The independent roles of PMCA1 and PMCA4 in the development and progression of left ventricular hypertrophy and failure

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

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Abstract

A thesis submitted to the University of Manchester by Nicholas Pierre Stafford for the degree of Doctor of Philosophy entitled

‘The independent roles of PMCA1 and PMCA4 in the development and progression of left ventricular hypertrophy and failure’

September 2013

Heart failure is responsible for one in twenty deaths in the UK, and as the average age of the general population increases that number is predicted to rise over the coming years. Hypertrophic growth is believed to be an adaptive response to a chronic increase in workload under circumstances such as hypertension, yet it is also known to contribute to the pathological progression into heart failure. Abnormal calcium handling is known to play a critical role in determining disease progression, not only through its function as the driving force behind myocardial contraction and relaxation but also through directing the signals which regulate hypertrophic growth. Both isoforms 1 and 4 of the diastolic calcium extrusion pump plasma membrane calcium ATPase (PMCA) are present in the heart, yet unlike in other cell types their contribution to overall calcium clearance is only small; however their role in the disease process is yet to be defined.

A novel mouse line was generated in which both PMCA1 and 4 were deleted from the myocardium (PMCA1:4^dko mice). Through comparison with PMCA1 knockout mice (PMCA1^cko) this thesis set out to identify the specific function of each pump under normal conditions and during the development of pathological hypertrophy induced by pressure overload through transverse aortic constriction (TAC).

Under basal conditions each isoform functioned independently, PMCA1 to extrude calcium during diastole and PMCA4 to regulate calcium levels during systole; however the loss of neither isoform impacted significantly on cardiac function. In response to TAC, PMCA1^cko mice progressed rapidly into decompensation and displayed signs of systolic failure after just 2 weeks, whilst cardiac function was preserved in TAC controls. Calcium handling analysis revealed that prior to the onset of failure PMCA1^cko mice displayed a distinct lack of adaptive changes to calcium cycling which were present in controls. In stark contrast, the additional loss of PMCA4 led to an attenuated hypertrophic response to TAC in PMCA1:4^dko mice which remarkably preserved cardiac function despite the absence of PMCA1. This adds to accumulating evidence which suggests that the inhibition of PMCA4 may be protective during the development of pathological hypertrophy, whilst highlighting the possibility for a novel role for PMCA1 in coordinating essential adaptations required to enhance calcium cycling in response to the increased demands imposed on the left ventricle during pressure overload.
Declaration

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Nicholas Pierre Stafford
School of Medicine
Faculty of Medical and Human Sciences
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I owe thanks to three students who have helped me out in aspects of this project; Sam Cook, Donna Page and Yasser Mushtaq. I couldn’t have wished for better students and I have no doubt your futures hold great successes in store.

Finally and most importantly I’d like to thank my parents for always being supportive of me in my decisions over the years and I dedicate this thesis to you both.
The Author

I studied medicine at the University of Nottingham, where in undertaking a research project studying the mechanisms involved in platelet aggregation I decided a career in cardiovascular research was more suited to my tastes and I left after 4 years with an intercalated Bachelor of Medical Sciences (BMedSci). In working towards this thesis that view has not changed, and I look forward to continuing on in the lab over the coming year.
### Abbreviations

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<td>0Na⁺/0Ca²⁺ solution</td>
<td>Nominally sodium and calcium-free solution</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial natriuretic factor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BNP</td>
<td>B-type natriuretic peptide</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca²⁺/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium-induced calcium release</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′, 6-Diamidino-2-phenylindole dihydrochloride</td>
</tr>
<tr>
<td>DBP</td>
<td>Diastolic blood pressure</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>dIVS</td>
<td>Intraventricular septum in diastole</td>
</tr>
<tr>
<td>dLVD</td>
<td>Left ventricular diameter in diastole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dP/dt&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximal change of left ventricular systolic pressure</td>
</tr>
<tr>
<td>dP/dt&lt;sub&gt;min&lt;/sub&gt;</td>
<td>Minimal change of left ventricular diastolic pressure</td>
</tr>
<tr>
<td>dPW</td>
<td>Posterior wall thickness in diastole</td>
</tr>
<tr>
<td>E&lt;sub&gt;a&lt;/sub&gt;</td>
<td>Arterial elastance</td>
</tr>
<tr>
<td>EC coupling</td>
<td>Excitation-contraction coupling</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiography</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemi-luminescence</td>
</tr>
<tr>
<td>EDV</td>
<td>End diastolic volume</td>
</tr>
<tr>
<td>E&lt;sub&gt;es&lt;/sub&gt;</td>
<td>End systolic elastance</td>
</tr>
<tr>
<td>EF</td>
<td>Ejection fraction</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular receptor kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ESPVR</td>
<td>End systolic pressure volume relationship</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FS</td>
<td>Fractional shortening</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome wide association study</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HEK 293</td>
<td>Human embryonic kidney cell line 293</td>
</tr>
<tr>
<td>HF</td>
<td>Heart failure</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse-radish peroxidise</td>
</tr>
<tr>
<td>HT</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>HW</td>
<td>Heart weight</td>
</tr>
<tr>
<td>HW/BW</td>
<td>Heart weight to body weight ratio</td>
</tr>
<tr>
<td>HW/TL</td>
<td>Heart weight to tibial length ratio</td>
</tr>
<tr>
<td>IGEPAL</td>
<td>Octylphenoxypolyethoxyethanol</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial methylated spirit</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal injection</td>
</tr>
<tr>
<td>LTCC</td>
<td>L type calcium channel</td>
</tr>
<tr>
<td>LVH</td>
<td>Left ventricular hypertrophy</td>
</tr>
<tr>
<td>MAGUK</td>
<td>Membrane-associated guanylate kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>Na$^+$/K$^+$ ATPase</td>
<td>Sodium/potassium ATPase</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>Na$_v$1.5</td>
<td>Voltage gated sodium channel</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium calcium exchanger</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NRCM</td>
<td>Neonatal rat cardiomyocytes</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>pc</td>
<td>Post coitum</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD95/Dlg1/zo-1</td>
</tr>
<tr>
<td>PE</td>
<td>Phenylephrine</td>
</tr>
<tr>
<td>$P_{es}$</td>
<td>End systolic pressure</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLN</td>
<td>Phospholamban</td>
</tr>
<tr>
<td>$P_{max}$</td>
<td>Maximal systolic pressure</td>
</tr>
<tr>
<td>PMCA</td>
<td>Plasma membrane calcium ATPase</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonylfluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin-angiotensin-aldosterone-system</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>Ras-associated factor 1 A</td>
</tr>
<tr>
<td>RIPA buffer</td>
<td>Radioimmunoprecitation buffer</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RV</td>
<td>Right ventricle</td>
</tr>
<tr>
<td>RWT</td>
<td>Relative wall thickness</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarcoendoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>shRNA</td>
<td>Small hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short-interfering RNA</td>
</tr>
<tr>
<td>sIVS</td>
<td>Interventricular septum in systole</td>
</tr>
<tr>
<td>sLVD</td>
<td>Left ventricle diameter in systole</td>
</tr>
<tr>
<td>sPW</td>
<td>Posterior wall thickness in systole</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>SV</td>
<td>Stroke volume</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>TAC</td>
<td>Transverse aortic constriction</td>
</tr>
<tr>
<td>Tau</td>
<td>Time constant</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris buffered saline containing Tween-20</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TL</td>
<td>Tibia length</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>T-tubule</td>
<td>Transverse-tubule</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cells</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
Chapter 1
1. GENERAL INTRODUCTION

When exposed to the stress of chronically increased workload the heart initiates an adaptive response designed to improve its pumping capacity and maintain adequate cardiac output. This response incorporates elements of myocardial growth, termed hypertrophy, and alterations to the cellular processes governing cardiac contractility; however whilst initially compensatory, in the long-term these changes can contribute to a progressive deterioration in cardiac function to the point that the heart can no longer meet the metabolic demands of the body, a syndrome known clinically as heart failure.

Through its function as the driving force for myofilament contraction and relaxation, as well as its ability to regulate the signalling events which dictate hypertrophic remodelling, intracellular calcium plays a fundamental role in determining disease progression. As a minor contributor to calcium extrusion during diastole the plasma membrane calcium ATPases (PMCAs) have traditionally been considered relatively insignificant in terms of normal calcium homeostasis; however their influence on the disease process is not well defined. The presence of two isoforms (PMCA1 and PMCA4) suggests specialized functions, yet the lack of specific inhibitors has hindered progress in elucidating these. In recent years, through the use of genetically modified mice an emergent role for PMCA4 in the regulation of signal transduction processes has been identified. In contrast little is known of the role of PMCA1. This thesis aims to identify the specific function of each isoform in the normal and hypertrophic heart.

