 cells from 3 animals, $p > 0.05$; fig 5.2).

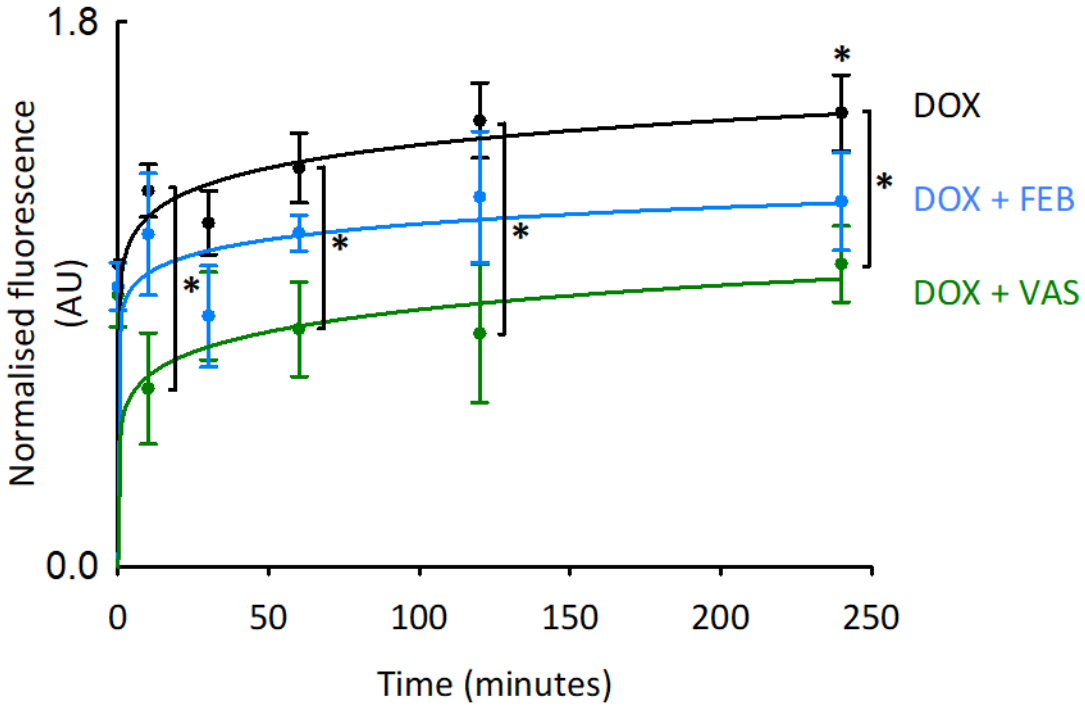


Figure 5.2: The time dependent effect of 0.1 µM DOX on OS production in sheep ventricular myocytes with and without XO and NOX inhibition. Statistically significant results were determined using a two-way ANOVA. n = 10-46 cells from 3–8 animals, * denotes statistical significance.

5.3.2 The effect of xanthine oxidase inhibition on the alterations to Ca handling and contractility produced by DOX

The specimen traces in figure 5.3 show the effect of 1 nM DOX on intracellular Ca and contractility in cardiac myocytes after pre-treatment with 10 µM FEB. On average, no significant effect on diastolic Ca (n = 19, p =0.082; fig 5.4A) or diastolic sarcomere length (n = 15, p = 0.605; fig 5.4B) was observed by DOX after FEB treatment. DOX did not significantly reduce Ca transient amplitude (n = 18, p = 0.527) after FEB pre-treatment however this was significantly reduced in wash by 18.8 ± 4.92 % (n = 19, p = 0.015; fig 5.4C). On average, FEB pre-treatment resulted in DOX reducing sarcomere length shortening by 16.47 ± 6.05 % however this was not significant (n = 14, p = 0.252; fig 5.4D). The rate constant of decay (k_{sys}) was not altered by DOX in the presence of FEB (n = 16, p = 0.414; fig 5.4E). On average, DOX did not alter sarcomere relaxation after FEB treatment (n = 14, p = 0.261) however a significant decrease of 14.53 ± 9.16 % occurred in wash (n =14, p = 0.01; fig 5.4E).

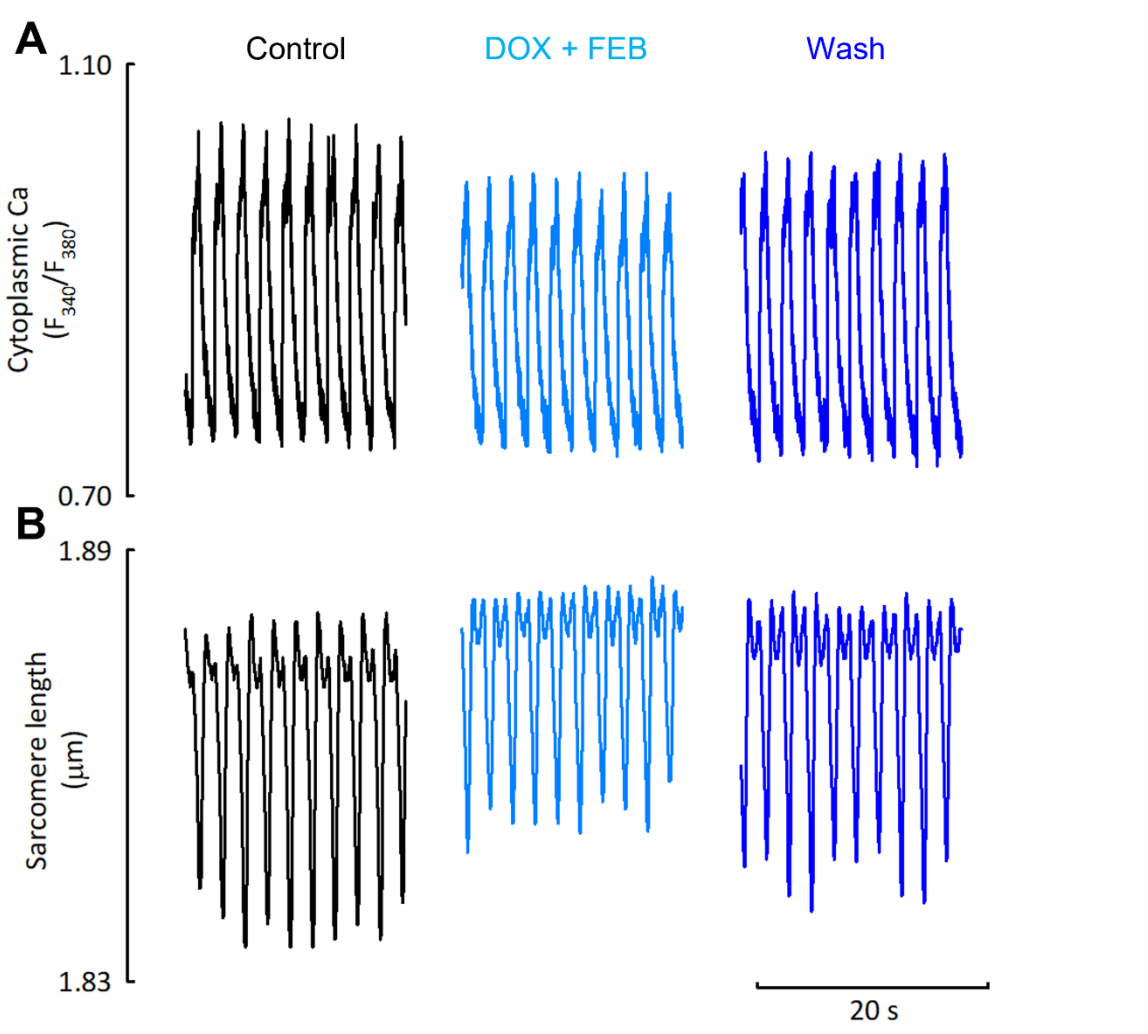


Figure 5.3: The effect of xanthine oxidase inhibition using 10 µM FEB and 1 nM DOX on cytoplasmic Ca and contractility in sheep ventricular myocytes. (A) specimen trace of Ca transients. (B) specimen trace of sarcomere length.

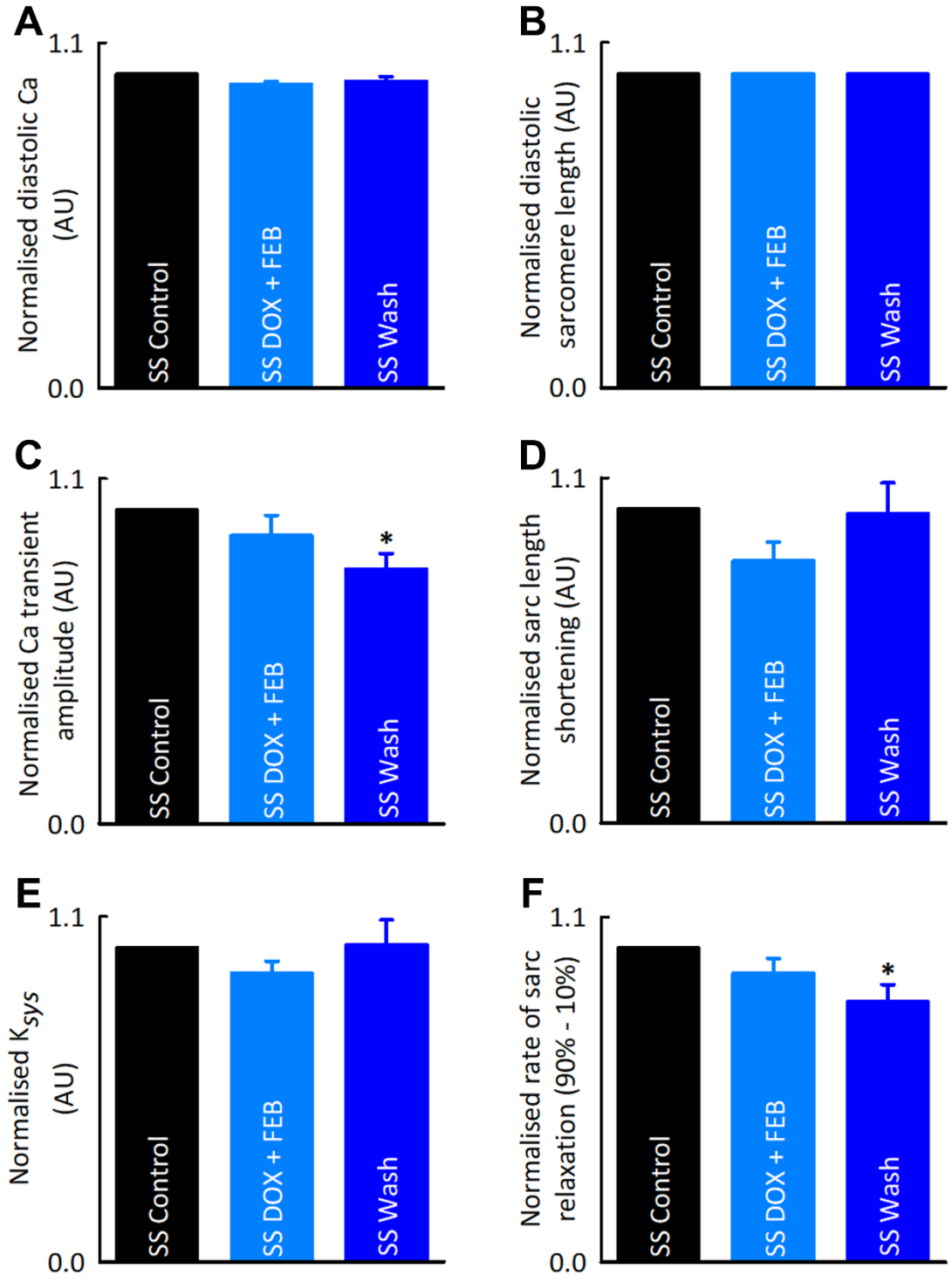


Figure 5.4: The effect of xanthine oxidase inhibition using 10 µM FEB and 1 nM DOX on intracellular Ca and contractility in sheep ventricular myocytes. (A) mean normalised diastolic Ca. (B) mean normalised diastolic sarcomere length. (C) mean normalised Ca

transient amplitude. (D) mean normalised sarcomere length shortening. (E) mean normalised Ca transient rate constant of decay (k_{sys}). (F) mean normalised 90 – 10 % decay time of sarcomere length. n = 14 – 19 (from 6 animals), * denotes statistical significance.

5.3.3 The effect of NOX inhibition on the alterations to Ca handling and contractility produced by DOX

The specimen traces in figure 5.5 show the effect of 1 nM DOX on intracellular Ca and contractility in cardiac myocytes after pre-treatment with 30 µM VAS. On average, no significant effect on diastolic Ca (n = 18, p = 0.216; fig 5.6A) or sarcomere length (n = 18, p = 0.916) (fig 5.6B) was observed. DOX decreased Ca transient amplitude by 21.58 % ± 4.24 % (n = 17, p < 0.001) and did not recover upon wash out after VAS pre-treatment (n = 17, p < 0.001; fig 5.6C). This was accompanied by a 15.37 ± 6.79 % decrease in sarcomere length shortening (fig 5.6D), however this decrease was not significant (n = 17, p = 0.063). On average, DOX did not alter the rate constant of decay (k_{sys}) following VAS pre-treatment (fig 5.6E; n = 18, p = 0.916). DOX did not alter sarcomere relaxation (n = 16, p = 0.636; 5.6F) after VAS treatment.

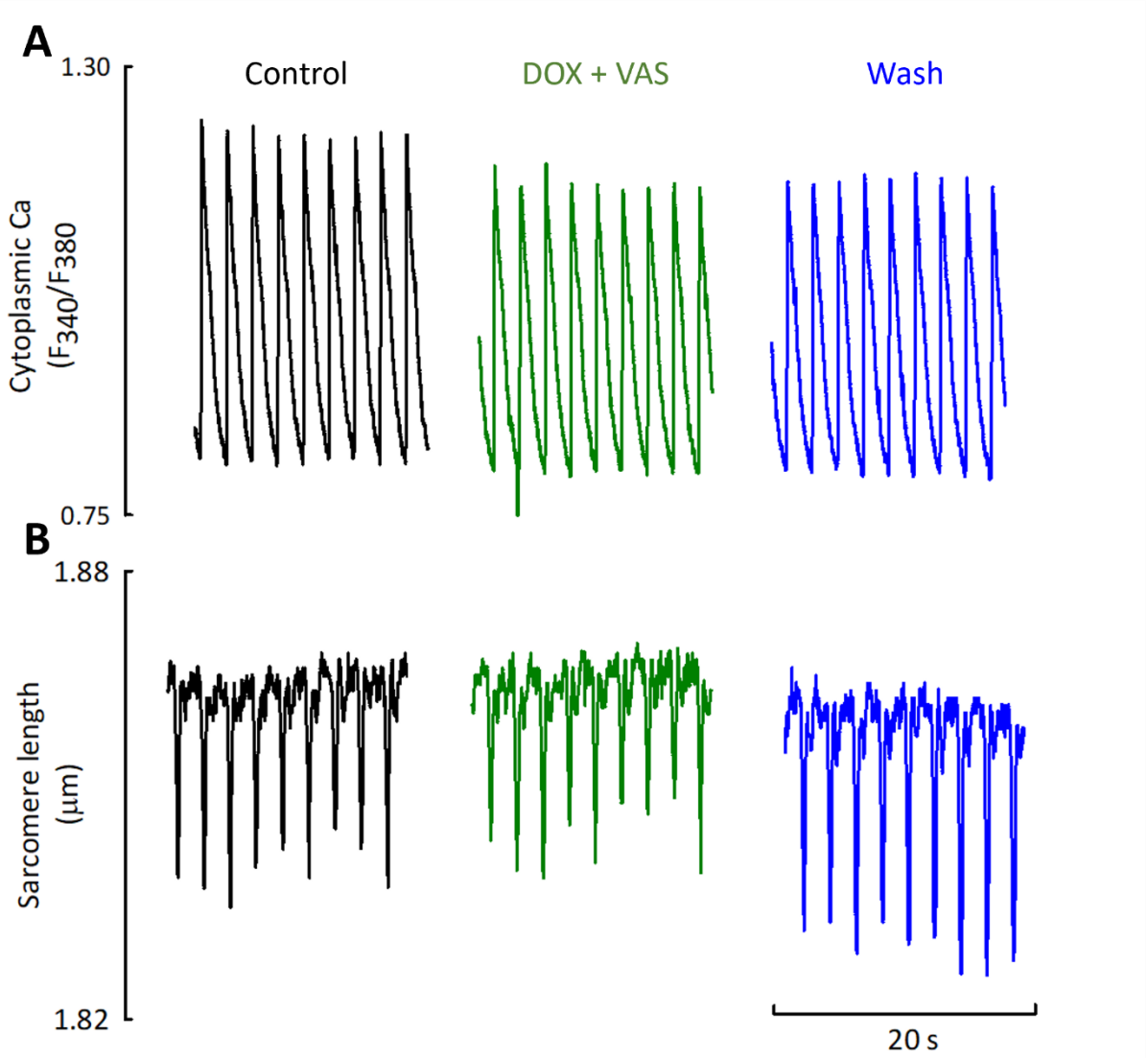


Figure 5.5: Specimen traces of the effect of NOX2 and NOX4 inhibition using 30 µM VAS and DOX on cytoplasmic Ca and contractility in sheep ventricular myocyte. (A) specimen trace of Ca transients. (B) specimen trace of sarcomere length.

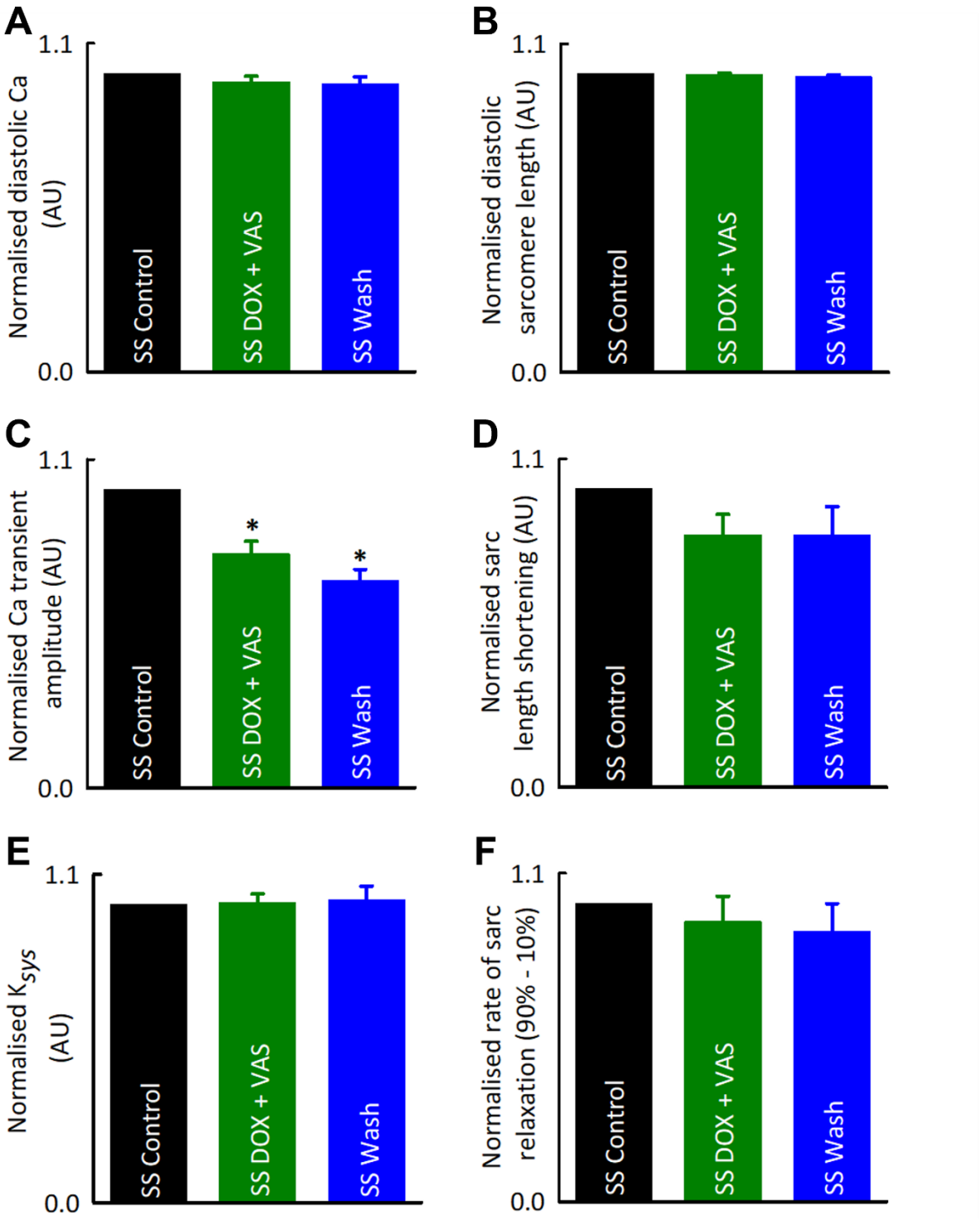


Figure 5.6: The effect of NOX2 and NOX4 inhibition using 30 µM VAS and 1 nM DOX on intracellular Ca and contractility in sheep ventricular myocytes. (A) mean normalised diastolic Ca. (B) mean normalised diastolic sarcomere length. (C) mean normalised Ca

transient amplitude. (D) mean normalised sarcomere length shortening. (E) mean normalised Ca transient rate constant of decay (k_{sys}). (F) mean normalised 90 – 10 % decay time of sarcomere length. n = 15 - 18 (from 5 animals), * denotes statistical significance.

5.3.4 The impact of NOX an XO inhibition on DOX-induced alterations to Ca handling and contractility

Though DOX still resulted in a decrease in Ca transient amplitude when cells were pre-treated with xanthine oxidase (FEB) and NOX (VAS) inhibitors (fig 5.4C and fig 5.6C) these effects were attenuated by 79 % and 45 % respectively (n = 18, $p < 0.001$ and n = 17, $p = 0.015$; fig 5.7A). The reduction in sarcomere length shortening produced by DOX was attenuated by 61 % and 64 % by FEB and VAS pre-treatment respectively (fig 5.7B; n = 14, $p = 0.004$ and n = 17, $p = 0.001$). The removal of Ca by all removal mechanisms was shown to be reduced by 1 nM DOX (fig 3.7A), this effect was attenuated by VAS 103 % (n = 18, $p = 0.003$). While FEB attenuated the impact of DOX on k_{sys} by 51 % this change was not significant (n = 16, $p = 0.244$). 1 nM DOX increased the rate of sarcomere relaxation (fig 3.5F), however pre-treatment with FEB and VAS fully attenuated this effect by 120 % and 115 % respectively (n = 14, $p = 0.008$ and n = 16, $p = 0.007$).

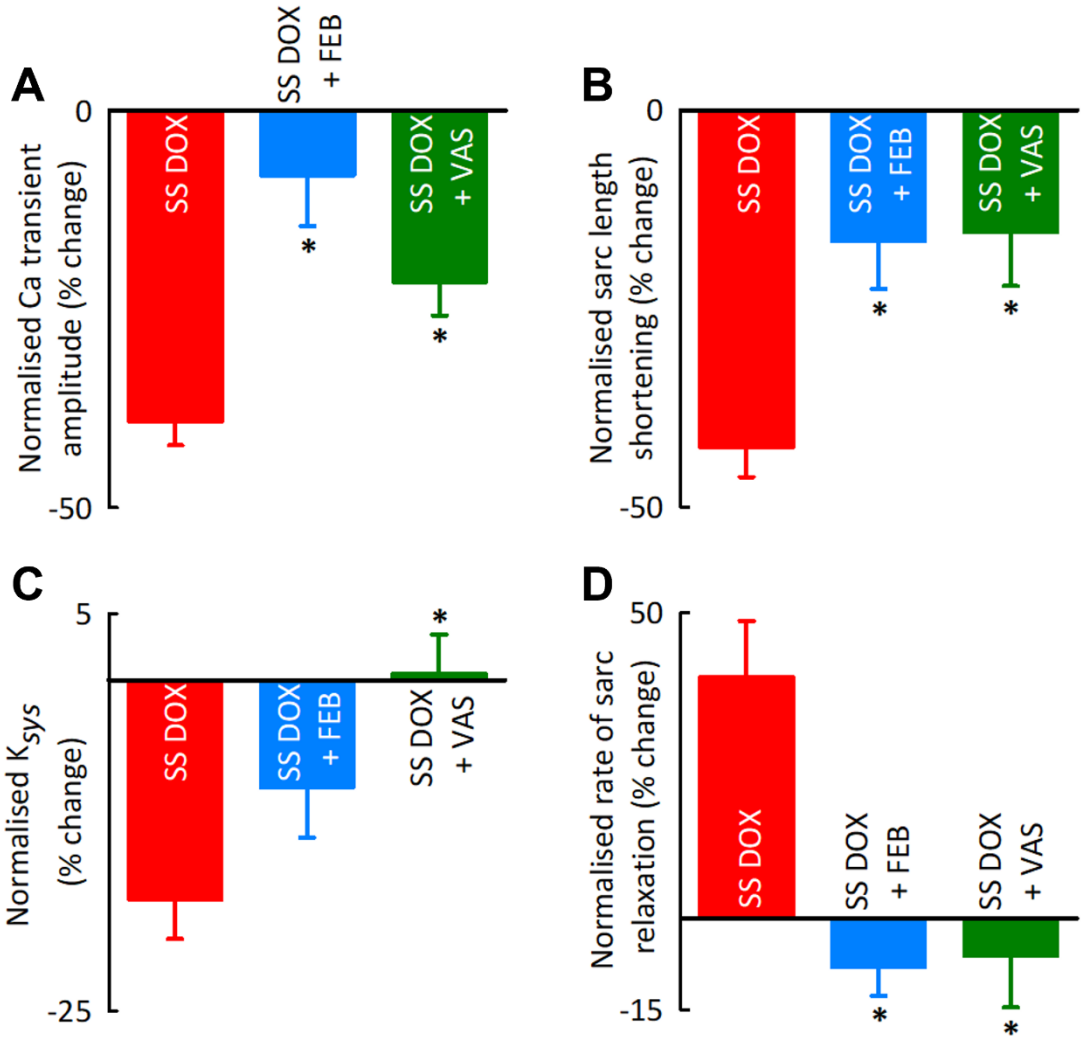


Figure 5.7: The percentage change compared to control in cardiac myocytes treated with 1 nM DOX only (n = 36 from 9 animals) and those treated with FEB (n = 14 from 6 animals) or VAS3947 (n = 15 from 5 animals) prior to DOX treatment. (A) mean percentage change compared to control of Ca transient amplitude. (B) mean percentage change compared to control of sarcomere length shortening. (C) mean percentage change compared to control of peak Ca. (D) mean percentage change compared to control of rate of sarcomere relaxation, * denotes statistical significance.

5.3.5 The effect of NOX and XOR inhibition on DOX-mediated alterations to myofilament sensitivity

Phase-plane loops proceed counter-clockwise with sarcomere relaxation depicted by the left-hand side of the loop. The EC_{50} represent the cytoplasmic Ca at 50 % sarcomere relaxation. A shift of the loop highlights a change in myofilament sensitivity, for example a shift to the left indicates an increase in myofilament sensitivity. Chapter 3 shows DOX shifts phase plane loop. When pre-treated with FEB or VAS, this shift was prevented (fig 5.8A & C). To quantify this the EC_{50} was calculated. There was no significant difference between the EC_{50} of cells in control compared to 1 nM DOX treatment when pre-treated with either FEB or VAS ($n = 15$, $p = 0.105$ and $n = 15$, $p = 0.43$, respectively) indicating no alteration in myofilament sensitivity.

For comparison, data was normalised to each cell's control EC_{50} and the average EC_{50} is displayed in figure 5.9. The EC_{50} of each individual cell treated with DOX only is illustrated in figure 3.10, presented in figure 5.9 is the normalised average of this data. Compared to control the EC_{50} of DOX only was statistically significantly lower indicating an increase in myofilament sensitivity as explained in section 3.3.8. FEB and VAS pre-treatment resulted in no change in myofilament sensitivity during DOX treatment.

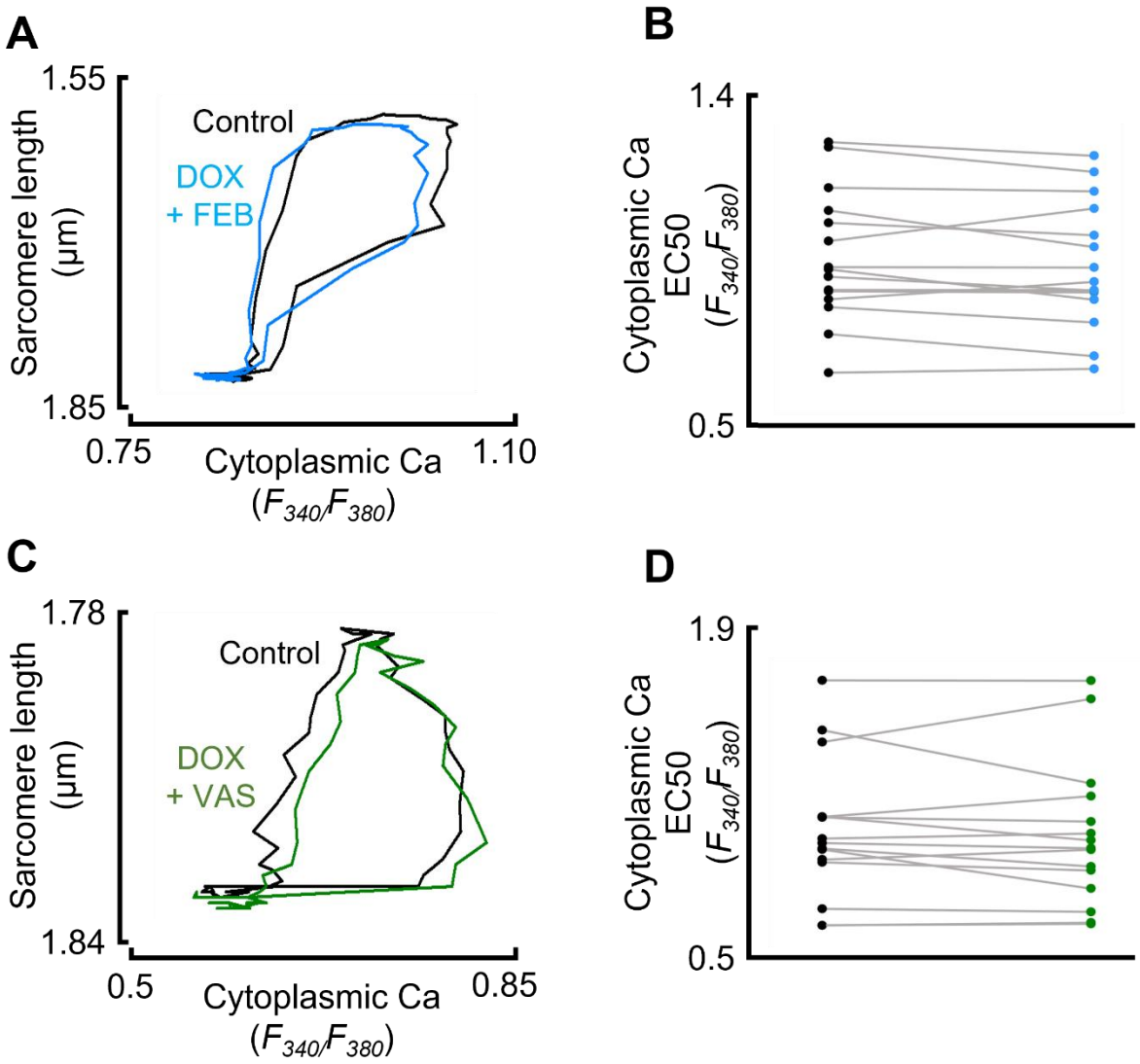


Figure 5.8: The effect of 1 nM DOX on myofilament sensitivity in sheep ventricular cardiac myocytes pre-treated with antioxidants. (A) phase-plane loop of sarcomere length versus cytoplasmic Ca in a cell pre-treated with 10 µM FEB (xanthine oxidase inhibitor) for 1 hour before and after DOX exposure. (B) EC_{50} (cytoplasmic Ca at 50 % sarcomere relaxation) of cells pre-treated with FEB in NT compared to 1 nM DOX. (C) phase-plane loop of sarcomere length versus cytoplasmic Ca in a cell pre-treated with 30 µM VAS (NOX inhibitor) for 30 minutes before and after DOX exposure. (D) EC_{50} (cytoplasmic Ca at 50 % sarcomere relaxation) of cells pre-treated with VAS in NT compared to 1 nM DOX treatment. Significant results ($p < 0.05$)

were determined by a paired T-test. n = 15 (from 5-6 animals), * denotes statistical significance.

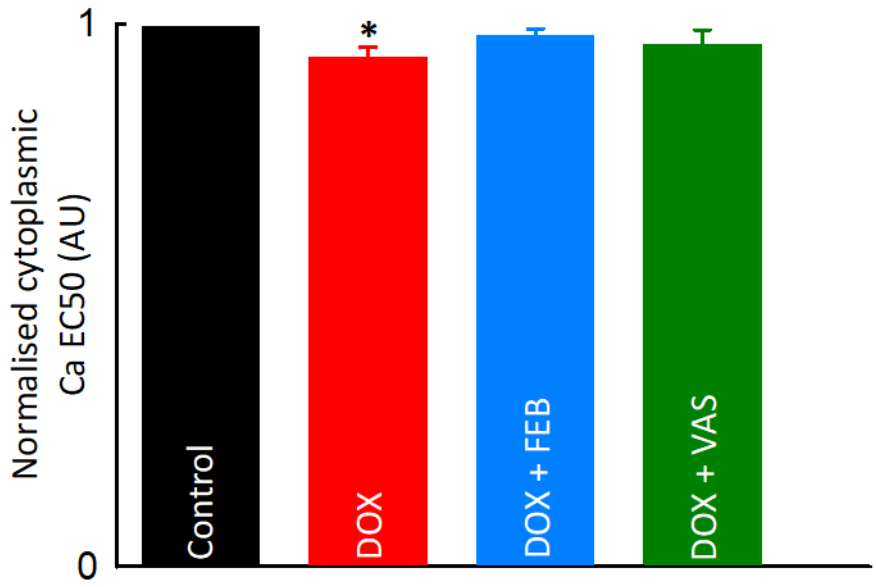


Figure 5.9: Mean normalised cytoplasmic Ca at 50 % sarcomere length relaxation (EC_{50}). n = 15-27 (from 4-6 animals), * denotes statistical significance.

5.4 Discussion

5.4.1 Does inhibition of XO and NOX2 and NOX4 reduce anthracycline-induced ROS?

Inhibition of XO and NOX2 and 4 reduces the levels of oxidative stress produced by 1 nM DOX (fig 5.2). Inhibition of XO resulted in a 22 % – 31 % reduction in DOX-induced OS on average over the stated incubation times (fig 5.2). While this is not statistically significant, given the magnitude of change we cannot exclude the possibility that XO contributes to the increase in OS caused by DOX. In mice treated with DOX for 7 days cardiac XO activity was found to be increased (Tanaka *et al.*, 2021). However, the cardiac XO activity in mice has been calculated to be greater than that of large mammals such as pigs and humans (Jong *et al.*, 1990). This

highlights the need for large animal model studies as it suggests that while DOX may increase XO activity, this increase would be to a lesser degree in humans than small rodents. It is likely that XO has a role in DOX-induced OS, however the extent of that role appears to be less than that of NOX from our findings.