1.1 Heart failure

Heart failure (HF) is a disease of ever increasing global importance, associated with significant morbidity and mortality whilst posing an immense financial burden on health authorities. Data from the Framingham Heart Study indicates the lifetime risk of developing HF to be 1 in 5 and that incidence rises sharply with age (Lloyd-Jones et al., 2002, Kannel, 2000), and it is suspected that UK prevalence stands at around 900,000 amongst those aged 45 and over, with a rate of over 15% in those over 85 (Petersen et al., 2002). As life expectancy continues to rise, especially as the developing world undergoes socioeconomic change, global prevalence is expected to increase dramatically in the
coming years (Mendez and Cowie, 2001). According to the latest figures published by the American Heart Association, this is anticipated to more than double the current financial costs of $32 billion associated with the disease by 2030 (Go et al., 2013). Despite being highly prevalent, current treatments are woefully inadequate and prognosis remains poor with a 5-year survival rate standing at around 50% (Levy et al., 2002), and it is predicted that HF now accounts for over 5% of total deaths in the UK (Petersen et al., 2002).

1.2 Current pharmacological treatments for heart failure

In an attempt to correct the deficit in cardiac output during heart failure, the body increases the levels of circulating neurohormonal factors which activate the renin-angiotensin-aldosterone system (RAAS) and sympathetic nervous system (SNS). RAAS activation increases blood volume through fluid retention, and in combination with catecholamines induces vasoconstriction to theoretically increase blood flow to tissues. RAAS activation, however, is also the leading cause of morbidity in HF patients, with fluid retention causing congestion in both the pulmonary and peripheral circulation. This is associated with severe dyspnoea, with patients experiencing shortness of breath upon minimal physical exertion, and in the worst cases at rest, contributing to quality of life amongst HF sufferers being poor relative to many other chronic conditions (Petersen et al., 2002).

Current pharmacological therapies for HF mainly target suppression of the RAAS and sympathetic nervous system to reduce myocardial workload. The latest guidelines from the European Society of Cardiology recommend that the frontline treatment regimen consists of a diuretic and angiotensin-converting enzyme (ACE) inhibitor to relieve congestion, in combination with a beta-blocker to prevent chronic sympathetic stimulation (McMurray et al., 2012). However, these drugs do not directly target the signalling pathways which drive hypertrophic remodelling. By understanding the mechanisms which regulate the development and progression of left ventricular hypertrophy (LVH) it may be possible to develop more efficacious therapies for the prevention of HF.
1.3 Left ventricular hypertrophy as a precursor to heart failure

The molecular phenotype of the failing heart is characterised by altered expression of calcium handling, contractile and cytoskeletal proteins in addition to remodelling of the extracellular matrix (Braunwald and Bristow, 2000). Almost invariably these changes are accompanied by cardiomyocyte hypertrophy and together these factors contribute to adverse remodelling which can lead to both systolic and diastolic dysfunction.

In the majority of cases in the developed world the primary underlying aetiology in HF is either ischaemia or hypertension (McMurray and Stewart, 2000). Each of these events exerts strain on the myocardium which if uncorrected would lead to a deficiency in cardiac output. To compensate, elevated levels of circulating catecholamines and angiotensin II from SNS and RAAS activation, amongst other secreted neurohumoral and endocrine factors, activate G-protein-coupled receptors (GPCR) at the cell membrane triggering a multitude of cross-talking signal transduction cascades ultimately resulting in the initiation of nuclear transcription factors and cardiomyocyte hypertrophy (Heineke and Molkentin, 2006). When activated following myocardial infarction (MI) or systemic hypertension hypertrophy will most commonly affect the left ventricle, thickening the wall and increasing muscle mass which in itself poses an independent risk factor for cardiovascular disease (CVD) and mortality (Levy et al., 1990).

1.3.1 Physiological and pathological cardiac hypertrophy

The increase in muscle mass is intended to improve cardiac function, which can be exemplified by considering the ‘athlete’s heart’ where growth leads to more efficient pumping and will typically lower the resting heart rate. In this instance of volume overload, sarcomeres are added predominately in series within myocytes causing an increase in cell length and chamber enlargement which may or may not be accompanied by increased wall thickness, hence relative wall thickness is largely unaltered (Bernardo et al., 2010). This eccentric pattern of hypertrophy witnessed in response to physiological stress is not accompanied by cardiac fibrosis, and is reversible upon termination of athletic training.
Conversely, when volume overload is induced by pathological insult, as is the case with valvular insufficiency and chronic MI, the eccentric response can often progress to decompensated hypertrophy (Sciarretta and Sadoshima, 2010). Similarly, pathological hypertrophy induced by chronic pressure overload, for example in hypertensive patients or those with aortic stenosis, can also lead to cardiac dysfunction. However, under these circumstances a concentric pattern of hypertrophy usually ensues, with sarcomeres added in parallel increasing myocyte width and no proportional increase in chamber size, causing an overall rise in relative wall thickness (Heineke and Molkentin, 2006, Bernardo et al., 2010).

1.4 The role of calcium in the development of cardiac hypertrophy and failure

Although the characteristics and long term consequences may vary greatly, physiological and pathological hypertrophy share a common goal of adapting to increased workload. As such, in each form of hypertrophy calcium handling is central to generating the required improvement in contractility, in addition to regulating many processes that govern growth itself.

1.4.1 Cardiac calcium homeostasis

The events leading to myocardial contraction and relaxation are dependent upon a respective rise and fall in the level of cytosolic calcium, and as the human heart beats roughly once every second this necessitates a dynamic mechanism for its control. The process through which these events occur is called excitation-contraction (EC) coupling and involves the coordinated efforts of a number of calcium transport systems which facilitate calcium movement both across the sarcolemma between the intracellular and extracellular space, and between the cytosol and membrane bound organelles within the cell (figure 1.1).
**Figure 1.1 Excitation-contraction coupling** Calcium movements in a ventricular cardiomyocyte leading to contraction (shown in red) and relaxation (shown in black). ATP, ATPase; I_{Ca}, L-type Ca^{2+} channel; Mito, mitochondria; Myofil, myofilament; Na-CaX, Na^{+}/Ca^{2+} exchanger; PLB, phospholamban; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; TnC, cardiac troponin C. Image adapted from (Bers, 2000)

**1.4.2 Excitation-contraction coupling**

With every heart beat the cardiomyocyte cytosolic calcium concentration cycles between its resting diastolic level of ~100nM and ~1μM during systole facilitating contraction and relaxation (Bers, 2000), a process known as excitation-contraction coupling. The rise in systolic calcium is initiated by entry through the voltage dependent L-type calcium channel (LTCC) which opens following membrane depolarisation during the action potential. This stimulates calcium release from sarcoplasmic reticulum (SR) stores via the ryanodine receptor (RyR), a process termed calcium induced calcium release (CICR), which accounts for the bulk of systolic calcium. The resultant calcium binds to myofilaments allowing cross-bridge formation to occur and thus initiating contraction.
To allow relaxation calcium must dissociate from the myofilaments, and hence cytosolic calcium declines. This is achieved via four mechanisms. The bulk of calcium, in the region of 70-92% dependent on species, is returned to the SR through reuptake via the sarcoplasmic reticulum Ca\(^{2+}\)- ATPase (SERCA), which transports two calcium ions per molecule of ATP hydrolysed (Reddy et al., 1996). The majority of the remaining calcium is extruded from the cell down an electrochemical gradient in exchange for three sodium ions per ion of calcium via the high capacity sodium calcium exchanger (NCX), and to a lesser extent via the lower capacity PMCA. It is no surprise that the NCX is the preferred route for efflux, as it is predicted to be capable of transporting up to 5000 ions per second (Hilgemann et al., 1991), in the order of 50 magnitudes higher than the rate via the PMCA (Blaustein and Lederer, 1999). Mitochondrial reuptake accounts for the residual calcium clearance, and it is believed that the combined contributions of this and the PMCA to global clearance total little more than 1%, hence they have been coined ‘slow’ clearance systems (Bers, 2000).

The rapid rise in calcium during CICR is facilitated by the close apposition of the LTCC and RyR in what are termed dyads, located in transverse or T-tubular regions of the sarcolemma (Brette and Orchard, 2003). T-tubules are invaginations in the sarcolemma which penetrate into the interior of the cell appreciably increasing cell surface area, and facilitate synchronous calcium release following excitation. As well as being the subcellular localisation for the majority of LTCC and RyR expression, over 60% of NCX activity and all extrusion via the PMCA are thought to occur at the T-tubule (Despa et al., 2003, Chase and Orchard, 2011).

1.4.3 Modulations to excitation-contraction coupling in health and disease

Given the relationship between calcium concentration and muscle contraction, modulations to the EC coupling process have the ability to profoundly affect cardiac performance. The biggest determining factor for the force of contraction during systole is the amount of calcium released from the SR during CICR, which in turn is dependent upon the amount of trigger calcium entering the cell and the concentration of calcium contained within the SR itself (Bassani et al., 1995a).
1.4.3.1 The response to β-adrenergic stimulation

To increase cardiac output in response to acute stress, for example during physical exertion, the binding of catecholamines to β-adrenoceptors enhances calcium mobilization from SR stores by influencing each of these processes. The activated GPCRs stimulate the production of cyclic adenosine monophosphate (cAMP) via adenylyl cyclase which in turn activates protein kinase A (PKA) (Bers, 2002). The LTCC and RyR each have amino acid residues which act as substrates for PKA-dependent phosphorylation, which serves to increase their activity and thus fractional release from the SR, causing a rise in the amplitude of systolic calcium. PKA activity also has the ability to increase lusitropic function through phosphorylation of phospholamban (PLN) (Li et al., 2000), the major regulator of SERCA function. PLN phosphorylation releases its inhibitory effects on SERCA, and thus enhances the rate of uptake into the SR (Kranias and Hajjar, 2012).

1.4.3.2 Alterations to EC coupling in the hypertrophic heart

During exercise training, where increased workload results from a greater demand for blood supply to the tissues, physiological cardiac growth is accompanied by alterations in the vascular system which reduce peripheral resistance (Dorn, 2007). Consequently in the resting trained heart, adequate cardiac output can be maintained at a lower pressure and the emphasis is placed on more efficient pumping. As such diastolic and systolic calcium levels are reduced and myofilament calcium sensitivities increase (Wisloff et al., 2001, Kemi et al., 2008b), whilst transient decay rates increase due to improved SR uptake via a more abundantly expressed SERCA (Kemi et al., 2008a).

In contrast, increased contractility during pathological hypertrophy is generally required to overcome increased resistance in the vasculature (as in hypertension) or due to valvular disease, or alternatively compensate for diseased tissue (as in MI). Consequently, the adaptive response in the pressure overloaded rat heart sees an increase in systolic calcium transient amplitude as early as two days following insult (Carvalho et al., 2006), which is sustained during the compensated stage of hypertrophy and may correlate to improved cell shortening (Sumida et al., 1998, Nagata et al., 1998). This is accompanied by enhanced calcium cycling at the SR, with increases in uptake, release and content noted in models of
both pressure overload and catecholamine induced hypertrophy (Carvalho et al., 2006, Ohkusa et al., 1997, Chorvatova et al., 2004). Conversely, calcium decay rates are typically unaltered during compensated hypertrophy (Carvalho et al., 2006, Chorvatova et al., 2004), despite numerous studies reporting altered expression of diastolic extrusion pumps. NCX transcript levels have been shown to be upregulated as early as 1 hour after the onset of pressure overload (Kent et al., 1993), and elevated expression and activity persists in the hypertrophic heart (Studer et al., 1997, Nakanishi et al., 1989, Chorvatova et al., 2004). Similarly, instances of increased sarcolemmal ATPase activity and SERCA:PLN ratio have also been noted (Nakanishi et al., 1989, Carvalho et al., 2006).

1.4.3.3 Alterations to EC coupling in the failing heart

Whilst calcium cycling may be improved during the early stages of cardiac hypertrophy, as a consequence of prolonged exposure to stressful stimuli during adverse remodelling, β-adrenergic responsiveness, SR content and thus release are reduced and hence force of contraction diminishes (Briston et al., 2011, Pieske et al., 1999). The loss of calcium from the SR is thought to be caused by a number of contributing factors including decreased uptake through reduced SERCA expression and phospholamban phosphorylation, SR leak via RyR phosphorylation and increased efflux via a more abundantly expressed NCX (Hasenfuss and Pieske, 2002, Fischer et al., 2013). SR calcium leak not only leads to a reduction in systolic calcium availability, but in combination with sodium imbalance resulting from calcium entry through reverse NCX can also result in potentially fatal arrhythmias (Lehnart et al., 2009). Furthermore, HF is associated with a loss of T-tubule structure which results in a reduction in the efficacy of CICR leading to a dyssynchronous rise in systolic calcium across the cell (Lyon et al., 2009). Possibly as a consequence of this the expression of many ion channels and transporters, including both isoforms of the PMCA, are significantly reduced in the failing left ventricle (Borlak and Thum, 2003).
1.4.4 Cardiac calcium signalling

Amidst the large and constant fluctuations in cytosolic calcium required for muscle contraction, the cell still manages to maintain the calcium signal at desirable levels for normal regulation of a host of other processes including mitochondrial energy balance, cell death via apoptosis and necrosis, hypertrophic growth and contractility itself (Bers, 2008). This is thought to be primarily achieved through compartmentalisation of the signal within cellular microdomains by a process which has been coined excitation-transcription coupling, where calcium-dependent signalling can occur unaffected by the global rise and fall in concentration during EC coupling (Molkentin, 2006, Frey et al., 2000, Wu et al., 2006).

It is believed that the calcium binding messenger protein calmodulin (CaM) has a significant role to play in achieving this, activating a number of calcium-dependent target proteins by significantly raising their affinity for calcium upon binding (Chin and Means, 2000). Hence these proteins are not simply calcium-dependent but rather calcium/CaM-dependent. Two good examples of this pertinent to the development of pathological cardiac hypertrophy and HF are the phosphatase calcineurin and calcium/CaM-dependent protein kinase II (CaMKII).

1.4.4.1 Calcium-dependent regulation of cardiac hypertrophy

Perhaps the best characterised hypertrophic signalling pathway is that of the calcium-dependent phosphatase calcineurin, which acts to induce nuclear translocation of the transcription factor NFAT. Transgenic mice expressing constitutively activated forms of either of these proteins develop substantial spontaneous hypertrophy from an early age (Molkentin et al., 1998). Similarly cardiac hypertrophy can result from overexpression of the calcium-dependent α- and β- isozymes of PKC, albeit to a lesser extent (Braz et al., 2002, Bowman et al., 1997), whilst further calcium-mediated regulation of hypertrophy can occur via a newly defined pathway involving CAMTA2, part of a family of calmodulin binding transcription activators (Song et al., 2006).
Calcium oscillations have also been shown to regulate the expression and activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Dolmetsch et al., 1998), which exists as an inactive dimer in the cytoplasm of most cell types bound to regulatory inhibitors (IκB). NF-κB activation occurs in response to a variety of stimuli, which cause the degradation of IκB allowing NF-κB nuclear translocation and subsequent regulation of transcription. In the heart the effects of this are wide ranging, but prolonged NF-κB activation is thought to contribute to disease progression in cardiac hypertrophy and failure (Gordon et al., 2011), and recently NF-κB has been shown to synergistically promote pathological remodelling in combination with NFAT (Liu et al., 2012).

The best insight into how excitation-transcription coupling regulates pathways during hypertrophy however comes from investigations into the actions of the calcium-calmodulin-dependent protein kinase II (CaMKII). Transgenic overexpression of the various isoforms of CaMK induces hypertrophy (Zhang et al., 2002, Passier et al., 2000), and in the case of CaMKII, activation has been shown to occur through local calcium release from the nuclear envelope via the inositol 1,4,5-trisphosphate receptor (IP₃R) (Wu et al., 2006). Thus it is hypothesised that similar calcium microdomains may regulate other signalling pathways, in order to overcome the ‘noise’ of global calcium transients during EC coupling.

1.4.4.2 Signalling and the progression from compensated hypertrophy to heart failure

Irrespective of whether eccentric or concentric in nature, pathological hypertrophy causes irreversible cardiac remodelling which in the presence of a sustained stimulus can result in progression from a compensated state into decompensation. Apoptotic, necrotic and autophagic cell death leading to extensive fibrosis, coupled with excessive hypertrophy and myocyte disarray are key features which contribute to this progression, and cause the ventricle to become dilated, inelastic and unable to meet the demands of the body (Diwan and Dorn, 2007, Ritter and Neyses, 2003).
The same pathways activated during the compensatory stage of hypertrophy can also contribute to the progression towards dilated cardiomyopathy at the molecular level through the transcription of a variety of genes, reverting the myocardial profile to a more immature state (Frey et al., 2000). For example, the calcineurin transgenic heart expresses high levels of β-MHC and skeletal α-actin which normally dissipate soon after birth, thus affecting the contractile machinery (Molkentin et al., 1998).

As exposure to stress continues, the remodelling process progresses and, possibly as a consequence of the metabolically unfavourable switch in contractile protein isoforms, eventually leads to myocyte death and replacement with fibrous tissue (Diwan and Dorn, 2007). The resulting myocardium becomes inelastic, dilated and incapable of pumping with sufficient force to eject enough blood during systole, and hence heart failure ensues.

Given the critical nature of the pathways over which calcium signalling governs, it is easy to see how improper regulation of the signal can lead to disease progression, and whilst the overall contribution of the calcium/CaM-dependent PMCAs to calcium clearance during diastole may be small, there is accumulating evidence to suggest that they may play a significant role in the regulation of cardiac calcium signalling.
1.5 The plasma membrane calcium ATPase

The presence of a calcium pumping ATPase located in the plasma membrane was first identified in erythrocytes nearly half a century ago (Schatzmann, 1966). The PMCA has since been classified a member of the family of P-type transport ATPases, due to a covalent intermediate state formed during its catalytic cycle upon phosphorylation of an aspartic acid residue that is highly conserved amongst the group (Pedersen and Carafoli, 1987). This occurs following the binding of a single intracellular calcium ion with high affinity (0.2-0.5μM under optimal conditions), and leads to conformational changes allowing transport of the ion through the plasma membrane and its subsequent extrusion to the extracellular space by lowering the pump affinity, thus promoting dissociation (Mangialavori et al., 2010, Brini and Carafoli, 2009). The PMCA therefore has a low capacity for extrusion, removing only one calcium ion per ATP molecule hydrolysed in what is thought to be electroneutral exchange for two protons (Thomas, 2009), whereas related ATPases such as SERCA have a second calcium binding site and exhibit a 2:1 stoichiometry (Guerini et al., 2000). In non-excitable cells the PMCA represents the main pathway for calcium extrusion, however in excitable tissues such as the heart the presence of the higher capacity NCX means that only a small amount of calcium is removed through the PMCA (Brini et al., 2013).