Inhibition of NOX significantly reduced levels of oxidative stress by 50 – 70 % on average across the time points measured (fig 5.2). This suggests that at least half of the OS induced by DOX is a result of NOX activation. This is likely the combined activity of both NOX2 and NOX4. NOX4 is constitutively active whereas NOX2 requires regulatory subunits for activation (Nisimoto *et al.*, 2010). NOX2 activation involves phosphorylation of the adaptor protein p47phox which then allows interaction between NOX2 and p67phox which leads to ROS production (Brandes *et al.*, 2014). Given the significant increase in ROS production by DOX (~ 50 %) it may be that DOX promotes the phosphorylation or interaction leading to NOX2 activation. This is supported by a study conducted on endothelial progenitor cells that found DOX to activate NOX2 (De Falco *et al.*, 2016). NOX4 is localised to the inner mitochondrial membrane and is a major source of mitochondrial oxidative stress (Kuroda *et al.*, 2010; Peoples *et al.*, 2019). Due to the abundancy of mitochondria in cardiac myocytes it is logical that NOX4 contributes to a large proportion of cardiac myocyte ROS. This is evidenced by the large decrease in OS induced by DOX when NOX is inhibited (fig 5.2). While it is possible DOX may increase the activity of NOX2 and NOX4, it is also plausible that NOX2 and NOX4 contribute to reducing DOX to a semiquinone and releasing ROS in the process (Yousefian *et al.*, 2021). Due to NOX4 being continuously active the latter explanation is likely the most plausible to explain the role of this enzyme in DOX-induced OS.

These data suggest that NOX-mediated ROS production is the dominant contributor to DOX-induced oxidative stress. XO may also play an important role but requires further investigation.

5.4.2 Does xanthine oxidase inhibition alter the effect of DOX on Ca handling and contractility?

As shown in figure 5.4 when pre-treated with FEB, DOX did not significantly alter any parameters of Ca handling, however in wash Ca amplitude and rate of sarcomere relaxation were significantly decreased. This suggests that at the least, the immediate effects of DOX are heavily dependent on XO. As mentioned, the relationship between ROS and Ca is bidirectional. ROS have been shown to alter the activity of Ca channels and exchangers, for example activation of voltage dependent channels such as L-type Ca channels may be facilitated by ROS (Görlach *et al.*, 2015). Therefore, the gradual reduction in Ca amplitude and sarcomere relaxation may not be a direct implication of DOX treatment but rather an effect of lack of ROS production. The lack of ROS production may result in a decrease in L type Ca channel activation resulting in a decrease in Ca amplitude (Tabet *et al.*, 2004). As shown in figure 5.2 OS is reduced by FEB by ~ 20 %, though not significant this still highlights a decrease in ROS which would support this theory.

When compared to DOX treatment alone the effect on Ca transient amplitude was attenuated by 79 % and the effect on sarcomere length shortening was attenuated by 61 % as shown in figure 5.7. This indicates that ROS production via xanthine oxidase contributes to more than half of the effect seen to Ca amplitude and therefore sarcomere length shortening. This is surprising given that the reduction in DOX-induced OS after XO inhibition was 22 % following a 10-minute treatment. The attenuation effects are large given the relatively modest reduction in OS. However, when pre-treated with FEB, the removal of Ca by NCX and SERCA was attenuated by 51 % and the effect of DOX on sarcomere relaxation was fully prevented. This may indicate SR Ca is not altered. Given the steep relationship between SR Ca and Ca amplitude (Dibb *et al.*, 2004) this may explain the attenuation effect on Ca amplitude. The reduction in OS though not statistically significant may allow activity of Ca channels and exchangers to be restored while not being reduced to the extent that they are negatively affected by the lack of ROS (Görlach *et al.*, 2015).

These studies in combination highlight that XO may contribute (at least partially) to DOX-induced OS, and that even a ~ 20 % reduction in OS may restore the alterations in Ca handling and contractility induced by DOX.

5.4.3 Does NOX2 and NOX4 inhibition alter the effect of DOX on Ca handling and contractility?

Cells pre-treated with VAS did not alter Ca handling or contractility in ventricular myocytes except for an irreversible decrease in Ca transient amplitude as shown in figure 5.6. The effect of DOX on Ca transient amplitude was attenuated by 45 % in the presence of VAS with sarcomere length shortening attenuated by 64 % (fig 5.7). Inhibition of NOX (via VAS) resulted in a 65 % reduction in DOX-induced OS at 10 minutes (fig 5.2). This suggests DOX-induced OS through NOX accounts for at least half of the alterations identified in Ca amplitude, this is supported by a study that found NOX inhibition restored contractility and improved Ca handling in an aged-rat model (Valdés *et al.*, 2018). ROS production via NOX has been shown to alter Ca handling and contractility (Szekeres *et al.*, 2021b) and our study supports that the alterations in Ca handling and contractility due to DOX are largely attributable to ROS production facilitated by NOX. Given that k_{sys} (removal of Ca by all mechanisms) was fully attenuated as was the rate of sarcomere relaxation in the presence of the NOX inhibitor this suggests the role of NOX in DOX-induced OS is one of the main mechanisms by which Ca removal is altered. As discussed in chapter 4 ROS may alter SERCA and NCX, NOX may contribute to ROS production either through an increase in activity due to DOX and/or by facilitating the reduction reaction of DOX leading to ROS formation.

Our data show that DOX-induced ROS is largely attributed to NOX and has a vital role in altering Ca handling and contractility.

5.4.4 Does XO and NOX inhibition alter the effect of DOX on myofilament sensitivity?

DOX was shown to increase myofilament sensitivity using phase-plane loops (fig 3.12) when cells were exposed to FEB and VAS this increase in sensitivity was not apparent (fig 5.8). ROS has been found to increase myofilament sensitivity (Haizlip *et al.*, 2012; Hiranandani & Janssen, 2009; Miura *et al.*, 2015). Whereas NOX inhibition has been shown to decrease myofilament sensitivity, likely due to the reduction in ROS (Valdés *et al.*, 2018). Xanthine oxidase inhibition with allopurinol was shown to increase myofilament sensitivity (Pérez *et al.*, 1998), given that ROS would be reduced this is surprising.

The reduction in ROS by both FEB and VAS as shown in figure 5.2 along with the lack of alteration to myofilament sensitivity (fig 5.8) indicate that DOX-induced OS through both xanthine oxidase and NOX causes an increase myofilament sensitivity. It is also possible that NOX and xanthine oxidase inhibition desensitise the myofilaments to Ca (Valdés *et al.*, 2018) therefore combating the increase in sensitivity caused by DOX.

5.5 Concluding remarks

In conclusion, xanthine oxidase inhibition resulted in a 22 % – 31 % reduction in DOX-induced OS on average. Despite this reduction not being *statistically* significant XO inhibition attenuated various DOX induced effects on Ca handling and contractility suggesting it does indeed play an important role. On average, NOX inhibition resulted in a 50 – 70 % reduction in DOX-induced OS highlighting the substantial role of this enzyme; NOX-mediated production of superoxide is clearly a major mechanism underlying DOX-induced OS. Inhibition of NOX attenuated effects of DOX on Ca handling and contractility. Ca amplitude was still reduced however this reduction was significantly less than DOX treatment alone. Myofilament sensitivity was not altered by DOX following ROS inhibition. These data highlight the potential role of XO in DOX cardiotoxicity however NOX has been shown to be a major contributor in DOX-induced OS.

Chapter 6
The effects of doxorubicin and daunorubicin on Ca handling and contractility in sheep ventricular myocytes from sheep with heart failure

6.1 Introduction

Heart failure (HF) is a leading cause of morbidity and mortality and can manifest as systolic or diastolic dysfunction (Lloyd-Jones *et al.*, 2002). This results in a variety of symptoms such as laboured breathing, fatigue and peripheral oedema (swelling due to fluid accumulation) (King *et al.*, 2012). Those with heart failure will often have an ejection fraction of < 40 % however new terminology for patients with HF with a mid-range ejection fraction (40 – 49 %) has emerged to aid research and treatment of the subpopulation (Ponikowski *et al.*, 2016). Ejection fraction may be used as a predictor of mortality. Diagnosis for HF requires a combination of examinations and tests such as a review of medical history, chest radiography and electrocardiography (King *et al.*, 2012). The cellular basis of heart failure is attributed to the loss of cardiac myocytes and altered Ca handling (Briston *et al.*, 2011a; Mittmann *et al.*, 1998).

6.1.1 Altered Ca handling and contractility in heart failure

This study looks at the response of cells isolated from a HF large animal model to DOX and DAUN. Chapter 3 shows and DOX and DAUN alter Ca handling and contractility in cardiac myocytes, we also know from the research of others that Ca handling is altered (Baartscheer *et al.*, 2003a; Clarke *et al.*, 2015; Sipido *et al.*, 1998). Therefore, those with pre-existing heart conditions who also develop cancer may be at a higher risk of anthracycline-mediated complications due to certain adverse cell changes already present. Treatment with anthracyclines may amplify already existing cell changes associated with HF or effects may already be saturated to a level that anthracyclines do not increase alterations. Furthermore, both cancer and HF are associated with aging, while this is not directly relevant to childhood cancer it is still possible for children to have pre-existing heart conditions and have existing cell changes. This study seeks to understand the effect of anthracyclines on cells that already have a HF phenotype, as currently patients with underlying heart conditions are considered higher risk for anthracycline-induced cardiotoxicity (Armenian *et al.*, 2017).

As previously discussed, excitation-contraction coupling underpins heart function and alterations in this can result in a variety of clinical manifestations. While an increase in diastolic Ca has been found in cardiac myocytes isolated from human patients with HF (Sipido *et al.*, 1998), the impact on diastolic Ca in animal models of HF are inconsistent. For example, diastolic Ca was increased in a rabbit animal model of HF (Baartscheer *et al.*, 2003a) but decreases in a sheep model of HF (Briston *et al.*, 2011a). In a canine model of heart failure no change in diastolic Ca was identified (Hobai *et al.*, 2004).

In models of heart failure Ca transient amplitude has been found to be reduced (Clarke *et al.*, 2015; Hobai *et al.*, 2004; Piacentino *et al.*, 2003; Sipido *et al.*, 1998). While some found this reduction in amplitude could be explained by a decrease in SR Ca content (Hobai *et al.*, 2004; Piacentino *et al.*, 2003), in atrial myocytes from a model of HF, SR Ca content was increased (Baartscheer *et al.*, 2003b; Briston *et al.*, 2011a; Clarke *et al.*, 2015). Alterations in sheep ventricular cardiac myocytes Ca amplitude was likely due to decreased L type Ca current rather than a reduction in SR Ca (Briston *et al.*, 2011a).

In myocytes isolated from a HF animal model, intracellular Na has been found to be increased (Despa *et al.*, 2002) which favours reverse mode NCX activity facilitating the influx of Ca (Schillinger *et al.*, 2003). This Ca influx in HF may offset the effects in SR Ca load (Bers *et al.*, 2006). The initial increase in Na may be due to a decrease in Na removal via Na/K pump or increased influx of Na (Bers *et al.*, 2006).

Evidence also exists that suggests in end stage chronic heart failure the expression of SERCA is reduced (Kubo *et al.*, 2001). Interestingly, in the large animal model for HF the expression of SERCA and phospholamban were not altered (Briston *et al.*, 2011a). An integrated approach must be taken when observing the alterations in individual channels involved in Ca handling in heart failure models to understand the pathophysiological changes.

6.1.2 Heart failure and anthracyclines

OS has been found to contribute to the progression of various cardiovascular diseases and development of heart failure (Moris *et al.*, 2017a). As discussed in 4.1.3 ROS have been shown to alter Ca handling and contractility. A decrease in Ca transient amplitude, reduced SR Ca content and decreased SERCA activity are key observations in cardiac myocytes as a result of ROS (Greensmith *et al.*, 2010; Kim *et al.*, 2006b; Kuster *et al.*, 2010; Sag *et al.*, 2013).

Anthracyclines increase oxidative stress via stimulation of ROS production as discussed in 1.5.2.2 and evidenced in 4.3.3. DOX-induced OS has been shown to play a vital role in alteration to Ca handling and contractility in this study (chapter 4 and 5). The already elevated level of ROS in the heart failure model may lead to no further alterations in Ca handling and contractility as effects may be saturated as discussed in 6.1.1.

The potential of cardiac dysfunction is discussed with patients prior to treatment and those considered at higher risk may undergo prevention and screening strategies to allow intervention if warranted (Armenian *et al.*, 2017). Patients considered high risk are those on a high dose of anthracycline, compromised cardiac function and more than two cardiovascular risk factors such as obesity and diabetes (Armenian *et al.*, 2017). While some factors such as smoking would not be relevant for children some may suffer from other cardiac conditions and the effect anthracyclines in these patients must be considered. It is generally agreed that pre-existing heart conditions would be worsened by anthracycline treatment. The use of a heart failure model allows the identification of the effect of anthracyclines on cardiac myocytes isolated from a diseased state.

6.1.3 Aims

- Use integrative analysis to measure the effects of DOX and DAUN on ECC in the dynamic setting of intact ventricular myocytes from a sheep model of heart failure.

- Investigate if DOX and DAUN result in altered myofilament sensitivity in cells from a sheep model of heart failure.
- Establish if the effects of DOX and DAUN are enhanced in cardiac myocytes isolated from a heart failure model compared to a control model.

6.2 Methods

6.2.1 Heart failure model

Sheep used to produce a heart failure model were ~ 18 months old (matched to healthy model in previous results). Transvenous right ventricular tachypacing was utilised to induce heart failure. A pacing lead was positioned at the apex of the right ventricle and was attached to a Medtronic Thera cardiac pacemaker (located in a cervical subcutaneous pocket). After surgical recovery (10 - 14 days) the pacemaker was activated and tachypacing commenced. Hearts were paced at 210 bpm for ~ 50 days. Sheep were monitored for symptoms of heart failure such as lethargy and shortness of breath. To determine end-stage heart failure alterations in fractional shortening were determined using echocardiography on conscious, non-sedated animals. Animals were sacrificed once end-stage heart failure was determined. The same model methods are described in other articles (Clarke *et al.*, 2015; Dibb *et al.*, 2009).

All procedures to generate heart failure models were carried out at the University of Manchester by the Trafford/Dibb research group and were in accordance with the Animals (Scientific Procedures) Act, UK, 1986 and Directive 2010/63/EU of the European Parliament. All procedures carried out at the University of Manchester have ethical approval from the University of Manchester Welfare and Ethical Review board and the experiments have ethical approval from the University of Salford ethical review board.

6.2.2 Cell preparation

Primary left ventricular cardiomyocytes were isolated from ~18-month-old sheep with induced heart failure (see section 2.2.2). Cells were loaded with fura-2 (see 2.6.2) and stored in 0.95 mM Ca by combining NT and taurine solution (see 2.6.2) in equal parts.

6.2.3 Excitation-contraction coupling measurements

As described in section 2.5 cells were paced at 0.5 Hz using field stimulation in NT. Ca and sarcomere length transients were obtained in NT once steady state was reached before switching to 1 nM DOX or DAUN. After a new steady stage was established, the reversibility of any effect was determined by washing out the drug using NT. Recordings were calibrated and analysed offline using custom software as described in section 2.8.

6.2.4 Measurement of sarcoplasmic reticulum Ca content and Ca removal mechanisms

10 mM caffeine was utilised to estimate changes in SR Ca content. Caffeine increases the open probability of RyR and results in mass Ca release from the SR (Eisner *et al.*, 2017).

The rate constant of decay of systolic Ca transients (k_{sys}) represents the removal of Ca via main mechanisms. In mammalian cardiac myocytes these are NCX and SERCA as discussed in 1.2.9.1. The RC of decay of the caffeine-evoked Ca transient (k_{caff}) represents the activity of NCX due to caffeine rendering the activity of SERCA ineffective. Subtraction of k_{caff} from k_{sys} represents the activity of SERCA (k_{SERCA}).

6.2.5 Myofilament sensitivity measurements

Phase-plane loops were plotted as described in 3.2.4. The loops proceed counter-clockwise with cell shortening depicted by the ascending limb (right hand side of loop). The EC_{50} for each cells phase-plane loop was determined to quantify any change in myofilament sensitivity. Statistical significance was taken as $p < 0.05$ and calculated using a paired T-test.

6.3 Results

6.3.1 A detailed investigation of the effects of DOX on Ca and contractility in a heart failure model

The specimen traces in figure 6.1 show the effect of 1 nM DOX on intracellular Ca and contractility from a heart failure model and are representative of the means shown in figure 6.2. On average, 1 nM DOX had no significant effect on diastolic Ca (n = 18, p = 0.144; fig 6.2A) or diastolic sarcomere length (n = 18, p + 0.718; fig 6.2B). However, diastolic Ca significantly decreased by 6.32 % ± 2.78 % in washout (n = 18, p = 0.03; fig 6.2A) accompanied by a significant decrease in diastolic sarcomere length by 0.58 % ± 0.26 % (n = 18, p = 0.021; fig 6.2B). On average, DOX reduced Ca transient amplitude by 22.73 % ± 2.95 % (n = 19, p < 0.001; fig 6.2C) and did not recover in washout with a decrease of 30.3 % ± 4.76 % (n = 19, p < 0.001; fig 6.2C). This effect on Ca was accompanied by a 27.51 % ± 4.98 % (n = 16, p = 0.025) decrease in sarcomere shortening which recovered upon washout (n =16, p = 0.741; fig 6.2D). On average, peak Ca decreased by 14.28 % ± 1.94 % (n = 19 < 0.001) and did not recover upon washout (n = 19, p < 0.001; fig 6.2E). On average, the rate of sarcomere relaxation decreased by 19.52 % ± 4.61 (n = 16, p = 0.02) and recovered upon washout (n = 16, p = 0.273; fig 6.2F).

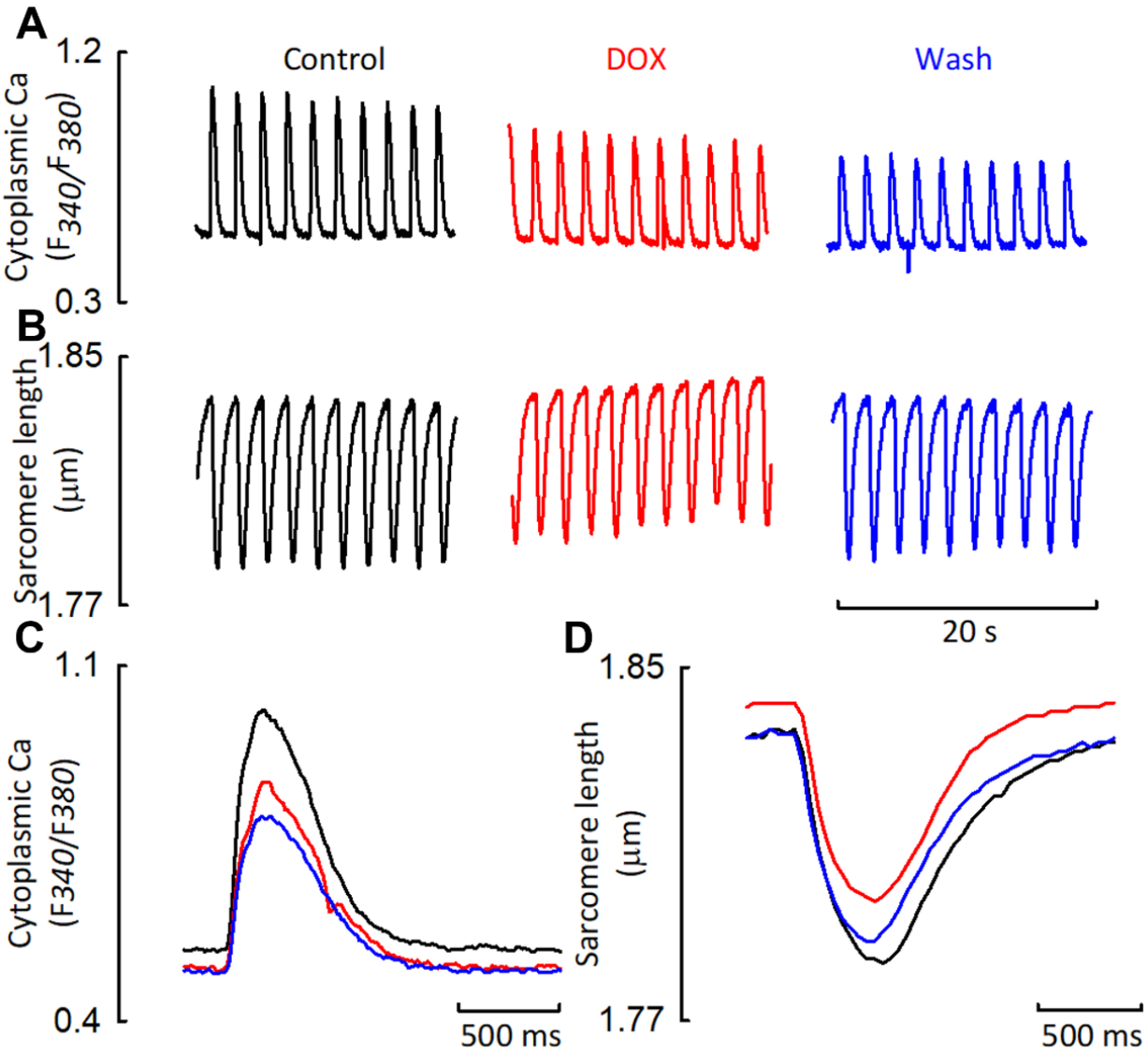


Figure 6.1: The effect of 1 nM DOX on cytoplasmic Ca and sarcomere shortening in cardiac myocytes from a heart failure model. (A) long time base record that shows specimen Ca transients. (B) specimen contractility transients. (C) Ca transient overlays. (D) contractility transient overlays. Long time base records represent steady state from which five Ca transients were averaged to produce the overlays. Data from this single cell is representative of the mean data shown in fig 6.2.

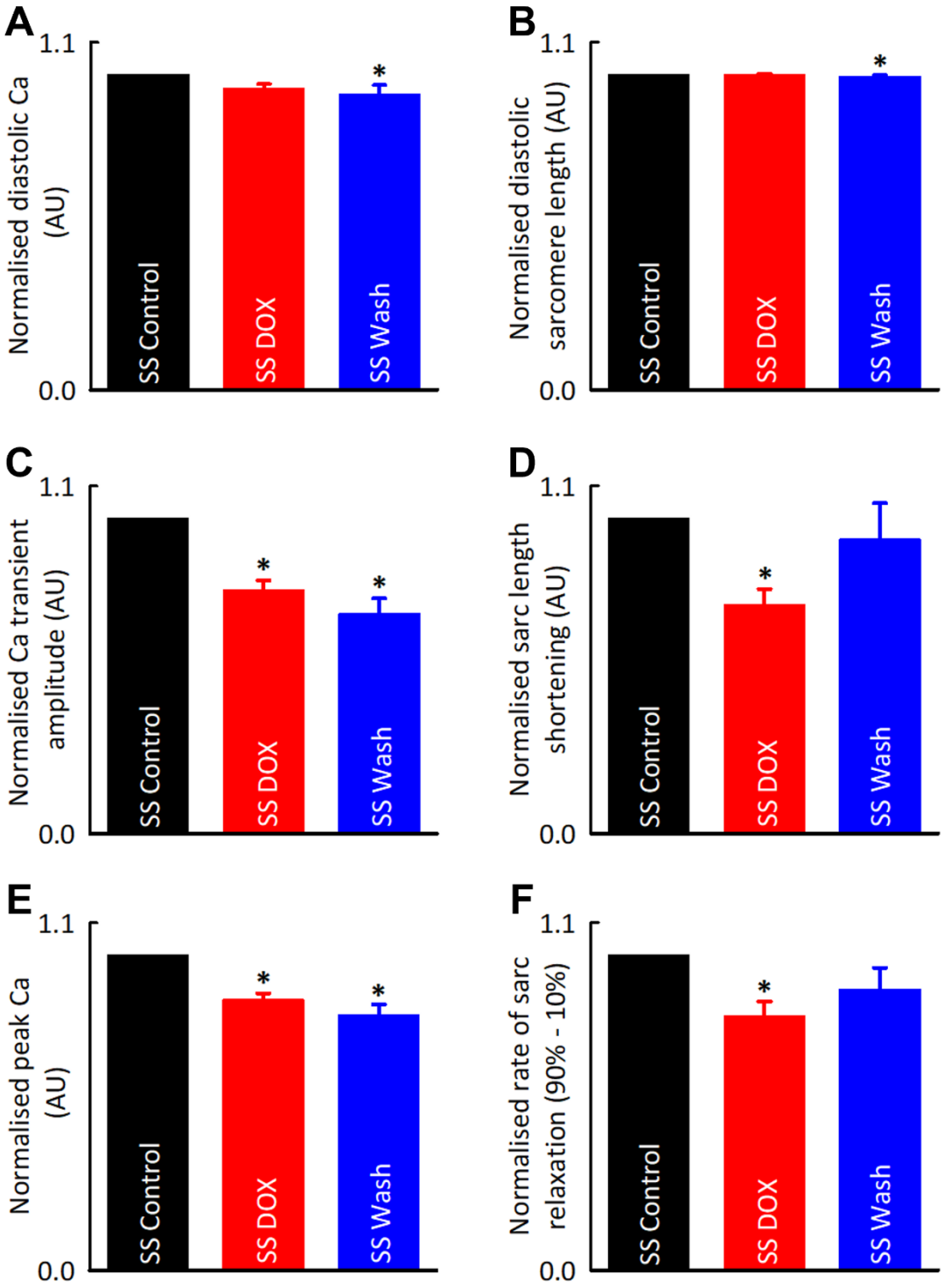


Figure 6.2: The effect of 1 nM DOX on intracellular Ca and contractility in sheep ventricular myocytes from a heart failure model. (A) mean normalised diastolic Ca. (B) mean normalised diastolic sarcomere length. (C) mean normalised Ca transient amplitude. (D) mean normalised

sarcomere shortening. (E) mean normalised peak Ca. (F) mean normalised 90-10 % decay time of sarcomere length. n = 16 - 19 (from 3 animals), * denotes statistical significance.

6.3.2 The effect of doxorubicin on SR Ca content and rate of Ca removal in a heart failure model

As described in section 6.2 caffeine enables the investigation of changes in SR Ca content. The specimen shown in figure 6.3A shows the effect of 1 nM DOX on the amplitude of the caffeine evoked Ca transient. On average, this reduced by 28.47 % ± 2.19 (n = 9, $p < 0.001$) progressing to a 48.28 % ± 4 % reduction in washout (n = 9, $p < 0.001$; fig 6.4A). Specimen figure 6.3B illustrates the effect of DOX on the Ca transient rate of constant decay (k_{sys}). k_{sys} was unaltered by DOX (n = 19, p = 0.673; fig 6.4B). The caffeine-evoked Ca transient rate constant of decay (k_{caff}) was not significantly altered (n = 9, p = 0.238; fig 6.4C). On average, k_{SERCA} was not significantly altered (n = 9, p = 0.572; fig 6.4D).

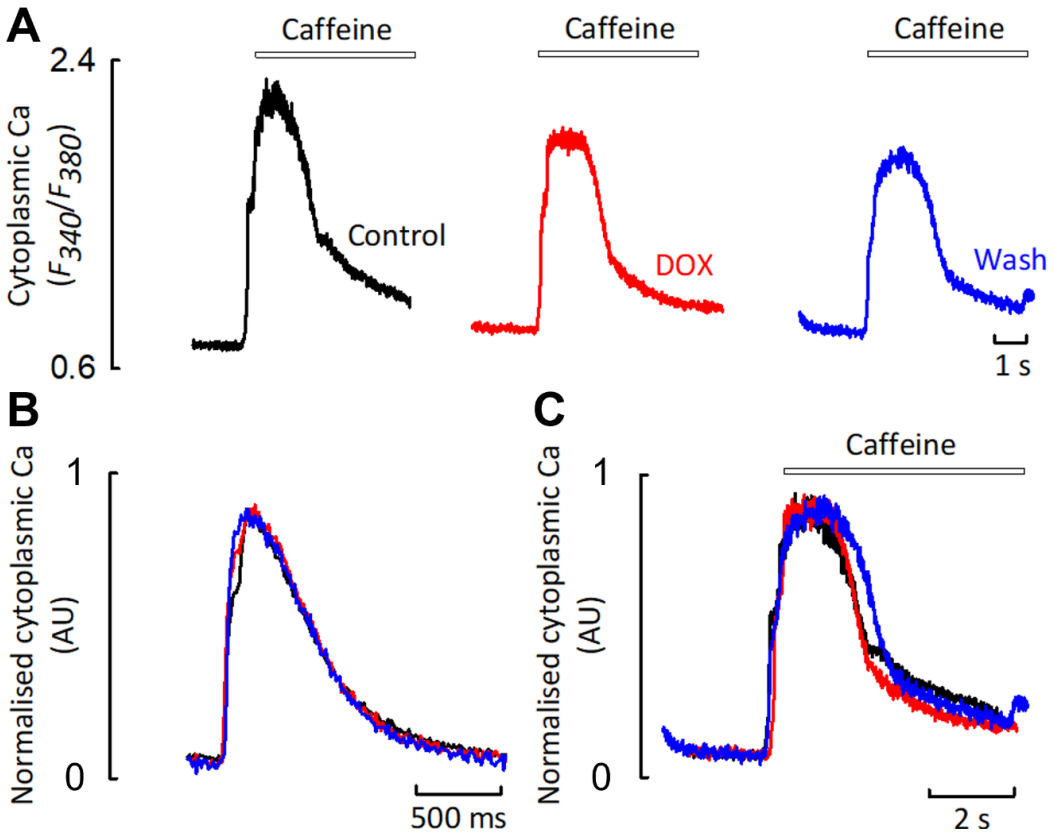


Figure 6.3: The effect of 1 nM DOX on SR Ca content in a heart failure model. (A) specimen Ca transients in the presence of caffeine. (B) specimen normalised Ca transients to permit direct comparison of the rate of Ca decay. (C) specimen normalised Ca transients to permit direct comparison of the rate of Ca decay in the presence of caffeine.

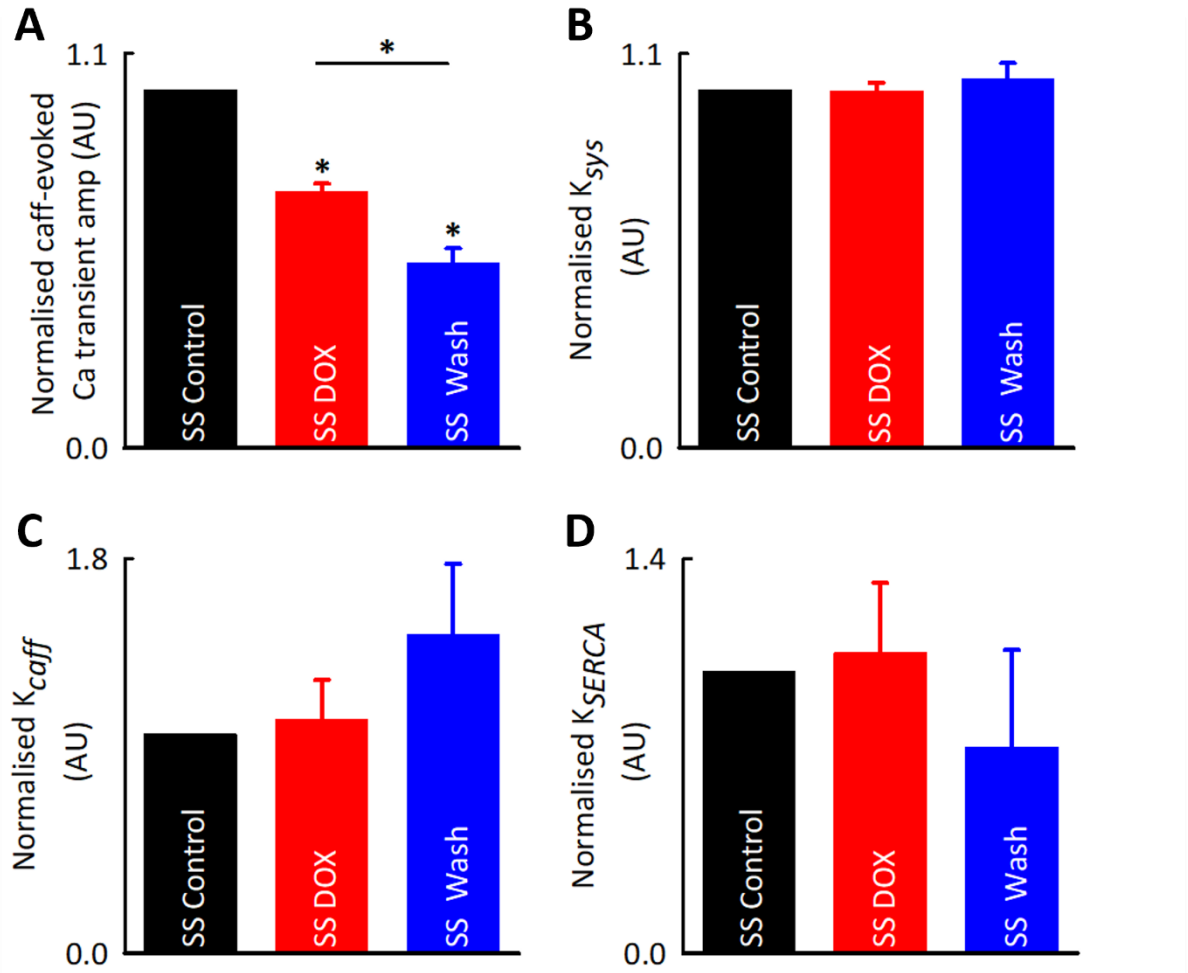


Figure 6.4: The effect of 1 nM DOX on Ca removal in sheep ventricular myocytes from a heart failure model. (A) mean normalised amplitude of caffeine evoked Ca transient. (B) mean normalised k_{sys}. (C) normalised mean k_{caff}. (D) mean normalised k_{SERCA}. n = 9 (from 3 animals), * denotes statistical significance.

6.3.3 A detailed investigation of the effects of daunorubicin on Ca and contractility in a heart failure model

The specimen traces in figure 6.5 show the effect of 1 nM DAUN on intracellular Ca and contractility. On average, diastolic Ca was not altered by 1 nM DAUN (n = 12, p = 0.249), however a significant decrease of 4.03 % ± 1.79 % was observed upon wash out (n = 12, p = 0.03; fig 6.6A). On average, diastolic sarcomere length was not significantly altered in DAUN (n = 11, p = 0.338; fig 6.6B). A significant decrease in Ca transient amplitude of 24.28 % ± 3.56 % (n = 12, p < 0.001) was produced by DAUN which did not recover upon wash (n = 12, p < 0.001; fig 6.6C). This was accompanied by a 33.34 % ± 9.74 % decrease in sarcomere shortening (n = 10, p < 0.01) that recovered upon washout (n = 11, p = 0.999; fig 6.6D). On average, on the Ca transient rate of constant decay (k_{sys}) was unaltered (n = 12, p = 0.353; fig 6.6E). On average, sarcomere relaxation was not significantly altered (n = 9, p = 0.156; fig 6.6F).