There are four known PMCA isoforms, encoded by separate genes \textit{ATP2B1-4} located at human chromosomal loci 12q21-q23, 3p25-p26, Xq28 and 1q25-q32, respectively (Strehler and Zacharias, 2001). These exhibit differential spatial patterns of expression, with isoforms 1 and 4 being more or less ubiquitous and thus present in the heart, whilst PMCA2 and 3 expression is restricted to specific cell types, most notably in the nervous system. In addition, a total of over 25 splice variants have been identified, most of which show cell specific distributions, and this has led to the consensus that each isoform and variant may perform a unique function. The purpose of this thesis was to study the role of isoforms 1 and 4 as a whole in the myocardium and not the functional intricacies of alternative splicing and therefore variants will not be discussed any further.
1.5.1 PMCA isoform diversity and expression

The determination of the complete nucleotide sequence for the PMCA by two groups in the late 1980s began to clarify the structure of the enzyme (Shull and Greeb, 1988, Verma et al., 1988). These discoveries also made it evident that multiple isoforms existed, whilst a continuation of Shull and Greeb’s work revealed the presence of at least two alternatively spliced variants (Greeb and Shull, 1989). The current consensus is that there are four isoforms (PMCA1-4), each encoded by a different gene (ATP2B1-4, respectively). The gene products amongst these isoforms share approximately 75-85% identity, whilst around 85-90% of the primary sequence is conserved (Strehler, 1991). Interestingly, a comparison between rat and human isoforms reveals around 99% sequence homology amongst species. The regions of the PMCA displaying least identity amongst isoforms are the N- and C- terminals (Strehler et al., 2007), which may be of note as the C-terminal is especially rich in interaction partners (see section 1.5.5).

1.5.1.1 PMCA expression during embryonic development

The developing mouse embryo displays evidence of PMCA1 expression at least as early as day 9.5 post coitum (pc) and displays abundant and ubiquitous expression thereafter, although more concentrated in the heart, nervous system, skeletal muscle and intestine. By day 12.5pc all 4 isoforms are expressed, which in the case of PMCA2 is confined to the brain and PMCA3 to the nervous system, lung and skeletal muscle. PMCA4 expression is highest in the brain, bladder, heart and spinal cord although it is significantly less abundant than PMCA1 in all tissues (Zacharias and Kappen, 1999). The early and widespread nature of PMCA1 expression has led to the opinion that this isoform is the major housekeeping isoform.

1.5.1.2 PMCA expression in adulthood

The ubiquitous pattern of PMCA1 expression persists after birth and throughout adulthood in both rat and human, whilst PMCA4 is also present in most cells. In human adult lung, liver, kidney, stomach and skeletal muscle isoform 1 transcript levels have been found to make up roughly two thirds of total PMCA abundance, with PMCA4 accounting for the
remainder (Stauffer et al., 1993). In the heart however, isoform 1 & 4 transcript levels are present in similar amounts (47% and 51% of total PMCA expression, respectively). Conversely, PMCA2 displays a highly specific expression pattern, being most prominent in Purkinje and inner ear cells, as well as being abundant in lactating mammary glands (Strehler and Zacharias, 2001). Isoform 3 displays the most restricted pattern of expression in the neonate and adult, exclusively neuronal in human.

1.5.2 Disease associations of the PMCA

In recent years, the relevance of the PMCA in a number of human disease processes has come to light. Through examination of whole genomes amongst populations it is now possible to identify particular genetic variants associated with a certain disease. Upon extensive data analysis genome wide association studies (GWAS) can detect particular disease causing single nucleotide polymorphisms (SNPs) located within specific loci in the genome. Genetic mutations in the gene encoding each isoform have now been associated with human disease, highlighting the significance of this family of calcium pumps.

1.5.2.1 PMCA1, hypertension and cardiovascular disease

In the past five years GWAS into systolic and diastolic blood pressure, as well as hypertension in general have reported the most significant SNPs associated with these diseases to occur at multiple loci in and around ATP2B1 (Levy et al., 2009, Cho et al., 2009, Takeuchi et al., 2010, Kato et al., 2011, Johnson et al., 2011). This finding is consistent amongst multiple ethnicities, and Levy et al. predict the risk of developing hypertension to increase by 17% and 37% dependent upon whether one or two respective alleles are affected. These findings have since been corroborated in transgenic mice, where PMCA1 silencing leads to the development of hypertension associated with an increase in intracellular calcium and vascular remodelling (Kobayashi et al., 2012, Shin et al., 2013). In addition, a recent study identified ATP2B1 as a candidate gene for both coronary artery calcification and MI (Ferguson et al., 2013) which when combined with the association to hypertension suggests that PMCA1 plays a critical role in calcium clearance in VSM and/or endothelial cells.
1.5.2.2 PMCA2 and hereditary deafness

Two studies have identified separate mutations in \textit{ATP2B2} in families with hereditary hearing loss (Schultz et al., 2005, Ficarella et al., 2007). In each case this seems to potentiate the effects of a mutation in the cadherin23 gene, resulting in severe impairment. A transgenic mouse known as ‘deafwaddler’, which as the name suggests has both deafness and motor imbalance, also contains a PMCA2 mutation, and it is believed that this affects sensory transduction in hair cells of the inner ear, in addition to neurotransmitter release from the basolateral membrane (Street et al., 1998).

1.5.2.3 PMCA3, cerebellar ataxia and adenoma

\textit{ATP2B3} mutations have been linked to two separate diseases affecting different organs. One was identified in a family with X-linked cerebellar ataxia, where 3 out of 3 descendants of a carrier female were found to be either affected (males) or a carrier (female) (Zanni et al., 2012). \textit{In vitro} studies found the mutation to impair calcium clearance in cell culture.

Surprisingly given the predominant expression of PMCA3 in the nervous system, an \textit{ATP2B3} mutation was recently found in tissue from a number of aldosterone-producing adenomas, which contribute to primary aldosteronism being the most common cause of secondary hypertension (Beuschlein et al., 2013). This highlights a highly specialised function for this isoform in non-neuronal tissue.

1.5.2.4 PMCA4 and malarial resistance

The calcium ATPase of the membrane of erythrocytes was the first to be characterised, and has since been found to consist predominately of PMCA4 (Stauffer et al., 1995). Recently a GWAS identified a novel locus within \textit{ATP2B4} where several SNPs conferred resistance to a severe form of malaria amongst a West African population (Timmann et al., 2012), which may make PMCA4 an interesting target for anti-malarial medication (Mohamed et al., 2013).
1.5.3 PMCA structure

The general structure of the PMCA is comparable to other members of the P-type ATPase family, having ten hydrophobic transmembrane (TM) domains and two major intracellular loops, flanked by cytosolic N- and C-terminals (figure 1.2) (Strehler and Zacharias, 2001, Di Leva et al., 2008). The N-terminal comprises of 80-90 amino acids and contains the most variation amongst isoforms. The two intracellular loops span TM domains 2-3 and 4-5, the second of which contains conserved aspartate and lysine residues critical for catalytic phosphorylation and ATP-binding respectively, hence this is the catalytic domain of the PMCA. The long carboxyl terminal contains the CaM-binding site which in the absence of CaM inhibits pump activity by interacting with a region on each of the intracellular loops to render the PMCA in a closed conformation (Falchetto et al., 1991, Falchetto et al., 1992).

![Figure 1.2 General structure of the PMCA](image)

**Figure 1.2 General structure of the PMCA** Cartoon of the PMCA structure. Sites A and C represent the major splice sites. Highlighted is the catalytic core of the pump containing conserved aspartate (D) and lysine (K) residues, as well as the autoinhibitory interaction between the intracellular loops and CaM-binding domain. Figure courtesy of Donna Page
1.5.4 PMCA regulation

Autoinhibition via the CaM-binding domain serves as the major regulator of pump activity. Upon CaM binding, autoinhibition is released and pump calcium affinity increases to sub-μM levels rendering it active at cellular concentrations (Enyedi et al., 1987). There is a distinct difference in CaM affinity between the ubiquitous and neuronal isoforms, being in the order of 5-10 magnitudes higher in PMCA2 and 3 compared to PMCA1 and 4 (Brini and Carafoli, 2009). In addition, PMCA2 displays unusually high basal ATPase activity in the absence of CaM compared to other isoforms, in which CaM binding will usually serve to increase pump activity 4-6 fold (Elwess et al., 1997, Hilfiker et al., 1993). These factors may explain why PMCA4 is much slower to respond to a rise in calcium than PMCA2 and 3 (Caride et al., 2001).

A number of further CaM-independent modes of pump activation have been identified, including the action of acidic phospholipids, low concentrations of unsaturated fatty acids and substances leading to partial proteolysis of the enzyme (Niggli et al., 1981). In addition, phosphorylation of the PMCA by protein kinase C (PKC) (Enyedi et al., 1996), and interestingly in the case of PMCA1 PKA (Guerini et al., 2003), increases basal activity. Another peculiarity of PMCA1 identified in this latter study was its sensitivity to degradation by calpain when compared to the other isoforms, with some speculation from the authors as to whether this may indicate a higher turnover rate for the pump in vivo. Hence, despite sharing a good degree of homology in structure, the pumps display substantial inter-isoform variation in certain biochemical properties.