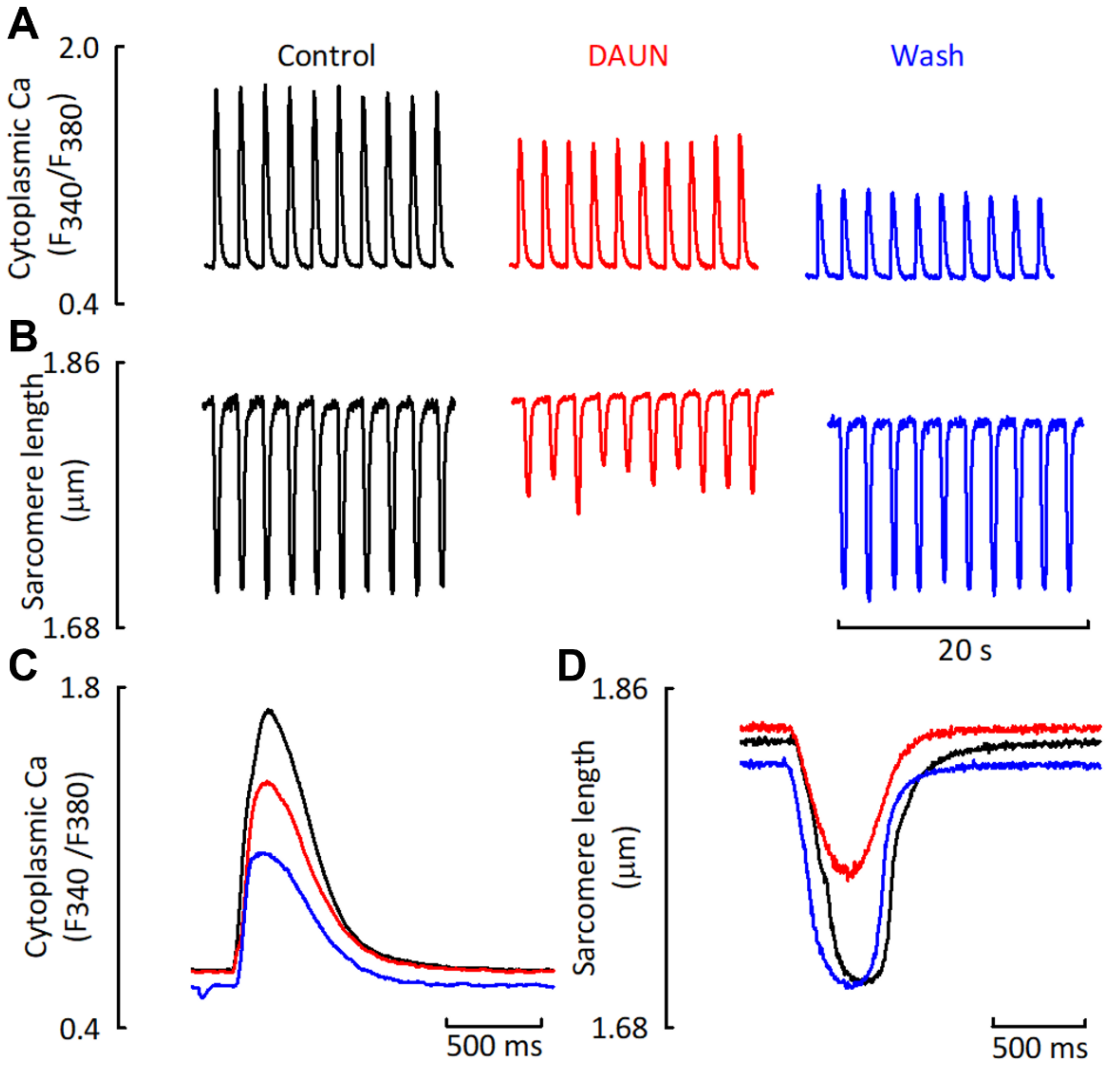


Figure 6.5: The effect of 1 nM DAUN on cytoplasmic Ca and sarcomere shortening in cardiac myocytes from a heart failure model. (A) long time base record that shows specimen Ca transients. (B) specimen contractility transients. (C) Ca transient overlays. (D) contractility transient overlays. Long time base records represent steady state from which five Ca transients were averaged to produce the overlays. Data from this single cell is representative of the mean data shown in figure 6.6.

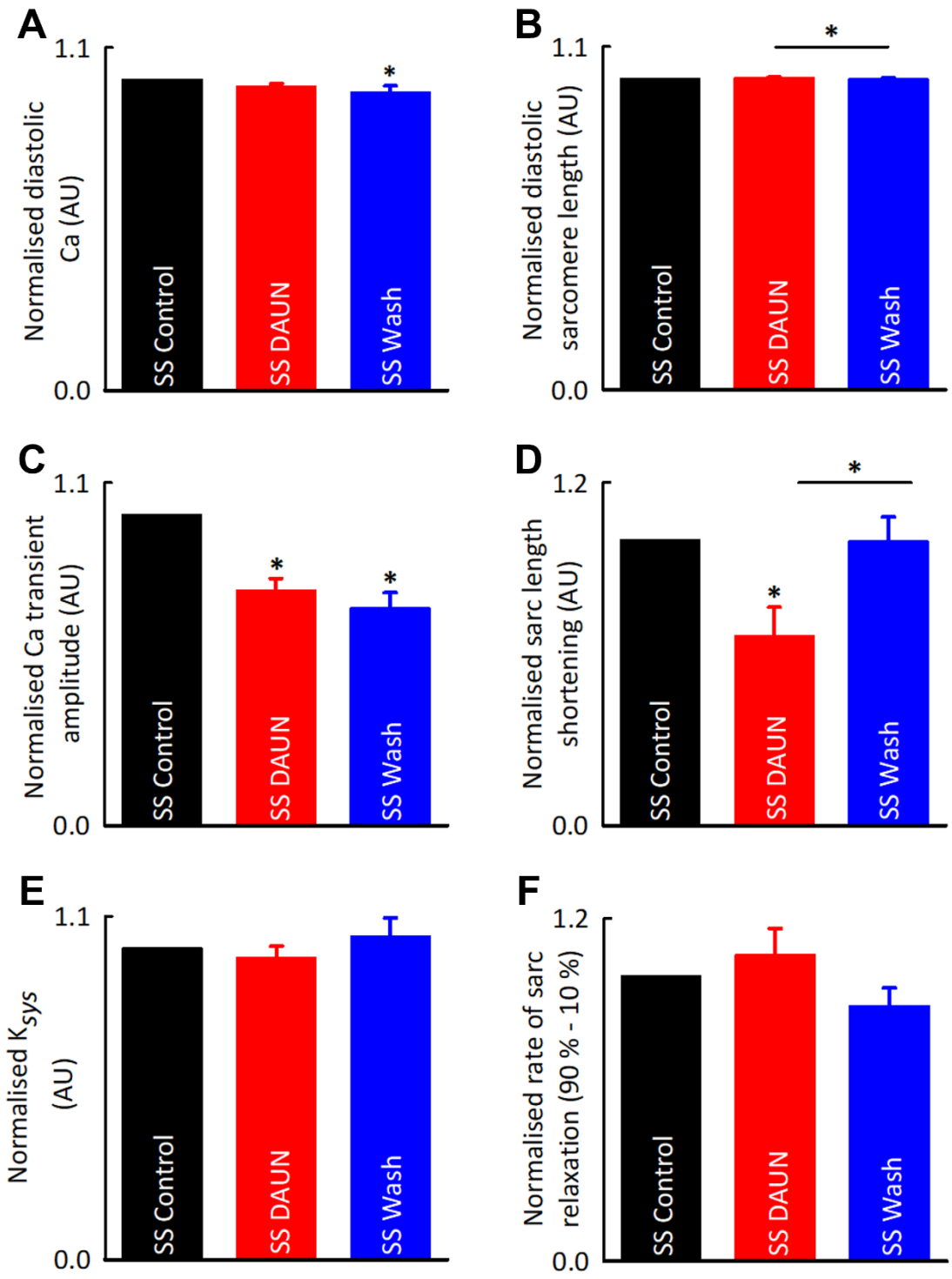


Figure 6.6: The effect of 1 nM DAUN on intracellular Ca and contractility in sheep ventricular myocytes from a heart failure model. (A) mean normalised diastolic Ca. (B) mean normalised diastolic sarcomere length. (C) mean normalised Ca transient amplitude. (D) mean

normalised sarcomere shortening. (E) mean normalised peak Ca. (F) mean normalised 90 – 10 % decay time of sarcomere length. n = 9 -12 (from 3 animals), * denotes statistical significance.

6.3.4 The effects of doxorubicin and daunorubicin on myofilament sensitivity in a heart failure model

Figure 6.7A and C show representative phase-plane loops for cells treated with 1 nM DOX and DAUN from a heart failure model. Phase-plane loops proceed counter-clockwise and plot the change of cytoplasmic Ca versus the change of sarcomere length in the absence and presence of DOX and DAUN. Neither DOX or DAUN resulted in a shift leftward or rightward in the loops (fig 6.7A & C). To quantify if a shift occurred the intracellular Ca concentration required for half relaxation (EC_{50}) was measured. On average, DOX and DAUN did not alter the EC_{50} (n = 18, p = 0.204; fig 6.7B) and (n = 12, p = 0.401; fig 6.7D) respectively.

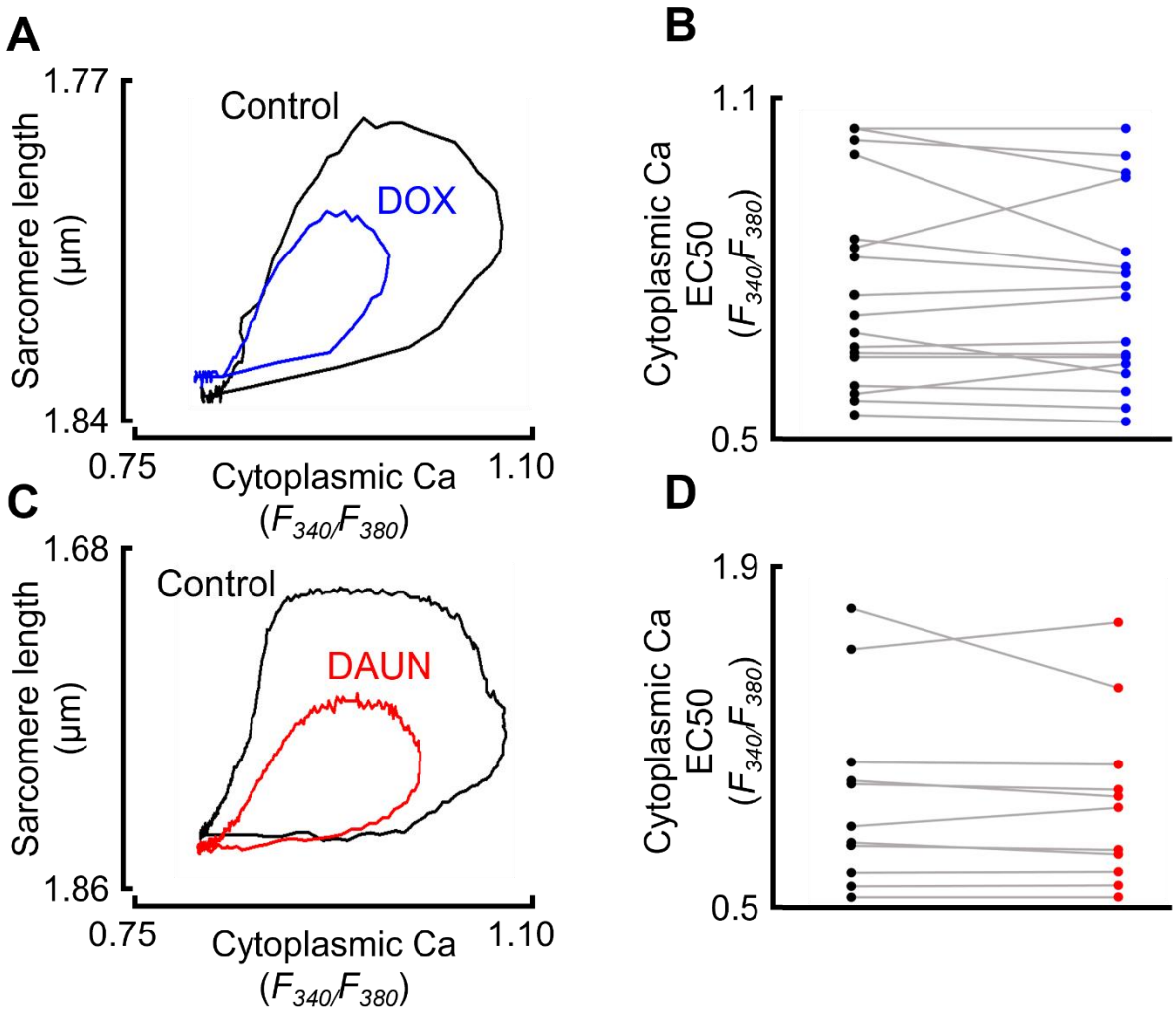


Figure 6.7: The effect of DOX and DAUN on myofilament sensitivity in sheep left ventricular myocytes from a heart failure model. (A) phase-plane loop of sarcomere length versus cytoplasmic Ca when treated with 1nM DOX. (B) EC_{50} (cytoplasmic Ca at 50 % relaxation) of cells in NT compared to 1nM DOX treatment. (C) phase-plane loop of sarcomere length versus cytoplasmic Ca when treated with 1nM DAUN. (D) EC_{50} of cells in NT compared to 1nM DAUN treatment. n = 12 - 18 (from 3 animals), * denotes statistical significance.

6.3.5 A comparison of the effects of DOX on global Ca and contractility in cells from healthy and HF sheep

Data from healthy sheep presented in chapter 3 was used for comparison purposes.

In cells from healthy sheep, the effect of DOX on the Ca transient amplitude was significantly greater than that in cells from HF sheep (healthy; - 39 %, HF; - 23 %, n = 19, p = 0.0023; fig 6.8A). On average, the effect of DOX on peak Ca was not significantly different between cells from healthy and HF sheep (n = 19, p = 0.355; fig 6.8C). In cells from healthy sheep, the effect of DOX on sarcomere length shortening was greater than that in cells from HF sheep (healthy; - 42 %, HF; - 28 %, n =16, p = 0.036; fig 6.8B). On average, the effect of DOX on the rate of sarcomere relaxation was greater in cells from healthy sheep than that of cells from HF sheep (healthy; + 39 %, HF; - 20 %, n = 16, p < 0.001; fig 6.8D).

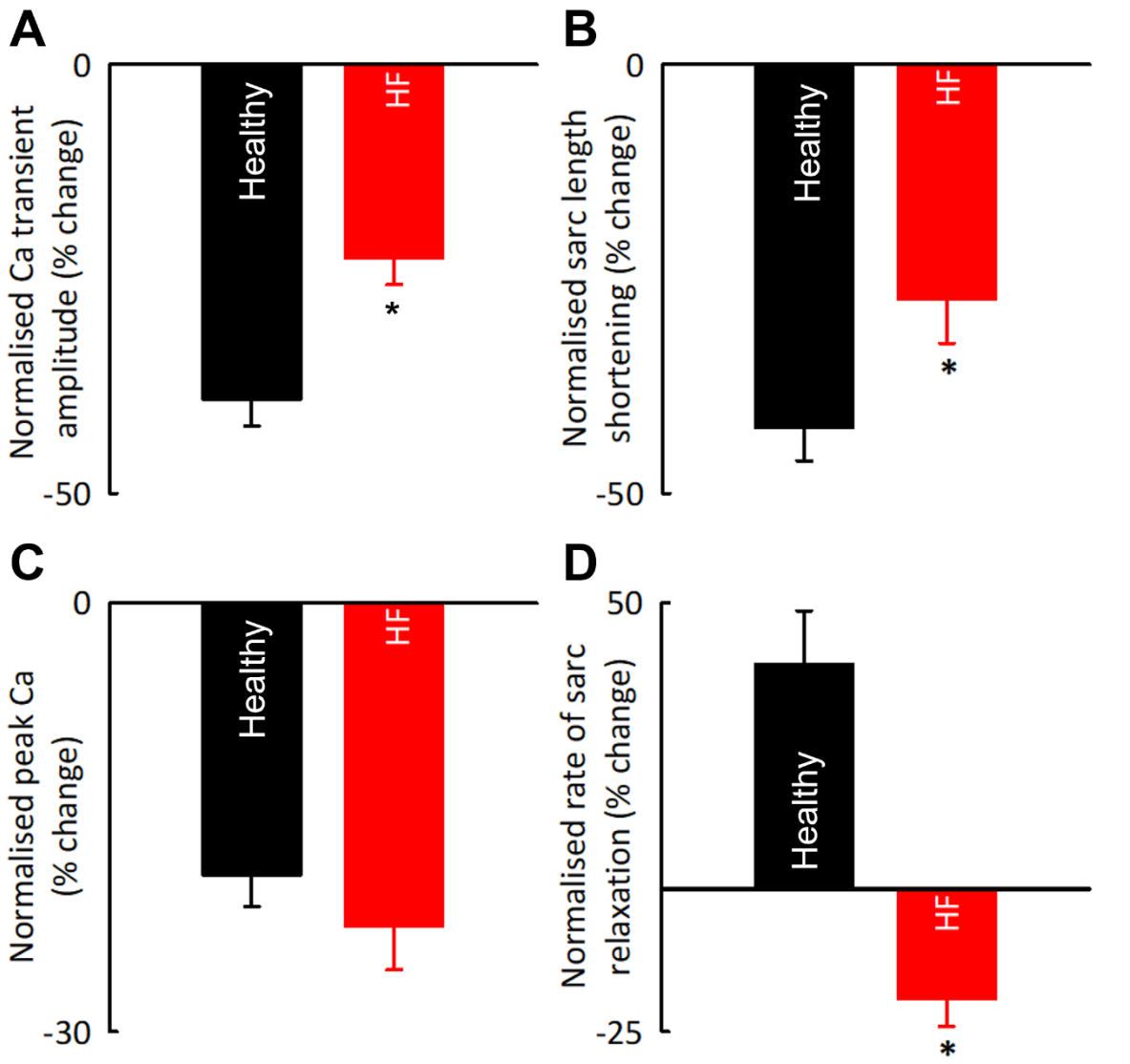


Figure 6.8: The effect of 1 nM DOX on Ca handling and contractility in healthy versus heart failure animal model. (A) mean normalised percentage change in Ca transient amplitude. (B) mean normalised percentage change in sarcomere length shortening. (C) mean normalised percentage change in peak Ca. (D) mean normalised percentage change in rate of sarcomere relaxation. n = 16 – 49 (from 3 – 9 animals), * denotes statistical significance.

6.3.6 A comparison of the effects of DOX on SR Ca and Ca removal in cells from healthy and HF sheep

No significant difference in the effect of 1 nM DOX on the amplitude of the caffeine evoked Ca transient between cells from healthy and those from HF sheep was identified (n = 9, p = 0.598; fig 6.9A). On average, the in cells from healthy sheep the effect of DOX on the Ca transient of constant decay (k_{sys}) was greater than that of cells from HF sheep (healthy; - 17 %, HF; - 0.4 %, n = 19, p = 0.0018; fig 6.9B).The effect of DOX on k_{caff} in cells from healthy sheep was greater than that of cells from HF sheep however this was not statistically significant (healthy; + 64 %, HF; + 7 %, n = 9, p = 0.19; fig 6.9C). The effect of DOX on k_{SERCA} was greater in cells from healthy sheep compared to HF however this was not statistically significant (healthy; - 34 %, HF; + 7 %, n = 9, p = 0.058; fig 6.9D).