1.5.5 PMCA interactions

A key feature of the PMCA is the existence of multiple interaction partners, which may lend itself well to the notion of its role as a signalling molecule. The majority of these have been found to occur via a PDZ domain located at the terminal end of the carboxyl tail. All human PMCA isoforms adhere to the minimum consensus for class I PDZ ligand binding by terminating in the consensus motif ETSX. In PMCA4 a final valine residue replaces leucine which is conserved amongst isoforms 1-3 (Strehler and Zacharias, 2001). Interacting partners at this domain include members of the membrane-associated guanylate kinase (MAGUK) family, homer proteins and neuronal nitric oxide synthase (nNOS).
(Schuh et al., 2003b, Kim et al., 1998, DeMarco and Strehler, 2001, Sgambato-Faure et al., 2006, Schuh et al., 2001). The latter of these interactions is found in a macromolecular complex at the plasma membrane with α-1 syntrophin and dystrophin, through binding the second intracellular loop of PMCA to a linker region on α-1 syntrophin (Williams et al., 2006). Further interactions on this loop occur between the PMCA and calcineurin, the tumour suppressor Ras-associated factor 1A (RASSF1A) and eNOS (Buch et al., 2005, Armesilla et al., 2004, Holton et al., 2010), whilst the N-terminal region has been shown to associate with an isoform of 14-3-3 (Rimessi et al., 2005).

It may in part be through the individualities of these interactions that isoforms can perform specific functions. For example, in identifying an inhibitory interaction between isoform ε of 14-3-3 and PMCA4, Carafoli and colleagues noted no association with PMCA2 (Rimessi et al., 2005). Similarly, whilst Strehler and colleagues found both isoforms 2 and 4 to interact with MAGUK family proteins, some family members bound selectively to PMCA4 (DeMarco and Strehler, 2001).

1.5.5.1 The interaction between PMCA4 and nNOS

Whilst the precise function is not yet known of some of these interactions, others have been well characterised to be of functional significance. Of particular note in the cardiovascular system, the binding of PMCA4 and nNOS at its PDZ domain has been shown to influence vascular tone, basal contractility and β-adrenergic responsiveness (Cartwright et al., 2007, Cartwright et al., 2009).

This interaction was first identified in cell culture, where PMCA4 overexpression resulted in a reduction of nNOS activity, postulated to occur due to the decreased availability of local calcium, whilst a mutant nNOS molecule lacking its PDZ domain was unaffected (Schuh et al., 2001). Shortly after this two independent groups found arteries isolated from vascular smooth muscle cell (VSMC)-specific PMCA4 overexpressing mice to exhibit increased agonist-induced vasoconstriction which was reversible upon NOS inhibition, which Schuh et al. found to correlate with increased left ventricular systolic pressure (Gros et al., 2003, Schuh et al., 2003a).
Similarly, a functional interaction has since been identified in the heart, whereby PMCA4 overexpression was found to attenuate the inotropic response to β-adrenergic stimulation to an extent comparable to that seen upon nNOS-specific inhibition in controls, but not in mice overexpressing a mutant form of PMCA4 unable to interact with nNOS (Oceandy et al., 2007). This pathway has since been characterised to affect local cGMP and hence cAMP levels, thus modulating PKA activity at the SR (Mohamed et al., 2009, Mohamed et al., 2011). Interestingly, the latter of these studies found PMCA4 knockout mice to display a similar attenuation of the β-adrenergic response, but have enhanced basal contractility correlating with an increase in systolic calcium due to phosphorylation of the RyR at PKA-dependent serine residues. Furthermore, the PMCA4-nNOS interaction may also have disease implications following MI, when nNOS in association with its adaptor protein CAPON (carboxy-terminal PDZ ligand of NOS1), have been shown to be recruited to the sarcolemma to bind to PMCA4 (Beigi et al., 2009).

1.5.5.2 The interaction between the PMCA and modulators of hypertrophy

The characterisation of functioning interactions between isoforms of the PMCA and the hypertrophic signalling molecules calcineurin and RASSF1A suggested that the pumps may have a role to play during cardiac hypertrophy. Each molecule binds to a domain on the catalytic second intracellular loop of PMCA. Upon overexpression of PMCA4 in cultured HEK293 cells, activity of RASSF1A was reduced, thus inhibiting the activation of Ras-mediated signalling (Armesilla et al., 2004). At the time RASSF1A, although a potential upstream regulator of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signalling, was not known to be associated with the hypertrophic process. However, knockout hearts have since been shown to have an exacerbated response to pressure overload, through mediating ERK1/2 signalling (Oceandy et al., 2009).

An association between the PMCA and calcineurin has been witnessed in multiple isoforms in a number of cell types. The interaction was first witnessed in PMCA4 overexpressing HEK cells and mapped to the catalytic subunit of calcineurin A, with consequential attenuation of calcineurin-NFAT (nuclear factor of activated T-cells) signalling (Buch et al., 2005). Calcineurin has since been noted to also interact with
PMCA2 and 4 in breast cancer cells and PMCA1,2 and 4 in endothelial cells (Holton et al., 2010, Holton et al., 2007), as well as with PMCA4 in the PC12 pheochromocytoma cell line (Kosiorek et al., 2011). Calcineurin-NFAT signalling is one of the best characterised pathways in the development of pathological hypertrophy and remodelling (Molkentin et al., 1998), and thus this has prompted research into the function of this interaction during the hypertrophic process. PMCA4 overexpression was found to significantly reduce the extent and progression of pathological remodelling through the attenuation of NFAT signalling (Wu et al., 2009), however the role of PMCA4 in the hypertrophic process in general has produced conflicting results (Oceandy et al., 2007, Abou-Leisa, 2013). These are discussed further in chapter 4, however what does seem clear is that isoform 4 has the ability to regulate hypertrophic signalling.

1.5.6 The PMCA in the cardiovascular system

In addition to its regulatory roles in contractile and hypertrophic processes in the heart, several roles have been identified for the PMCA in non-cardiac cell types in the cardiovascular system. In contrast to cardiac muscle, it has been demonstrated that the PMCA accounts for the bulk of calcium extrusion from vascular smooth muscle, and its inhibition prolongs aortic relaxation to a similar extent to that of SERCA whilst NCX inhibition has little effect (Pritchard et al., 2010), and in myometrial resistance arteries PMCA inhibition mimics the phenotype of preeclamptic vessels (Wimalasundera et al., 2010). It is perhaps not surprising therefore that both PMCA1 and 4 have been shown to influence vascular tone (Gros et al., 2003, Schuh et al., 2003a, Shin et al., 2013), and as previously mentioned PMCA1 has recently been identified as a leading candidate gene for the development of hypertension (Hirawa et al., 2013). The development of hypertension in PMCA1 VSMC-specific knockout mice was found to correlate with increased intracellular calcium (Kobayashi et al., 2012), whereas PMCA4 overexpressing mice were found to elevate blood pressure through the negative regulation of nNOS (Gros et al., 2003, Schuh et al., 2003a). More recently a functional interaction between isoforms 1, 2 and 4 of the PMCA and eNOS, the major producer of the vasodilator nitric oxide in the vasculature, has been identified in endothelial cells, providing further evidence of the potential significance of the PMCA in this tissue (Holton et al., 2010).
Outside of the vasculature the PMCA has also been found to influence calcium homeostasis in platelets, being identified as the major responder to a rapid rise in intracellular calcium (Juska, 2010), and PMCA inhibition has been found to reduce platelet aggregation responses, a phenomenon which was also witnessed in PMCA4 knockout mice (Jones et al., 2010).

1.5.7 The potential for specific roles for PMCA1 and 4 in the heart

Given the pattern of expression, the differences in CaM affinity and dependence, and the preferential associations with some interacting partners it is fairly clear that the neuronal isoforms have evolved to have independent and specialised roles. The question is what is the requirement for two ubiquitously expressed PMCA isoforms? The presence of a PKA phosphorylation site in PMCA1 and substitution of a single amino acid residue in the PDZ domains may highlight two potential differences for pump function, but surely this cannot explain the need for separate gene products.

The early presence of PMCA1 in the developing mouse embryo and its predominant expression over PMCA4 in the majority of tissues has led to the opinion that PMCA1 may be the major housekeeping isoform involved in calcium clearance, whilst accumulating evidence suggests that PMCA4 has an alternative role in calcium signalling highlighted by the functional interactions with nNOS and calcineurin described in the sections 1.5.5.1-2. Indeed, our group and others have shown global deletion of PMCA1 to cause embryonic lethality suggesting an important developmental role in calcium clearance (Shaheen, 2010, Okunade et al., 2004). Evidence from other tissues certainly suggests that PMCA1 is crucial to calcium homeostasis in non-cardiac cell types, exemplified by its link to hypertension (Hirawa et al., 2013). Meanwhile the localisation of PMCA4 in tissues including the heart to invaginations in the cell membrane known as caveolae, believed to be major sites for signal transduction, supports its role as a regulator of the calcium signal (Fujimoto, 1993, Hammes et al., 1998). Furthermore, PMCA4 knockout and transgenic cardiomyocytes have not displayed an altered rate of calcium decay suggesting that they indeed play a negligible role in extrusion of the global transient (Oceandy et al., 2007, Wu et al., 2009, Mohamed et al., 2011).
Few studies have managed to distinguish between PMCA1 and PMCA4 function due to the lack of specific inhibitors. However, through the use of PMCA4 knockout mice, in combination with mice heterozygous for PMCA1 deletion, Liu et al were able to deduce that in bladder smooth muscle reducing PMCA1 expression causes a rise in intracellular calcium, whereas deletion of PMCA4 leads to a reduced response to agonist stimulation (Liu et al., 2007). Hence the authors drew the conclusion that in the bladder calcium extrusion is mainly mediated by PMCA1, and PMCA4 regulates signalling events. Further support for this notion comes from a study conducted in breast cancer cells which found knockdown of PMCA1 to impair calcium clearance but not PMCA4 (Curry et al., 2012).