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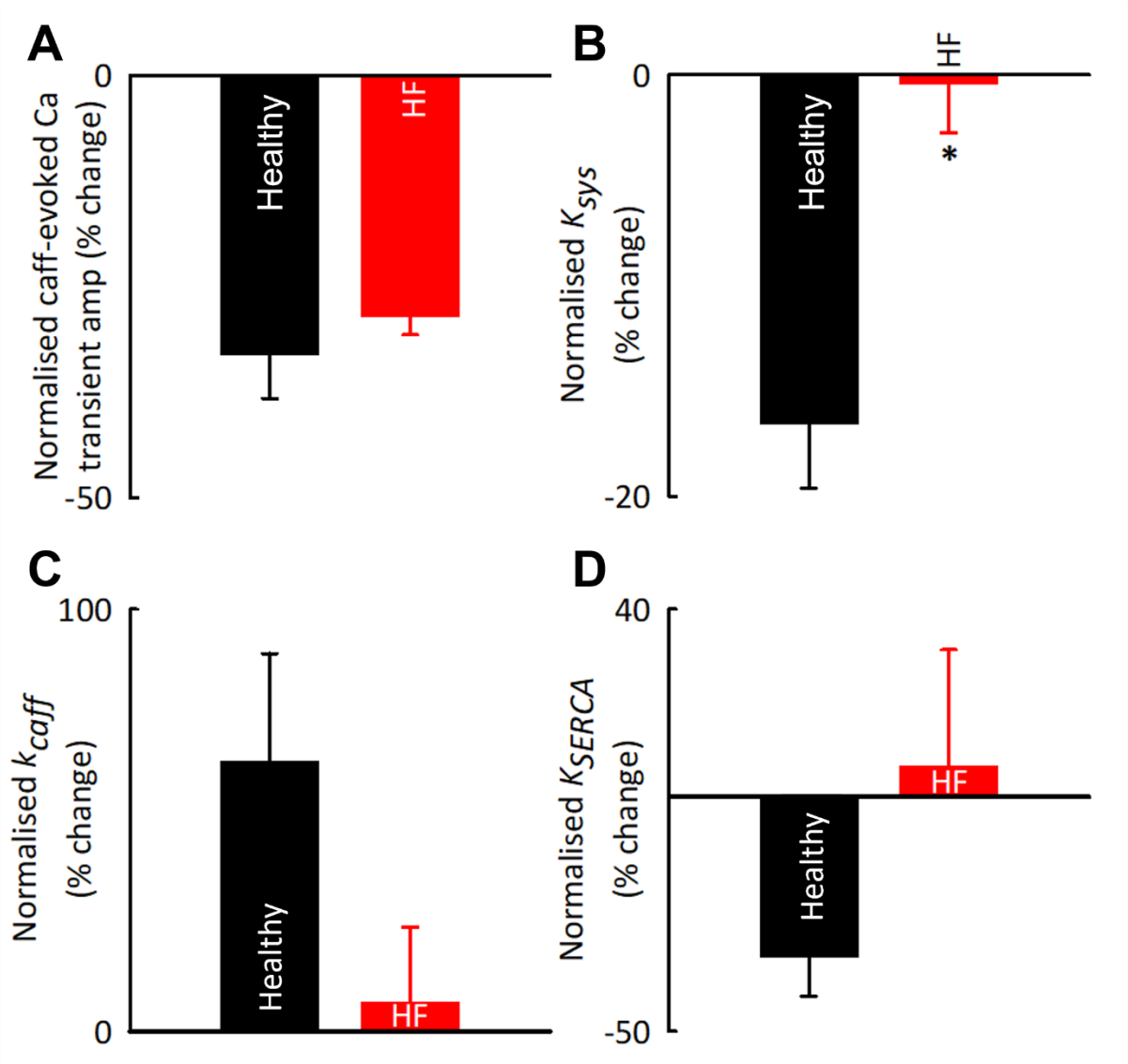

Figure 6.9: The effect of 1 nM DOX on Ca removal in sheep ventricular myocytes isolated from healthy versus heart failure animal model. (A) mean normalised percentage change in the amplitude of caffeine evoked Ca transient (B) mean normalised percentage change k_{sys}. (C) mean normalised percentage change k_{caff}. (D) mean normalised percentage change in k_{SERCA}. n = 9 – 19 (from 3 - 5 animals), * denotes statistical significance.

6.3.7 Alterations in response to daunorubicin in Ca handling and contractility between a healthy and heart failure model

In cells from healthy sheep, the effect of DAUN on the Ca transient amplitude was significantly greater than that in cells form HF sheep (healthy; - 45 %, HF; - 25 %, n = 12, $p < 0.001$; fig 6.10A). On average, the effect of DAUN on Ca transient of constant decay (k_{sys}) was greater in cells from healthy compared to cells from HF sheep (healthy; - 23 %, HF; - 3%, n = 12, p = 0.0035; fig 6.10C). In cells from healthy sheep, the effect of DAUN on sarcomere length shortening was greater than that in cells from HF sheep (healthy; - 64 %, HF; - 33 %, n = 10, p = 0.0026; fig 6.10B). The rate of sarcomere relaxation was affected to greater extent by DAUN in cells form healthy sheep compared to cells from HF sheep (healthy; + 88 %, HF; + 7 %, n = 9, p = 0.042; fig 6.10D).

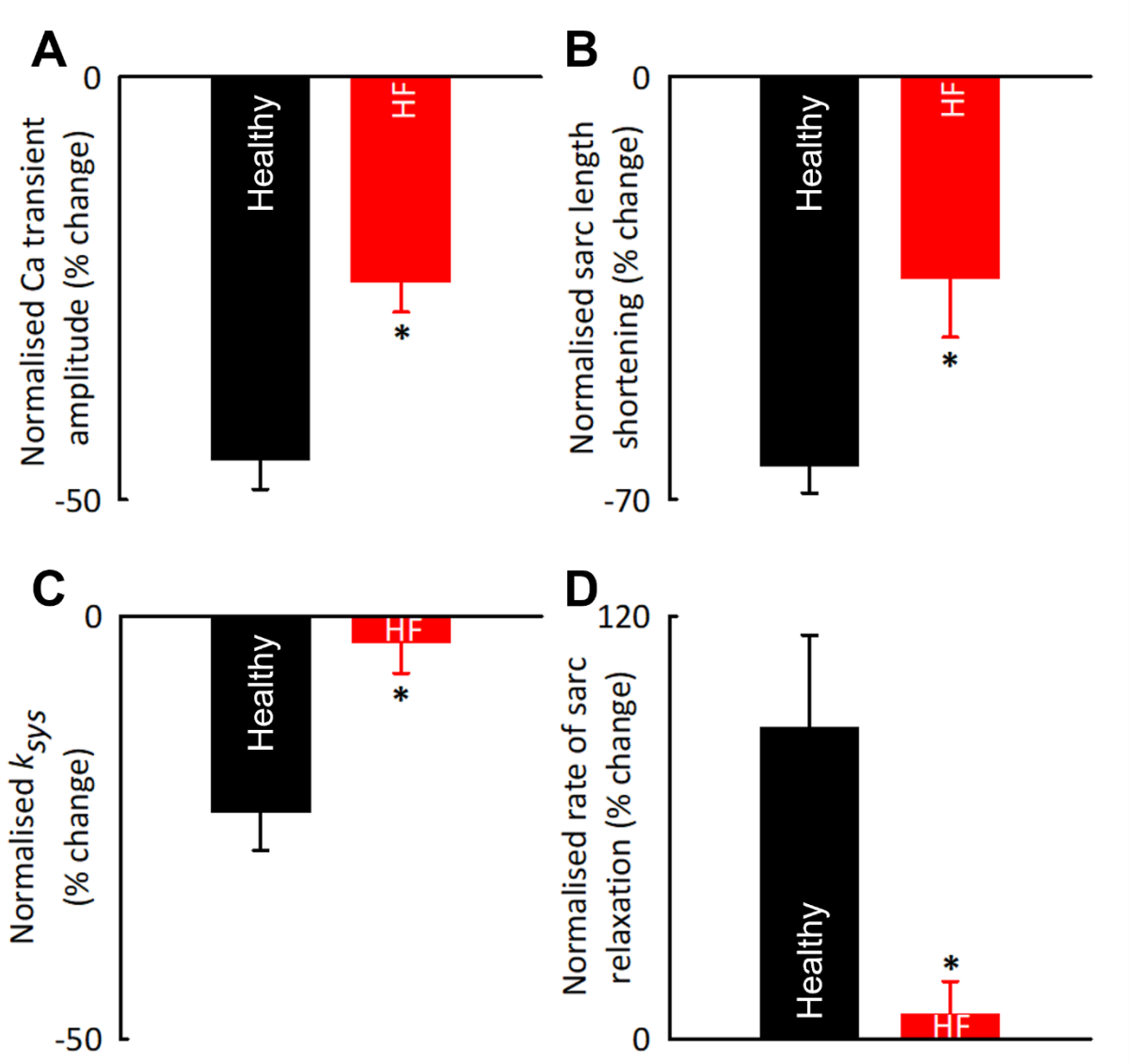

Figure 6.10: The effect of 1 nM DAUN on Ca handling and contractility in healthy versus heart failure animal model. (A) mean normalised percentage change in Ca transient amplitude. (B) mean normalised percentage change in sarcomere length shortening. (C) mean normalised percentage change in k_{sys}. (D) mean normalised percentage change in rate of sarcomere relaxation. n = 9 – 20 (from 3 – 6 animals), * denotes statistical significance.

6.4 Discussion

6.4.1 Does 1 nM doxorubicin alter intracellular Ca and contractility in sheep cardiomyocytes isolated from a heart failure model?

1 nM DOX did not alter diastolic Ca or sarcomere length in sheep ventricular myocytes isolated from a heart failure model (fig 6.2), however the decrease upon washout was significant. Briston *et al* (2011) found a decrease in diastolic Ca in the sheep model of HF. If a decrease in diastolic Ca occurs our findings suggest that DOX has an irreversible effect on diastolic Ca leading to a further reduction upon washout.

In HF cells DOX resulted in an irreversible decrease in Ca transient amplitude (fig 6.2C) which was accompanied by a decrease in sarcomere shortening that did recover upon washout (fig 6.2D). As discussed in 6.1.1 Ca transient amplitude is known to reduced in HF (Clarke *et al.*, 2015; Hobai *et al.*, 2004; Piacentino *et al.*, 2003; Sipido *et al.*, 1998). ROS are also known to be increased in HF (Moris *et al*., 2017) and can result in a decrease in Ca amplitude (Goldhaber & Liu, 1994; Greensmith *et al.*, 2010; Kuster *et al.*, 2010) (see 4.1.2). The effect of DOX on Ca amplitude was not saturated by the effects of HF alone and so the rise in ROS by DOX may not be the only influence on Ca amplitude and sarcomere shortening. This is supported by a reduction in Ca amplitude and sarcomere shortening still being evident due to DOX after ROS inhibition (see 4.3.5).

The change in Ca transient amplitude is supported by a sustained decrease in peak Ca (fig 6.2E). Peak Ca represents absolute systolic Ca levels whereas Ca transient amplitude represents the difference between peak Ca and diastolic Ca (Greensmith, 2014b). Therefore, a change in diastolic Ca may offset any change to peak Ca resulting in amplitude being unaltered. The alteration in both amplitude and peak Ca supports no alteration in diastolic Ca.

The rate of sarcomere relaxation was decreased by 1 nM DOX in cells from HF (fig 6.2F) which suggests an increase in Ca removal however this was not detected as discussed in 6.4.2.

6.4.2 Is SR Ca and the activity of the Ca removal mechanisms altered by 1 nM doxorubicin in a heart failure model?

DOX decreased SR Ca content in cardiac myocytes isolated from HF model. The amplitude of the Ca transient is proportional to the third power of SR Ca (Dibb *et al.*, 2007). DOX reduced SR Ca content to 71.5 % of control (fig 6.4A). This would be expected to decrease the Ca transient amplitude by $(0.715)^3$ = 36.6 %. The Ca transient amplitude decreased to 77 % indicating that SR Ca content may not be the only factor responsible for the reduction in Ca transient amplitude. This is supported by (Clarke *et al.*, 2015). Clarke *et al* (2015) found HF SR Ca content was increased but Ca transient amplitude was reduced, they determined that a decrease in the L-type Ca current underpinned the alteration to Ca transient amplitude. Therefore, it may be in this model that the L-type Ca channel plays a greater role in reducing the Ca transient amplitude than SR Ca content.

K_{sys}, k_{caff} and k_{SERCA} were not significantly altered by DOX in HF cells (fig 6.4). This suggests that Ca removal is not responsible for the decrease in SR Ca content. It is possible that an increase in RyR leak results in a reduction in SR CA content (Bers *et al.*, 2003).

6.4.3 Does 1 nM daunorubicin alter intracellular Ca and contractility in sheep cardiomyocytes isolated from a heart failure model?

Diastolic Ca and sarcomere length were unaltered in 1 nM DAUN (fig 6.6A & B). This response is similar to that seen in cells isolated from control animals (see fig 3.9). This suggests that DAUN does not alter diastolic Ca or sarcomere length regardless of model. An irreversible decrease in Ca transient amplitude was detected (fig 6.6C) and this was accompanied by a reversible decrease in sarcomere shortening (fig 6.6D). As discussed, Ca amplitude is reduced in HF models (see 6.1.1). Treatment with DAUN prevents recovery of Ca transient amplitude in HF cells suggesting that the effect is too severe to be reversed. Sarcomere shortening recovers upon wash which may be due to an increase in myofilament sensitivity (discussed in

6.4.4). k_{sys} and rate of sarcomere relaxation were not altered by DAUN (fig 6.6E & 6.6F). While the effect on Ca removal could not be tested due to the previously mentioned interaction between DAUN and caffeine (see 3.3.6), the lack of change in k_{sys} suggests no change in Ca removal. This was the case for DOX (see 6.4.2).

6.4.4 Do doxorubicin and daunorubicin alter myofilament sensitivity in a heart failure model?

DOX and DAUN did not alter myofilament sensitivity in cells isolated from HF (fig 6.7) however in cells from healthy cells an increase in myofilament sensitivity was detected (see figure 3.12).

Myofilament sensitivity has been found to be increased in cardiac myocytes isolated from mammalian models of heart failure (van der Velden *et al.*, 2003) but the increase in sensitivity to Ca does not result in an increase in force due to changes in pH (van Der Velden *et al.*, 2001). This increase in sensitivity is thought to be due to reduction of β-adrenergic phosphorylation of troponin 1 (Wolff *et al.*, 1996). However, no change in myofilament sensitivity regardless of HF severity has also been found (Denvir *et al.*, 1995). While these studies provide insight into the possible mechanisms of increased myofilament sensitivity in HF, the issue with human samples is the lack of a control due to treatment already carried out in patients (Marston & de Tombe, 2008).

If in the case of the studies discussed myofilament sensitivity is increased in the sheep HF model, then our results suggest that the effect is saturated to the extent that neither DOX nor DAUN increase sensitivity further.

6.4.5 Do the effects of doxorubicin on Ca handling and contractility differ between a healthy and heart failure model?

In cells from the HF model Ca amplitude was reduced to a lesser extent by DOX than in control (fig 6.8A). This may be due to the fact the baseline Ca transient amplitude is already reduced in HF model as discussed in 6.1.1. ROS has been found to be elevated in HF (Hill & Singal,

1996; Ide *et al.*, 1999; Moris *et al.*, 2017a). ROS reduced Ca transient amplitude in cardiac myocytes (Greensmith *et al.*, 2010; Kuster *et al.*, 2010) therefore is it logical that Ca transient amplitude will be at a reduced state in cells from HF compared to control. This may mean that the effects of DOX on Ca transient amplitude are saturated in the HF model.

DOX altered sarcomere shortening and sarcomere relaxation to a lesser extent in cells from the HF model (fig 6.8B & D). As discussed in 6.4.4 myofilament sensitivity may be increased in the HF model. DOX has also been shown to increase myofilament sensitivity (fig 3.12). The effects on myofilament sensitivity may be saturated in the HF model meaning that the effect of DOX on sarcomere shortening is to a lesser extent than would be expected.

There was no statistically significant difference in the reduction of SR Ca content between cells from healthy animals and cells from the HF model (fig 6.9A). However, k_{sys} was not altered in HF. This suggests that the effect of DOX on Ca removal channels such as SERCA and NCX differs in the HF model. This is not surprising given the notable difference in activity and expression of both channels in HF models. In HF SERCA expression has been found to be reduced (Zhihao *et al.*, 2020) resulting in a decrease in SERCA activity while NCX activity is increased but in reverse mode (Schillinger *et al.*, 2003). This suggests that less Ca is actively transported into the SR however more Ca is brought in through the reverse mode of NCX. Overall, these findings may explain why k_{sys} is not altered by DOX in the HF model as NCX and SERCA activity is not altered possibly due to the effect on the channels being saturated and/or altered expression.

In healthy cells DOX resulted in a decrease in SERCA activity (fig 3.7). The effects in HF suggest that the decrease in SERCA activity is saturated due to the alterations already present and aren't worsened by DOX as the activity of SERCA was not altered (fig 6.4D). NCX activity was not altered in HF cells by DOX (fig 6.4C) whereas in healthy cells the activity was increased (fig 3.7).

The decrease in SR Ca content in HF due to DOX cannot be accounted for by removal of Ca as is the case for cells from healthy sheep (see chapter 3). Therefore, as discussed in 3.1.1 DOX may alter L-type Ca channel activity as well as the open probability of RyR which can impact SR Ca content.

6.4.6 Do the effects of daunorubicin on Ca handling and contractility differ between a control and heart failure model?

The change in Ca transient amplitude was altered to a lesser extent in the heart failure model (fig 6.10A). The explanation for this may be similar to that discussed in 6.4.5, however results from chapter 4 found that DAUN only increased OS at a higher concentration (10 μM). As previously discussed, SR Ca content is a major determinant of Ca transient amplitude. Given the apparent interaction between caffeine and DAUN we were unable to investigate this. However, k_{sys} was unaltered in HF cells by DAUN indicating the combined activity of SERCA and NCX is unaltered. DAUN may alter the open probability of RyR and/or activation of L-type Ca channels as discussed in 3.1.1 which in turn may alter SR Ca content.