In light of this evidence it seems sound reasoning to assume that in cardiomyocytes similarly distinct roles exist for PMCA1 and PMCA4. If the role for PMCA1 is to extrude calcium, then the minor contribution of the PMCAs as a whole to global calcium clearance would suggest that PMCA1 therefore plays a largely insignificant role in the heart. However, studies in SERCA knockout mice and NCX knockdown cells have each shown substantial increases in PMCA expression, whilst calcium homeostasis has been relatively well maintained, at least for a certain period of time (Louch et al., 2010, Hurtado et al., 2005). Although specific PMCA antibodies were not used in these studies, this suggests that at least one isoform of the PMCA is capable of compensating in part for the loss of either of the faster systems, and in conjunction with a decrease in L-type calcium current and increased gain of SR calcium release these adaptations are sufficient to preserve adequate cardiac function in adult NCX knockout hearts (Henderson et al., 2004). Therefore, perhaps the major role of PMCA1 in cardiomyocytes is a reserve capacity for calcium clearance during times of stress?

Given the multi-faceted nature of the PMCA’s actions during calcium homeostasis, and their associations to a number of disease processes in other tissues, it seems pertinent to fully characterise their role in an organ in which calcium homeostasis is so crucial to the maintenance of health and development of disease. This thesis will examine the specific
role of PMCA1 and PMCA4 in the normal heart, and during the development of left ventricular hypertrophy.

1.6 Hypothesis

Isoforms 1 and 4 of the PMCA perform specific roles in the heart. At rest PMCA1 accounts for the bulk of the calcium cleared via the PMCA, whilst PMCA4 influences systolic calcium levels. Following pressure overload PMCA1 will have a greater contribution to calcium clearance than at rest, whilst PMCA4 gene ablation will regulate the hypertrophic response.

1.7 Aims

To characterise the specific roles of PMCA1 and 4 in the heart two knockout mouse models will be used. The first of these has been previously generated in our laboratory and carries cardiomyocyte-specific deletion of PMCA1. The second will be generated by crossing PMCA1 knockout and PMCA4 targeted mice to create a PMCA1:4 double knockout, with deletion restricted to the myocardium.

The basal cardiac phenotype in double knockout mice will be fully characterised using in vivo techniques such as echocardiography and pressure volume loop analysis as mice develop throughout adulthood. Calcium handling protein expression will be analysed by western blot. Intracellular calcium handling will be assessed by field stimulation, and compared to that of PMCA1 knockout mice to assess the specific role of each isoform, as well as their relative contributions to global calcium clearance by inhibiting SERCA and NCX.

To assess the roles of each isoform during the development of pathological hypertrophy the common technique of transverse aortic constriction will be used to induce pressure overload for a period of 2 weeks in each mouse line. Again the cardiac phenotype will be analysed in vivo in addition to histologically. The contribution of PMCA1 to calcium
clearance following pressure overload will be assessed in the same manner as basal calcium handling.

Lastly, the response of double knockout mice will be assessed during a physiological form of hypertrophy induced by exercise, given the vastly different pathway through which it acts when compared to pathological hypertrophy. This will be achieved by subjecting mice to an intense program of swimming for a period of 4 weeks, following which the hypertrophic response will be assessed. All experiments will be performed alongside appropriate controls incorporating the different transgenes used in the generation of the knockout mice.
Chapter 2
2. GENERAL METHODS

2.1 Animal subjects

All mice were housed in a specific pathogen free environment in the animal housing unit of the University of Manchester, and procedures performed adhered to the United Kingdom Animals (Scientific Procedures) Act 1986 and were approved by the University of Manchester Ethics Committee. For the purposes of this study, all experiments were performed using male mice due to the protective effects of oestrogens during pathological hypertrophy (Knowlton and Lee, 2012).

All surgical procedures were performed under general anaesthesia following IP injection of ketamine and xylazine (100 & 5 mg/kg respectively), with 0.1mg/kg IP buprenorphine administered as analgesia upon recovery of consciousness. In vivo analysis of cardiac function was conducted under general anaesthesia induced by 250mg/kg of Avertin (tribromoethanol, Sigma) delivered by IP injection, with the exception of experiments involving recovery in which 3% isofluorane supplemented by 1% oxygen was used. All mice in this study were killed by cervical dislocation, an approved schedule 1 method.

2.2 Generation of mice carrying cardiomyocyte-restricted dual deletion of PMCA1 and PMCA4

Cardiomyocyte-specific PMCA1:4 double knockout mice (referred to hereafter as PMCA1:4\(^{dcko}\) mice) were generated using Cre/loxP technology driven by the \(\alpha\) myosin heavy chain (\(\alpha\)MHC) promoter (Agah et al., 1997). This promoter has been shown to catalyse recombination events in around 80% of targeted cardiomyocytes by 3 weeks of age. PMCA1 and PMCA4 floxed mouse lines (PMCA1\(^{ff}\) and PMCA4\(^{ff}\)) bred on a mixed C57Bl/6:Sv/129 background had been previously established in our laboratory, the constructs of which are shown in figure 2.1. In both cases the \(loxP\) sites flanked regions containing the ATG start codon, spanning exon 2 and exons 2-3 of the PMCA1 and 4 genes respectively, which would be predicted to prevent transcription upon recombination. Thus, when these mice are crossed with heterozygous animals expressing Cre-recombinase under the control of the \(\alpha\)MHC promoter the region is excised and the open reading frame
disrupted, producing cardiomyocyte-specific single knockout mice (PMCA1\textsuperscript{cko} and PMCA4\textsuperscript{cko} mice). The previously generated PMCA1\textsuperscript{cko} mouse line would also be used in this study (Shaheen, 2010).

PMCA1:4\textsuperscript{dcko} mice were generated by firstly mating the PMCA1\textsuperscript{cko} and PMCA4\textsuperscript{f/f} mouse lines, and subsequent crossing of heterozygous offspring. The breeding strategy used to generate double knockout mice, along with control subjects carrying either the floxed construct or αMHC-Cre transgene is described in the results section of chapter 3.

**Figure 2.1 Sites of \textit{loxP} insertion in PMCA1 and 4 floxed alleles** Cartoon demonstrating the location of \textit{loxP} sites in regions flanking the ATG start codon within exon 2 of the PMCA 1 and 4 genes, and the excised allele remaining upon Cre-mediated recombination.
2.3 Molecular analysis

A number of molecular analysis techniques were used during the generation and characterisation of PMCA1:4\textsuperscript{dcko} and PMCA1\textsuperscript{cko} mice. Mouse genotypes and evidence of Cre-mediated excision events were determined by polymerase chain reaction (PCR), calcium handling protein expression was examined by western blot and biomarkers for fibrosis and induction of the foetal gene program were analysed by quantitative real-time PCR (qPCR).

2.3.1 PCR

The polymerase chain reaction amplifies targeted regions of DNA through stages of thermal cycling, allowing the denaturation, annealing and elongation of a specific DNA fragment. The size of the PCR product can subsequently be determined upon electrophoresis, enabling the identification of inserted \textit{loxP} constructs and Cre-excision events in PMCA1:4\textsuperscript{dcko} mice.

2.3.1.1 DNA extraction

DNA was extracted from ear snips to allow genotyping of litters, and from tissue or cardiomyocytes taken from euthanized animals to examine whether cardiomyocyte-specific excision events had occurred in PMCA1:4\textsuperscript{dcko} mice.

Samples were incubated overnight at 56°C following the addition of 200μl of lysis buffer (containing 50mM Tris-HCl pH8, 100mM EDTA, 0.5% SDS) and 10μl of proteinase K (10mg/ml). Samples were then centrifuged for 10 minutes at 13000rpm and the lysate removed to a fresh tube. 300μl of propan-2-ol was then added and samples inverted 30 times in order to precipitate the DNA, followed by further centrifugation (13000rpm for 10 minutes). The supernatant was discarded and the DNA pellet washed through a final centrifugation (5 minutes at 13000rpm) following the addition of 100μl of 70% ethanol, which was subsequently removed and the pellet resuspended in 50-100μl of TE buffer.
(10mM Tris-HCl pH 7.5, 1mM EDTA pH 8) dependent on yield. Extracted DNA was stored at -20°C until required.

2.3.1.2 PCR genotyping

In order to genotype each mouse three separate reactions had to be performed to determine whether mice were wild type (wt), heterozygous or homozygous for floxed PMCA1 and 4 alleles, and whether or not they expressed the gene sequence for Cre recombinase. The reaction mix for each PCR differed only in the primers used, and contained 15μl ReddyMix PCR master mix (Thermo scientific), 11.9μl PCR water, 0.9μl forward primer, 0.9μl reverse primer, 0.3μl MgCl₂ and 1μl of DNA. Reactions were run on either an MJ Research PTC-200 or Veriti™ 96-well thermal cycler (Applied biosystems). The primers used and expected PCR product sizes for each reaction are listed in Table 2.1, and reaction conditions in Table 2.2.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence 5’-3’</th>
<th>Expected PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type allele</td>
<td>Floxed allele</td>
</tr>
<tr>
<td>PMCA1flex – forward</td>
<td>CTGGGCTCACCTAGTTTGCTAAACC</td>
<td>754</td>
</tr>
<tr>
<td>PMCA1flex – reverse</td>
<td>CTGGGAGTAGATGCCTGTCGTGC</td>
<td></td>
</tr>
<tr>
<td>PMCA4flex – forward</td>
<td>GCAAATTCTGGCAAATGACCCGAAC</td>
<td>702</td>
</tr>
<tr>
<td>PMCA4flex – reverse</td>
<td>GGATCTGAGTGTCCTGGGCTGTCG</td>
<td></td>
</tr>
<tr>
<td>MHC 5</td>
<td>ATGACAGACAGATCCCTCTATCTCC</td>
<td>Absent</td>
</tr>
<tr>
<td>Cre 3</td>
<td>TCTATCGTGCATCGAC</td>
<td>Absent</td>
</tr>
</tbody>
</table>

Table 2.1 Primer pair sequences and PCR product sizes for genotyping reactions

<table>
<thead>
<tr>
<th>Step</th>
<th>PMCA1flex</th>
<th>PMCA4flex</th>
<th>αMHC-Cre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Temp (°C)</td>
<td>Time (s)</td>
<td>Cycles</td>
</tr>
<tr>
<td>Denaturing</td>
<td>94</td>
<td>420</td>
<td>1</td>
</tr>
<tr>
<td>Denaturing</td>
<td>94</td>
<td>30</td>
<td>35</td>
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<td>Annealing</td>
<td>65</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>68</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>Completion</td>
<td>68</td>
<td>300</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.2 Cycling conditions for genotyping reactions
2.3.1.3 Detection of Cre-mediated excision events by PCR

DNA extracted from cardiomyocytes and other organs taken from 8 week old mice was amplified by two PCRs to detect whether Cre-mediated excision of the targeted regions of PMCA1 and 4 had occurred. Volumes used in the reaction mix were identical to those for genotyping reactions, with ReddyMix extensor PCR master mix (Thermo scientific) used in place of the standard ReddyMix, and reactions were run on a RoboCycler® gradient 40 machine (Stratagene). Primer sequences and PCR product sizes are listed in Table 2.3 and reaction conditions in Table 2.4.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence 5’-3’</th>
<th>Expected PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>wild type allele</td>
</tr>
<tr>
<td>PMCA1&lt;sup&gt;1st&lt;/sup&gt; – forward</td>
<td>CCAGAGACCATTCATGGCTTCTACC</td>
<td>3449</td>
</tr>
<tr>
<td>PMCA1&lt;sup&gt;1st&lt;/sup&gt; – reverse</td>
<td>AATGCTCTCTGACGATGGTCTGG</td>
<td></td>
</tr>
<tr>
<td>PMCA4&lt;sup&gt;4th&lt;/sup&gt; – forward</td>
<td>TCGTCCAGACCTTCCATCAG</td>
<td>5505</td>
</tr>
<tr>
<td>PMCA4&lt;sup&gt;4th&lt;/sup&gt; – reverse</td>
<td>AGAGACCTGAAGATGACGAGGCACGC</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3 Primer sequences and PCR product sizes for detection of Cre-mediated excision

<table>
<thead>
<tr>
<th>Step</th>
<th>PMCA1&lt;sup&gt;1st&lt;/sup&gt;</th>
<th>PMCA4&lt;sup&gt;4th&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Temp (°C)</td>
<td>Time (s)</td>
</tr>
<tr>
<td>Denaturing</td>
<td>95</td>
<td>300</td>
</tr>
<tr>
<td>Denaturing</td>
<td>95</td>
<td>30</td>
</tr>
<tr>
<td>Annealing</td>
<td>65</td>
<td>30</td>
</tr>
<tr>
<td>Extension</td>
<td>68</td>
<td>300</td>
</tr>
<tr>
<td>Completion</td>
<td>72</td>
<td>600</td>
</tr>
</tbody>
</table>

Table 2.4 Cycling conditions for the detection of Cre-mediated excision

Amplified PCR products were run at 100V on 0.8-1.5% agarose (agarose dissolved in TAE containing 40mM Tris base, 20mM acetic acid, 1mM EDTA) gel containing 0.015μl/ml of ethidium bromide alongside HyperLadder™ I (Bioline) DNA ladder and viewed using a ChemiDoc™ XRS+ imaging system (Bio-Rad).
2.3.2 Protein analysis by western blot

Western blot is a commonly used technique for examining protein expression. It involves the separation of proteins according to size by electrophoresis followed by immunological analysis using specific antibodies.

2.3.2.1 Protein extraction

Isolated adult cardiomyocytes were lysed in 300-700μl RIPA buffer (containing 1x PBS, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 0.5mM PMSF, 500ng/ml Leupeptin, 1μg/ml Aprotinin, 2.5μg/ml Pepstatin A) dependent on yield through rotation for 30 minutes at 4°C. Cellular debris was removed by centrifugation (3000rpm for 5 minutes at 4°C) and the proteinaceous supernatant pipetted off and stored at -80°C. 20μl was stored separately to determine concentration.

Tissues were harvested from sacrificed animals and immediately snap frozen in liquid nitrogen for storage at -80°C. To extract protein, tissues were washed in PBS to remove blood clots and cut into small pieces, which were then placed in a Dounce homogeniser. 400-1000μl (dependent on amount of tissue) of ice-cold RIPA buffer was added and tissue manually homogenised. As with cardiomyocyte extracts, cellular debris was removed and samples stored at -80°C.

2.3.2.2 Determination of total protein concentration

Protein concentration in all samples was quantified using a bicinchoninic acid (BCA) assay kit (Pierce), compatible with the detergents present in the RIPA buffer. This works on the principle that digested proteins reduce copper sulphate solution in the assay to copper ions under alkaline conditions, which bind to BCA forming a product of measurable absorbance at 562nm. The concentration of unknown samples can therefore be determined through their comparison to the absorbance of a range of known BSA protein standards (0-2mg/ml in RIPA buffer). Standards and samples were loaded in triplicate onto a 96 well plate, incubated for 30 minutes at 37°C following the addition of the BCA reagent and mean absorbance was measured on an optical plate reader (Thermo Labsystems).
2.3.2.3 Western blot

To analyse specific protein abundance, samples were separated by SDS-PAGE using 4-15% acrylamide gels dependent on target protein size (sodium dodecyl sulphate polyacrylamide gel electrophoresis – for buffers see table 2.5). 5-50μg of total protein per sample, again dependent on target protein, was loaded into the stacking gel together with 10μl of 2x Laemmli buffer (Sigma Aldrich) alongside a molecular weight marker (Bio-Rad). Gels were then placed in an electrophoresis tank containing Tris glycine buffer (25mM Tris Base, 0.25M glycine, 0.1% SDS) and run for 90 minutes at 127V. Following separation, proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane (Millipore) using wet or semi-dry apparatus transfer apparatus (Invitrogen) by applying a current of 200mA for 2 hours or 200mA/gel for 1 hour, respectively in the presence of a transfer buffer containing 25 mM Tris Base, 0.25 M glycine and 20% methanol. To prevent non-specific binding when probed, membranes were blocked for a minimum of 1 hour in TBS (Tris buffered saline containing 150 mM NaCl, 10 mM Tris base) containing 1-3% bovine serum albumin (BSA – Sigma Aldrich) or 1-5% non-fat milk (Sigma Aldrich).