6.5 Concluding remarks

In summary, DOX and DAUN decrease Ca transient amplitude which may be partially explained by a decrease in SR Ca content. In HF this decrease does not recover upon washout as was the case for healthy cells suggesting cells from HF are less able to recover from the effects of the DOX and DAUN. In HF models DOX and DAUN may lead to an increase in the open probability of RyR or alteration in L-type Ca channel activation which could account for a reduction in SR Ca content that cannot be accounted for NCX and SERCA. An increase in myofilament sensitivity was detected in healthy cells treated with DOX and DAUN however this was not altered in the HF model. The potential shift in myofilament sensitivity already induced in the HF model may mean the effects are saturated to a point were DOX and DAUN cause no further alteration. The effects of DOX and DAUN on cells from a HF sheep are not amplified in comparison to the cells form the healthy model suggesting that rather than having an additive effect the alterations in Ca handling and contractility are saturated.

Chapter 7
General Discussion

7.1 The effects of doxorubicin and daunorubicin on excitation-contraction coupling

7.1.1 The effect of doxorubicin and daunorubicin on intracellular Ca and contractility

This study examined the effects of both DOX and DAUN on Ca handling and contractility in intact sheep cardiac myocytes. This is because, while other studies have previously investigated the effects of DOX on aspects such as Ca transient amplitude and contractility very few have studied the effects of DAUN with the assumption being the mechanism of cardiotoxicity is the same. Furthermore, most of these studies were conducted using artificial planar membranes or small animal models and look at components individually. Our experiments overcome this by using cardiomyocytes from a large animal model along with an integrative approach to study aspects of ECC in a dynamic setting.

In sheep cardiomyocytes DOX resulted in a decrease in Ca transient amplitude and sarcomere shortening (fig 3.5). This is supported by studies using other models (Maeda *et al.*, 1997; Sag *et al.*, 2011; Temma *et al.*, 1996; Zheng *et al.*, 2011). While these studies provide insight there are limitations. As mentioned most studies assume that DAUN will behave in the same way as DOX. While DAUN also decreased Ca transient amplitude and contractility it was to a greater extent than that observed with DOX (fig 3.9). This is interesting given that DAUN has been shown to result in less cardiotoxicity in childhood cancer survivors (Feijen *et al.*, 2015b). The effects of both DOX and DAUN on Ca amplitude and sarcomere shortening were reversed upon washout. This suggests that any interactions with transporters are reversible upon washout and do not cause permanent changes to channels and are able to recover.

Previous studies show DOX increases diastolic Ca (Mijares & López, 2001; Sag *et al.*, 2011). However, we are aware of no studies where the associated effects on cell / sarcomere length have been studied in parallel. In our study, DOX did not alter diastolic Ca or sarcomere length. Both studies that identified an increase in diastolic Ca were conducted in rat cardiac

myocytes. Although the fundamental processes of ECC are highly conserved it is recognised that small animal models may respond differently. This is due to evolutionary differences that result in changes in heart rate, cardiac output and contractile kinetics that are underpinned by variation in excitation and Ca handling (Milani-Nejad & Janssen, 2014) which may explain the difference in diastolic Ca.

7.1.2 The effect of doxorubicin and daunorubicin on SR Ca content

Other studies provide *indirect* evidence that suggests DOX reduces SR Ca content (Ondrias *et al.*, 1990; Sag *et al.*, 2011), though an effect of DAUN on SR Ca has yet to be reported. To our knowledge, we are the first to use a direct method to measure the effect of DOX *and* DAUN on SR Ca and show both produce a decrease.

As discussed in 1.2.9.2 this decrease in SR Ca content can account for the reduced Ca transient amplitude as shown in 3.4.3 and 3.4.5. We acknowledge that we do not know if DOX or DAUN alter intracellular Ca buffering; thus, the relationship between total SR Ca release and free Ca (to which fura–2 fluorescence is proportional), therefore it is not possible to quantify the absolute change of SR Ca content (Trafford *et al.*, 1999a; Varro *et al.*, 1993). Nonetheless, as the relative change of caffeine-evoked $[Ca]_I$ transient amplitude is greater than could be accounted for by a change to buffering, we are confident that a reduction of SR Ca is a key contributor to the reduced $[Ca]_I$ transient.

Our study also highlights a potential interaction between caffeine and DAUN. Others note that DAUN can bind to the RyR and this results in reduced SR Ca release in response to caffeine application (Abramson *et al.*, 1988). In effect, DAUN acts as a non-competitive inhibitor. If this were the case in our experiments, it would suggest that the reduction in SR Ca content we identified in figure 3.10 is underestimated. This potential interaction was brought to our attention by a lack of change in k_{sys} in the presence of caffeine (that was reduced without caffeine) (see figure 3.11). This suggests that in the presence of caffeine DAUN no longer alters the overall removal of Ca.

7.1.3 The effect of doxorubicin and daunorubicin on the mechanisms of Ca removal

DOX was found to decrease SERCA activity and Hanna e*t al* (2014) have identified DOX can bind to SERCA, therefore it may alter SERCA activity through this interaction. The effect of DOX on NCX has been investigated to a lesser extent with some studies identifying no change in protein expression (Llach *et al.*, 2019) and others finding a decrease in expression (Olson *et al.*, 2005). However, alterations to the activity of the transporter remain unknown. As discussed in 1.2.9.4 SERCA and NCX are powerful regulators of SR Ca content (Eisner, 2014). Our study identified an increase in NCX activity that, along with the decrease in SERCA activity can explain the decrease in SR Ca content. The effect of DAUN on Ca removal could not be investigated due to the apparent interaction with caffeine as discussed in 7.1.2.

7.1.4 The effect of doxorubicin and daunorubicin on myofilament sensitivity to Ca

There are limited studies investigating the effects of DOX or DAUN on myofilament sensitivity as discussed in 3.4.6. Alteration in myofilament sensitivity alters the Ca-force relationship. For example, increased myofilament sensitivity leads to a leftward shift in the force versus Ca curve meaning that for any given Ca concentration the force of contraction is increased. Both DOX and DAUN showed evidence of increased myofilament sensitivity (see fig 3.12). This is supported by a study in female rats that found long term treatment with DOX (10 weeks) increased myofilament sensitivity (Rattanasopa *et al.*, 2019), however the Ca – force relationship was not altered by DOX in rabbit cardiac myocytes (Boucek *et al.*, 1993). There are very few studies that investigate the effect of DOX on myofilament sensitivity and to the best of our knowledge none that investigate the impact of DAUN on myofilament sensitivity. For all things being equal the reduction in Ca transient amplitude ought to produce a decrease in sarcomere shortening (see fig 1.8). In 3.4.2 I discuss that the decrease in Ca transient amplitude likely accounts for the reduction in sarcomere shortening however the effect of

myofilament sensitivity may make this relationship more complex. It appears that DOX and DAUN are acting as Ca sensitisers which would shift the relationship between Ca amplitude and force leftward. Clearly the net effect of DOX and DAUN is negative inotropy however given the effect on myofilament sensitivity that negative inotropic effect is likely offset.

7.1.5 An integrated model

Our study shows that in a large animal model DOX and DAUN decrease cardiac myocyte contractility which is underpinned by a reduction in systolic Ca. This is largely attributable to a decrease in SR Ca content. In DOX we identified a decrease in SERCA activity and an increase in NCX activity which can account for the overall reduction in SR Ca content. The same could not be established for DAUN due to an apparent caffeine interaction meaning further investigation is required. Both DOX and DAUN showed evidence of acting as Ca sensitisers as myofilament sensitivity was shown to be increased.

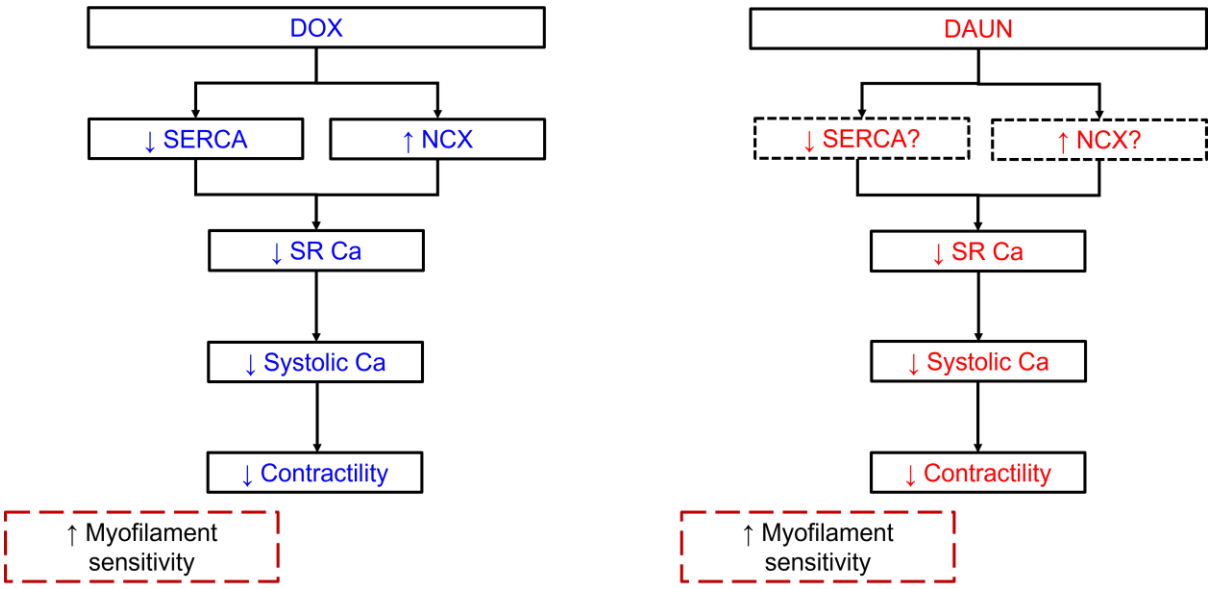

Figure 7.1: The effects of DOX and DAUN on Ca handling and contractility in sheep ventricular myocytes.

7.2 The role of oxidative stress in the anthracycline-mediated changes to excitation-contraction coupling

7.2.1 Do doxorubicin and daunorubicin increase oxidative stress?

DOX increased OS in a concentration-dependent manner (fig 4.2D) which is supported by others that have identified DOX results in an increase in ROS (Kim *et al.*, 2006a; Ma *et al.*, 2013; Zhou *et al.*, 2001). These studies are the first to show DOX not only increases ROS in cardiac myocytes but that this leads to an increase in OS and is evidenced by both the concentration and incubation time experiments as well as the addition of NAC which removed this OS response. An increase in OS was only present at the highest concentration of DAUN (10 µM) (fig 4.2C). Studies conducted to identify ROS production in cardiac myocytes due to DAUN are extremely limited with one study confirming that 1 µM DAUN did not increase ROS (Sawyer *et al.*, 1999).

The studies that show DOX increases ROS support our findings that DOX increases OS. This provides a clear basis for altered ECC as discussed in 7.2.3. Interestingly the OS response following DAUN treatment differs indicating that OS may not be responsible for the effects on ECC identified. It is also possible that the lack of OS via DAUN contributes to this anthracycline being associated with less cardiotoxicity in childhood cancer survivors (Feijen *et al.*, 2015b).

7.2.2 What is the source of DOX-induced oxidative stress?

As shown in chapter 4 DOX increased OS in a concentration-dependent manner however the mechanism by which this is achieved is unknown. As discussed in 4.1.1 ROS may be produced by various mechanisms in cardiac myocytes including the mitochondrial electron transport chain, XO and NOX. While the mitochondrial electron transport chain may be impacted by drugs this study focused on the enzymatic sources and investigated the role of XO and NOX on DOX-induced OS.

7.2.3 The role of xanthine oxidase in DOX-induced oxidative stress

XO is an enzyme involved in the production of uric acid and ROS as a biproduct of hypoxanthine metabolism (Kang & Ha, 2014). Clinically XO inhibitors such as allopurinol and febuxostat are used for gout treatment (Frampton, 2015). Inhibition of this enzyme has also been shown to improve contractility and left ventricular function in heart failure models (Ekelund *et al.*, 1999; Ukai *et al.*, 2001). Our study identified DOX resulted in a reduction in contractility due to a decrease in systolic Ca as shown in figure 7.1 as well as a rapid increase in OS. As discussed in 4.1.3 ROS may alter Ca handling and contractility and the attenuation of these effects by XO inhibition suggests this enzyme may have vital role. We sought to investigate if XO has a role in DOX-induced OS and if inhibition of this enzyme attenuated the effects on ECC.

Inhibition of XO via febuxostat reduced OS in the presence of DOX at all time points (though not significantly). Ca transient amplitude was reduced by 8 % in DOX with a significant decrease of 18.8 % being evident upon washout (fig 5.4). The rate of sarcomere relaxation was significantly reduced in wash. All other aspects of Ca handling and contractility measured were not altered by DOX following XO inhibition (fig 5.4).
These results indicate that XO contributes (at least partially) to DOX-induced OS production that then alters Ca handling and contractility in cardiac myocytes.

7.2.4 The role of NADPH oxidase in DOX-induced oxidative stress

NADPH oxidases produce ROS as part of the immune response with NOX2 and NOX4 being expressed in cardiac myocytes (Kuroda & Sadoshima, 2010; Szekeres *et al.*, 2021a). Our study showed inhibition of NOX2 and 4 reduced dox-induced OS for all incubation times (fig 5.2). These data highlight the vital role NOX has in mediating production of ROS in cardiac myocytes and thence OS due to DOX treatment. Furthermore, with the exception of Ca transient amplitude the effects of DOX on Ca handling and contractility were attenuated (fig 5.6).

This suggests that NOX ROS production is involved in altering the removal of Ca possibly through altering the activity of channels such as SERCA (Greensmith *et al.*, 2010; Kuster *et al.*, 2010; Morris & Sulakhe, 1997; Reeves *et al.*, 1986; Scherer & Deamer, 1986). The reduction in Ca transient amplitude is likely due a decrease in SR Ca content (as discussed in 1.2.9.2), however k_{sys} was unaltered suggesting the overall removal of Ca was not impacted. This suggests there may be an alteration to the L type Ca current and/or RyR which is not ROS dependent and this leads to a reduction in SR Ca content and therefore Ca amplitude.

7.2.5 Can DOX-induced oxidative stress account for the alterations to excitation-contraction coupling?

We have established that DOX-induces OS and perturbs ECC, we must now consider the role of DOX-induced OS on the ECC components.

In the presence of NAC sarcomere length shortening was reduced to a lesser extent by DOX as was Ca transient amplitude (fig 4.5 & 4.6). It is important to note that even though these parameters were still reduced in the presence of NAC it was to a lesser extent than DOX treatment alone; with approximately half of the effect of DOX on Ca amplitude and systolic shortening attributable to OS (see section 4.4.4). These findings are supported by others that show ROS reduces Ca amplitude and contractility (Goldhaber & Liu, 1994; Greensmith *et al.*, 2010; Kuster *et al.*, 2010).

The decrease in Ca transient amplitude that is present after OS reduction can be accounted for by a decrease in SR Ca content (fig 4.8). NAC pre-treatment attenuated the effects of DOX on SR Ca content by 45 % (fig 4.8A), however the reduction identified can account for the decrease in Ca amplitude. The attenuation effects highlight the impact of OS and others have shown ROS reduces SR Ca content (Boraso & Williams, 1994; Greensmith *et al.*, 2010; Kuster *et al.*, 2010).

NAC pre-treatment fully attenuated the effect of DOX on k_{sys} and k_{SERCA} (fig 4.8), this suggests that DOX-induced OS is the cause of reducing SERCA activity. ROS reduces the activity of SERCA (Greensmith *et al.*, 2010; Kuster *et al.*, 2010; Morris & Sulakhe, 1997; Reeves *et al.*, 1986; Scherer & Deamer, 1986) which supports this observation. The effect of DOX on NCX activity was partially attenuated by NAC pre-treatment (fig 4.8C) which suggests that 79 % of the increase in NCX activity can be attributed to DOX-induced OS. This is logical given that ROS has been shown to increase NCX activity (Eigel *et al.*, 2004; Goldhaber, 1996; Kuster *et al.*, 2010; Reeves *et al.*, 1986).

NAC pre-treatment still resulted in a reduction in SR Ca content following DOX exposure (though attenuated by 50 %) and may be explained by the 14 % increase in k_{caff}. Although this increase in NCX activity was not significant the effect on NCX was not fully attenuated by OS reduction suggesting that DOX is altering the transporter activity by another mechanism. This increase in NCX activity though not statistically significant may still contribute to the reduction in SR Ca content as NCX has been shown to play a greater role in SR Ca content than SERCA (Bode *et al.*, 2011). Though not investigated in this study the role of L type Ca channel activity and open probability of RyR may be altered by DOX and also contribute to the decrease in SR Ca content through a mechanism that is not OS dependent.

Studies have found myofilament sensitivity to be increased by ROS (Haizlip *et al.*, 2012; Hiranandani & Janssen, 2009; Miura *et al.*, 2015) which supports the evidence in this study that found the increase in myofilament sensitivity due to DOX was attenuated by the reduction in OS (fig 5.8). This suggest that DOX-induced OS is the primary mechanism that leads to an increase in myofilament sensitivity.

The inhibition of NOX showed similar finding to those identified with NAC pre-treatment and fully attenuated various effects of DOX on Ca handling and contractility with partial attenuation of Ca transient amplitude (fig 5.6 & 5.7). Inhibition of XO decreased DOX-induced

OS but to a lesser extent than NOX, however still resulted in attenuation of various parameters (fig 5.7). These findings suggest that NOX has a vital role in DOX-induced OS which in turn perturbs various aspects of ECC and that XO may also contribute to the increase in OS. Both enzymes may increase ROS in cardiac myocytes via reduction of DOX resulting in semi-quinone formation and ROS (Bates & Winterbourn, 1982; Huang *et al.*, 2022; Menna *et al.*, 2010). Given that DOX has been shown to accumulate in the mitochondria of cardiac myocytes (Ichikawa *et al.*, 2014a) and this is where NOX4 is expressed (Ichikawa *et al.*, 2014a; Kuroda *et al.*, 2010; Peoples *et al.*, 2019) it is logical that this enzyme is located in a prime position to facilitate the DOX reduction reaction leading to ROS formation.

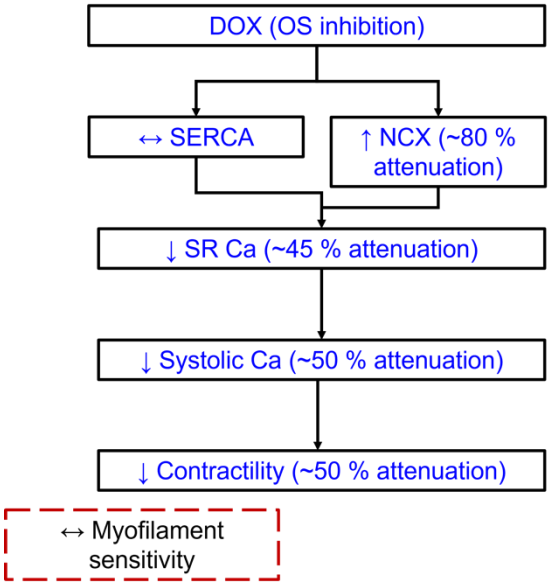

Figure 7.2: The role of DOX-induced OS on Ca handling and contractility in sheep ventricular myocytes.

7.2.6 Can DAUN-induced oxidative stress account for the alterations to excitation-contraction coupling?

Chapter 3 shows that DAUN reduces contractility of cardiac myocytes which is underpinned by reduction in systolic Ca due to a decrease in SR Ca content. However, OS was only increased at the highest concentration of DAUN. These findings suggest that DAUN alters ECC components mainly via a mechanism other than OS. This is not to say that ROS don't have a

role in DAUN cardiotoxicity but that the increase in ROS production does not seem to result in OS in acute exposure at low concentrations.

This is surprising given the similar chemical structures of DOX and DAUN (see fig 1.14). Our studies also identified a potential interaction between DAUN and caffeine which may explained by the binding both compounds have with caffeine (Abramson *et al.*, 1988). This interaction was not evident with DOX which may suggest the two anthracyclines have different binding properties to channels in the cardiac myocytes.

Overall, the mechanism by which DAUN alters ECC remains elusive. Given that many studies focus on the cardiotoxicity of DOX with the assumption that DAUN will behave in a similar manner our study provides evidence that this may not be the case and more biochemical investigation is required to understand the differences in protein interaction. These differences may account for the differences observed in cardiotoxicity between the compounds (Feijen *et al.*, 2015a).

7.3 The effects of doxorubicin and daunorubicin in heart failure

7.3.1 The effect of doxorubicin and daunorubicin on excitation-contraction coupling in a model of heart failure

Both DOX and DAUN reduced Ca transient amplitude in cardiac myocytes from a sheep model of HF (see chapter 6). Unlike in cells from the healthy model this reduction was not reversed upon washout. As discussed in 6.1.1 Ca transient amplitude is known to reduce in HF. (Clarke *et al.*, 2015; Hobai *et al.*, 2004; Piacentino *et al.*, 2003; Sipido *et al.*, 1998). This indicates that the recovery mechanisms of cells are disturbed within the HF model and may indicate a worse outcome in cardiac myocytes from a disease state, due to the lack of recovery following anthracycline treatment. Furthermore, the overall removal of Ca was not altered in the HF model by DOX indicating that the effects on SERCA and NCX were saturated, however SR Ca content was reduced (fig 6.4). While the reduction in SR Ca content from the HF model cannot

be explained by alteration in SERCA and NCX activity it may be that other ECC components are altered by DOX and DAUN. For example, as discussed in 3.1.1 L type Ca channel activity as well as the open probability of RyR may be altered by the anthracyclines which can impact SR Ca content.

7.3.2 Are the nature of the effects of doxorubicin and daunorubicin in health different to those in heart failure?

For both DOX and DAUN the percentage change for Ca transient amplitude and sarcomere length shortening were lower in cells isolated form HF model than the healthy model (see fig 6.8 & 6.10). This may be explained by the already increased state of ROS in HF models (Wagner *et al.*, 2011) meaning the impact of anthracycline-induced ROS is to a lesser extent. Both cells from healthy and HF models showed a decrease in SR Ca content which can account for the decrease in Ca amplitude. However, in cells from healthy animals this change can be accounted for by the increase in NCX activity and decrease in SERCA activity. In the cells from the HF model neither SERCA nor NCX activity was found to be altered by DOX. The altered states of SERCA and NCX in the HF model as discussed in 6.1.1 may be saturated meaning DOX does not induce a further change, however the fact SR Ca content is still reduced suggests other components may be altered such as L-type Ca channels and RyR.

Interestingly oxidative stress is associated with HF (Dubois-Deruy *et al.*, 2020; Grieve & Shah, 2003; Martins *et al.*, 2022). If the cells from the HF model have an increased basal level of OS it may be that these effects are also saturated and that DOX does not increase the levels further. This may explain why the effects on SERCA and NCX are saturated as results from chapter 4 show OS impacts SERCA and NCX activity.

7.4 Clinical implications in anthracycline-induced cardiotoxicity

7.4.1 Do the cellular effects of doxorubicin and daunorubicin account for clinical dysfunction?

As discussed in 1.6.1 treatment with anthracyclines can result in a decrease in left ventricular ejection fraction, decreased contractility and patients may develop heart failure (Lipshultz *et al.*, 1991; Nakayama *et al.*, 2020).

Both DOX and DAUN reduced systolic Ca which is the cellular basis of stroke volume/ejection fraction thus inotropy as discussed in 1.2.9. This decrease in systolic Ca was due to a decrease in SR Ca content. DOX resulted in a decrease in SERCA activity and increase in NCX activity which can account for a decrease in SR Ca content. The apparent interaction between DAUN and caffeine prevented exploration of channel activity. These cellular changes in Ca handling and contractility by DOX and DAUN provide a basis for systolic dysfunction and may contribute to the reduction in left ventricular contractility and overall function that is identified in anthracycline cardiotoxicity.

As shown in figure 1.11 the acute reduction in contractility due to DOX recovered only to decline again years after diagnosis (Lipshultz *et al.*, 2005). The experiments in this study were acute with the effects on Ca handling and contractility due to both DOX and DAUN recovering fully or partially upon washout. This cellular recovery may occur clinically after anthracycline treatment only to degrade again years later. Patients that suffer from acute cardiotoxicity due to anthracyclines are more likely to suffer from late cardiotoxicity (Grenier & Lipshultz, 1998).

7.4.2 How may the response to anthracycline treatment differ in patients considered high risk of cardiac complications?

Patients are considered high risk of cardiac complications from anthracycline treatment if they suffer from other cardiac conditions or have cardiovascular risk factors such as obesity

and diabetes (Armenian *et al.*, 2017). Both the risk of cancer and heart failure increase with age (Laconi *et al.*, 2020; Stewart *et al.*, 2003; Triposkiadis *et al.*, 2019; White *et al.*, 2014). This suggests that there is a risk of comorbidity in adults meaning they could suffer from both diseases (Bertero *et al.*, 2018; Finke *et al.*, 2021) meaning careful consideration of the use of anthracyclines in this patients is required. In children the risk of this comorbidity is lower but the fact that some children may have underlying heart conditions has to be considered. The use of a heart failure model allowed us to investigate the effects of anthracyclines on cardiac myocytes from a disease state and consider how pre-existing heart conditions would be impacted by anthracycline treatment.

When treated with DOX Ca transient amplitude was irreversibly reduced in cells isolated from HF and the percentage decrease was significantly less than from healthy animals at (HF; -23 %, healthy; - 39 %; fig 6.2 & 6.8). The percentage change in sarcomere length shortening as a result of DOX treatment was also lower in HF cells than healthy cells (fig 6.8). Results were similar for DAUN Ca transient amplitude is reduced in cells from the HF model compared to cells from the healthy model (Briston *et al.*, 2011b). These data suggest that the change inflicted by DOX may appear to be to a lesser extent in terms of percentage change from control but that the overall reduction is similar. The cells from HF do not recover in terms of Ca transient amplitude in wash unlike cells from the healthy model suggesting that recovery would be worse in patients with underlying cardiac conditions.

The overall response appears similar in cells from HF compared to healthy cells however many of the effects appear to have been saturated (at least partially) in the HF model prior to DOX or DAUN treatment. However, recovery upon washout was less successful in cells from the HF model suggesting that DOX and DAUN would worsen the heart function.
While these experiments are reductionist and acute the cellular mechanisms can provide insight into the different responses that may occur. These data suggest that pre-existing conditions that result in reduced systolic Ca and contractility may not be worsened acutely however the long-term implications are unknown.

7.5 Implications for alternative treatment strategies

7.5.1 Compensatory treatment strategies for anthracycline-induced cardiotoxicity

Current treatment of anthracycline-induced cardiotoxicity involves the use of angiotensin-converting enzyme inhibitors however there is no specific treatment strategy in terms of length of treatment; particularly following recovery (Henriksen, 2018). These enzymes are used as they have been shown to slow down the progression of ventricular dysfunction following anthracycline treatment (Kobza, 2021).

Our findings show DOX increased NCX activity while reducing SERCA activity therefore reducing systolic Ca and contractility. The inotropic state is shown to be altered as is the case in heart failure which can result in a larger amount of Ca being released from the SR via RyR, a decrease re-uptake of Ca into the SR via SERCA and an increase in Ca removal from the cell via NCX therefore reducing SR Ca content (Hasenfuss & Teerlink, 2011). Positive inotropic drugs increase the force of contraction ultimately by increasing free intracellular Ca (Scholz, 1984) and may be one avenue of investigation to combat anthracycline cardiotoxicity.

These agents include cardiac glycosides, β- adrenoreceptor agonists, phosphodiesterase inhibitors and calcium sensitisers (Hasenfuss & Teerlink, 2011). Glycosides such as digitalis raise intracellular Ca, reducing Ca efflux via NCX and favour SR Ca uptake (Reuter *et al.*, 2002). β-adrenoreceptor agonists stimulate β-adrenergic receptors and promotes increased force of contraction (Hasenfuss & Teerlink, 2011). Phosphodiesterase inhibitors prevent degradation of cAMP therefore allowing the downstream effects (see figure 1.10) such as promoting the opening of L type calcium channels (Feneck, 2007) and phosphorylation of SERCA (Rao & Xi, 2009) which promotes reuptake of Ca into the SR. These positive inotropic drugs may combat the effects that anthracyclines have on protein channels and restore SR Ca content therefore restoring Ca amplitude and contractility.

Ca sensitiser are another class of positive inotropic drug and as discussed in 3.4.6, they increase the force of contraction by increasing the affinity of troponin C to Ca or increasing the effectiveness of this attachment (Lee & Allen, 1997). These agents may be used to restore the regulation of ECC, and the alterations seen on NCX and SERCA therefore reducing the toxic cardiac side effects. However, our study shows DOX and DAUN increase myofilament sensitivity but the effects on Ca handling and contractility are still pronounced, which indicates the use of Ca sensitisers would not be beneficial in reducing anthracycline-induced cardiotoxicity.

The use of these positive inotropic agents are only recommended short term due to the potential proarrhythmic effects (Tisdale *et al.*, 1995). While these drugs may be beneficial in treating anthracycline-induced cardiotoxicity the ideal situation would be to prevent the cardiotoxicity from occurring.

7.5.2 Prevention treatment strategies for anthracycline-induced cardiotoxicity

Currently, dexrazoxane (DEX) may be given clinically alongside anthracycline treatment in an attempt to reduce the chances of cardiotoxicity (Muthuramalingam *et al.*, 2013). DEX is an iron chelating agent and may reduce mitochondrial iron accumulation therefore preventing ROS production, in addition it may degrade topoisomerase II which is involved in another potential mechanism of action of anthracyclines (Ichikawa *et al.*, 2014a). As anthracyclines are known to increase ROS, it is believed DEX works to combat this production therefore reducing cardiotoxicity. As discussed in 1.6.5 this treatment strategy has been shown to reduce cardiac events due to anthracycline treatment but the long-term implications for children are debated.

Our findings show DOX increase OS in cardiac myocytes. Alternative treatment strategies may involve reducing the production of ROS in the heart. Inhibition of XO and NOX2/NOX4 attenuated many of the effects of DOX on Ca handling and contractility (see chapter 5).

However, DAUN did not appear to increase ROS in the same manner as DOX and so this strategy may not be useful for all anthracycline treatment (see chapter 4). XO inhibitors are used clinically to treat gout and have been shown to reduce the risk of cardiovascular events (Saito *et al.*, 2021), highlighting their potential usefulness for anthracycline-induced cardiotoxicity prevention.

A concern with implementing the use of ROS inhibitors with anthracycline use is reducing the effectivity of the drugs on cancer. However, ROS production is not the only mechanism of action of anthracyclines as discussed in 1.5.2 indicating that overall reduction in anthracycline-induced ROS may be beneficial. Furthermore, cardiac myocytes are abundant in mitochondria and have fewer antioxidant enzymes (Barth *et al.*, 1992; Costa *et al.*, 2013) making them particularly vulnerable to ROS. One study found the use of the generic antioxidant NAC did not reverse the effects of DOX-induced cardiomyopathy, (Dresdale *et al.*, 1982) however this was given after the disease state had occurred which indicates ROS inhibitors would not be useful as a compensatory treatment. However, the use of ergothioneine which has antioxidant properties was found to have cardioprotective effects in mice treated with DOX and did not interfere with the treatment's efficacy (Cheah *et al.*, 2023). This suggest that ROS inhibitors may be useful given alongside anthracycline treatment as a preventative measure. The use of antioxidants with anthracyclines is not well studied and their use to support anthracycline treatment is not well known (Vincent *et al.*, 2013). Further investigation is required to ensure the efficacy of anthracycline treatment is not impacted by co-treatment with antioxidants and that cardioprotective benefits occur.

7.6 Limitations

The methodology used in this study allowed identification of the individual effects of DOX and DAUN on Ca handling and contractility in intact cardiomyocytes in a robust way. While the effects on various ECC components was investigated the use of patch clamping would have allowed further insight into the effects on L-type Ca channels and NCX activity as well as overcoming the caffeine interaction phenomenon with DAUN. The inhibition of OS resulted

in a decrease in SR Ca content due to DOX that could not be explained by the relative activity of the components investigated therefore studying activity of the L-type Ca channel may elucidate further mechanisms. Unfortunately, the equipment was not available during the course of these studies.

Furthermore, these studies are acute and while allowing an insight into the mechanistic effects of DOX and DAUN the implications of long-term treatment may differ. As shown in figure 1.15 the effect of anthracycline treatment on contractility recovers only to reduce again years later (Lipshultz *et al.*, 2005). Investigating the implications of treatment on ion channels over longer time periods may elucidate this mechanism. Primary cardiac myocytes are an invaluable resource but unfortunately are not viable long-term.

7.7 Future work

Valuable insight was obtained from these studies including the effect of DOX and DAUN on intracellular Ca and contractility, OS and the potential attenuating effects of ROS inhibitors. Future work will investigate the effects of DOX and DAUN on the other components of ECC such as LTCC using patch clamping. Furthermore, we will co-load cells with Ca and OS-sensitive fluorophores to more directly correlate the changes of OS to those of ECC. This will support that the effects on Ca handling due to DOX are ROS induced due to the bidirectional nature of this relationship. It would also be advantageous to investigate mitochondrial ROS production for which specific fluorophores exist. The effect by which DAUN evokes cardiotoxicity requires further investigation as does the interaction with caffeine which may elucidate mechanistic effects. We have characterised the effect of DOX and DAUN on various aspects of ECC (e.g., SERCA and NCX), however we don't know how the anthracyclines interact with these proteins. While our OS work suggests this may be through ROS signalling the interaction of DAUN and caffeine may suggest there is direct binding occurring with the RyR. To investigate this a collaboration has been secured which will incorporate the use of solid-state NMR to examine the molecular dynamics between anthracyclines and cardiac calcium handling proteins.

In terms of establishing if the long-term effects of anthracyclines are induced in a similar manner to the acute, a cultured cell line will be utilised. As previously mentioned, primary cardiac myocytes are not suitable for long-term experiments due to the reduction in viability. The HL-1 cell line have similar characteristics to primary cardiac myocytes and allow for electrophysiological studies while having the ability to be kept in culture (White *et al.*, 2004). This means that cells can be treated with anthracyclines for a longer period and the effects on Ca handling explored.

7.8 Summary

1 nM DOX reduced the activity of SERCA and increased the activity of NCX resulting in a reduction in SR Ca content. 1 nM DAUN also reduced SR Ca, but apparent interactions of DAUN with caffeine meant the mechanisms could not be fully elucidated. The reduction in SR Ca content underpinned a reduction in Ca transient amplitude by both DOX and DAUN which resulted in a decrease in contractility. DOX and DAUN did not appear to alter diastolic Ca acutely. Both DOX and DAUN increased myofilament sensitivity to Ca which may have potentially offset the effects seen to contractility.

DOX increased cytoplasmic OS in a concentration and time-dependent manner. DAUN also increased OS at higher concentrations (10 µM). This indicates potential differences in the mechanism of cardiac toxicity and may also account for DAUN being associated with reduced cardiotoxicity.

Removal of OS using NAC attenuated the majority of the effects of DOX on Ca handling and contractility however SR Ca content was still reduced but attenuated by ~45 %. This suggest that ~50 % of the effects of DOX on heart cells are OS-dependent. Further investigation showed NOX plays a vital role in ROS production associated with the increase in OS and that XO has a partial role. Inhibition of both enzymes attenuated various effects of DOX on Ca handling and contractility with the exception of Ca transient amplitude.

In cells from heart failure DOX and DAUN produced similar effects however the impact on Ca transient amplitude was not reversible upon wash. Furthermore, the Ca transient amplitude appeared to be reduced to a lesser extent in cells from a HF model compared to cells from the healthy model. While the SR Ca content was reduced by DOX in cells from HF the activity of NCX and SERCA was not altered (unlike in the healthy model), this suggests the effects on NCX and SERCA are saturated in the HF model and other channels such as L-type Ca channels may have a role in the reduction of SR Ca content.

This study considerably advances our understanding of the cellular basis of HF that is associated with the use of anthracyclines for cancer treatment. We are the first to show that elevations in OS contribute to DOX (and to potentially DAUN) mediated changes to ECC. NOX and XO were revealed as key sources of OS and may present as novel therapeutic targets.

Chapter 8
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