<table>
<thead>
<tr>
<th>Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-15% acrylamide/bis-acrylamide (37.5:1)</td>
<td>5% acrylamide/bis-acrylamide (37.5:1)</td>
</tr>
<tr>
<td>0.375 M Tris-base (pH 8.8)</td>
<td>0.375 M Tris-base (pH 6.8)</td>
</tr>
<tr>
<td>0.1% SDS</td>
<td>0.1% SDS</td>
</tr>
<tr>
<td>0.1% ammonium persulphate</td>
<td>0.1% ammonium persulphate</td>
</tr>
<tr>
<td>0.04% TEMED</td>
<td>0.04% TEMED</td>
</tr>
</tbody>
</table>

Table 2.5 Buffers used in preparation of gels for SDS-PAGE

The blocking agent was removed by washing 3 times in TBST (TBS containing 0.05% Tween-20), after which membranes were probed with a primary antibody (1:1000 dilution in TBS) for a minimum of 2 hours. Unbound antibody was washed 3 times in TBST and membranes were then incubated with the relevant horseradish peroxidise (HRP)-linked secondary antibody (Cell Signalling Technology anti-rabbit or anti mouse IgG, 1:5000 dilution in TBST for 2 hours), targeted against the same species as the primary.
Membranes were then washed 3 times in TBST before enhanced chemiluminescence (ECL) western blotting reagents (GE healthcare) containing 1μl/ml H$_2$O$_2$ were used to detect targeted protein bands upon imaging (ChemiDoc™ XRS+ imaging system - Bio-Rad). Antibodies were stripped from the membrane using a 0.1M glycine solution, pH 2.5 for 30 minutes at room temperature, which was subsequently washed 3 times in TBST before probing with antibodies to detect a housekeeping gene to which the protein of interest level could be normalised. The mean protein of interest:loading control ratio for the wild type control group in each blot was subsequently set to 1, and all other values and errors adjusted proportionally to this for ease of determination of fold change of expression. Band intensities were determined using Image Lab™ software (Bio-Rad). Antibodies used are listed in table 2.6.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Manufacturer</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMCA1</td>
<td>Abcam</td>
<td>Anti-rabbit</td>
</tr>
<tr>
<td>PMCA4</td>
<td>Abcam</td>
<td>Anti-mouse</td>
</tr>
<tr>
<td>NCX1</td>
<td>Swant</td>
<td>Anti-rabbit</td>
</tr>
<tr>
<td>SERCA2a</td>
<td>Thermo Fisher</td>
<td>Anti-mouse</td>
</tr>
<tr>
<td>LTCC</td>
<td>Abcam</td>
<td>Anti-mouse</td>
</tr>
<tr>
<td>RyR</td>
<td>Abcam</td>
<td>Anti-mouse</td>
</tr>
<tr>
<td>pPLN$_{ser16}$</td>
<td>Badrilla</td>
<td>Anti-rabbit</td>
</tr>
<tr>
<td>PLN</td>
<td>Millipore</td>
<td>Anti-mouse</td>
</tr>
<tr>
<td>Dystrophin</td>
<td>Sigma Aldrich</td>
<td>Anti-mouse</td>
</tr>
<tr>
<td>Na$_x$1.5</td>
<td>Alomone</td>
<td>Anti-rabbit</td>
</tr>
<tr>
<td>Na/K ATPase</td>
<td>Abcam</td>
<td>Anti-mouse</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Abcam</td>
<td>HRP-linked primary</td>
</tr>
<tr>
<td>β-actin</td>
<td>Abcam</td>
<td>HRP-linked primary</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>Abcam</td>
<td>HRP-linked primary</td>
</tr>
</tbody>
</table>

Table 2.6 Antibodies used in immunoblotting
2.3.3 mRNA analysis by qPCR

qPCR allows the quantification of mRNA expression of a target gene following its conversion to single stranded complimentary DNA (cDNA) by reverse transcription (RT). This is achieved by adding a fluorescent dye into the PCR reaction mixture which binds to the amplifying nucleotide fragment as it forms allowing measurement of its fluorescence in real-time.

2.3.3.1 RNA extraction

Frozen heart tissue was firstly cut into small pieces and transferred to a Dounce homogeniser. 1ml/whole heart of ice-cold TRIzol (Invitrogen) was added and the tissue manually homogenised before being poured back into an Eppendorf. 200μl of chloroform per ml TRIzol used was added and tubes shaken vigorously for 15 seconds, after which they were incubated at room temperature for 2 minutes and then centrifuged at 13000rpm for 15 minutes at 4°C. The clear aqueous upper phase was removed to a fresh tube and 0.5ml of propan-2-ol per ml TRIzol was added to precipitate the RNA. Tubes were inverted 30 times, incubated for 10 minutes at room temperature and then centrifuged at 13000rpm for 10 minutes at 4°C. The supernatant was discarded and pelleted RNA washed via the addition of 150μl of 70% ethanol and centrifugation (13000rpm for 5 minutes at 4°C), after which the supernatant was removed and the pellet resuspended in ~50μl (dependent on yield) of diethyl pyrocarbonate (DEPC) – treated water. RNA concentration was quantified using a NanoDrop 1000 spectrophotometer (Thermo scientific), following which samples were stored at -80°C.

2.3.3.2 RNA conversion to cDNA by reverse transcription

2μg of total RNA was converted to cDNA using a high-capacity cDNA reverse transcription kit in the presence of RNAase inhibitors (Applied Biosystems) as per the manufacturer’s instructions. Additional samples also underwent the same reaction but without the addition of the reverse transcriptase enzyme. These would act as -veRT controls during the qPCR reaction to account for the possibility of DNA contamination during the quantification process.
2.3.3.3 qPCR

cDNA was diluted to a final concentration of 10ng/μl with RNAse-free water. The 10μl qPCR reaction mix contained 5μl SYBR Green (Applied Biosystems), 1μl quantititect primer assay (Qiagen), 1μl cDNA and 3μl DEPC water. Samples were loaded in triplicate into a 96-well plate (Starlab) alongside -veRT and –ve cDNA controls and run on a 7500 Fast Real Time PCR machine (Applied Biosystems) using the cycling conditions listed in table 2.7. A melt curve stage was included to ensure that only one PCR product was present. Gene expression was then calculated by the ΔΔCT method of relative quantification (Livak and Schmittgen, 2001), by comparing gene of interest levels to those of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This works on the principle that the amplifying nucleotide strand doubles in length with each cycle, thus producing twice the fluorescence. This causes an exponential rise in fluorescence as the reaction progresses, and by determining at what cycle a certain threshold is reached compared to the housekeeping gene the relative abundance of the gene of interest can be calculated.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp (°C)</th>
<th>Time (s)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Initiation</td>
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<td>120</td>
<td>1</td>
</tr>
<tr>
<td>Denaturing</td>
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<td>600</td>
<td>1</td>
</tr>
<tr>
<td>Denaturing</td>
<td>95</td>
<td>15</td>
<td>40</td>
</tr>
<tr>
<td>Annealing</td>
<td>60</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Melt curve</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>15</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.7 Cycling conditions for qPCR
2.4 In vivo analysis of cardiac structure and function

The in vivo cardiac phenotype of PMCA1:4\(^{\text{dcko}}\) and PMCA1\(^{\text{cko}}\) mice was characterised using the techniques of cardiac ultrasound, invasive left ventricular catheterisation and 3-lead electrocardiogram (ECG). All experiments were performed under terminal anaesthesia with 250mg/kg of Avertin (tribromoethanol, Sigma) delivered by a single intraperitoneal injection unless stated otherwise. This is a popular choice of anaesthetic in cardiac research due to its modest cardiodepressive effects; however its use is discouraged in experiments involving recovery due to reports of fibrous adhesions in the abdominal cavity and death (Chu et al., 2006, Norris and Turner, 1983). Echocardiographic and haemodynamic procedures were performed by Min Zi.

2.4.1 Echocardiography

Cardiac ultrasound provides a non-invasive means with which to assess chamber dimensions, wall thickness and cardiac contractility in vivo through visualisation of the ventricle as it cycles between systole and diastole. Cardiac morphology and function was analysed using an Acuson Sequoia C256 ultrasound machine fitted with a 14MHz transducer (Siemens).

Mice were placed on a heat pad (set to 37°C) and a depilatory cream was applied to the left hemithorax. Warmed ultrasound transmission gel was then applied to the transducer for optimal resolution and the heart imaged in the two-dimensional short-axis view, whereupon an M-mode image was produced. Left ventricular diameter (LVD), posterior wall (PW) and interventricular septal (IVS) thicknesses in both systole (s) and diastole (d) were recorded using the leading-edge method over a minimum period of three cardiac cycles as depicted in figure 2.2. From these values an estimate of the measures of cardiac performance and structure listed in table 2.8 were made.
Figure 2.2 Imaging of the left ventricle through cardiac ultrasound

A) Two-dimensional short-axis view demonstrating the plane through which M-mode measurements were taken. B) Typical M-mode view from which the indicated measurements were obtained. LVD, left ventricular diameter; PW, posterior wall thickness; IVS, interventricular septal thickness; s, systole; d, diastole.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Formula</th>
</tr>
</thead>
</table>
| Fractional shortening (FS%)   | \[
\frac{[(dLVD-sLVD)/dLVD] \times 100}{\text{ }}
\]                                    |
| Relative wall thickness (RWT) | \[
\frac{(dLVD+dPW)/dLVD}{\text{ }}
\]                                       |
| Left ventricular mass (LVM)   | \[
1.055 \times \left[ \left(\frac{(dLVD+dPW+IVS)}{dLVD}\right)^3 - dLVD^3 \right]
\]                                 |

Table 2.8 Formulae derived from echo data for the assessment of cardiac structure and function

1.055 is the myocardial specific gravity (g/ml)
2.4.2 Left ventricular catheterisation and haemodynamic analysis

Through the insertion of a catheter into the left ventricle it is possible to obtain data pertaining to pressure and volume changes during the cardiac cycle. This enables the dynamic measurement of systolic and diastolic left ventricular performance.

Mice were placed in a supine position and a midline cervical incision was made, following which the sternohyoid muscles were retracted to expose the right carotid artery. This was tied at the bifurcation point preventing regurgitation from the periphery, and then occluded proximally allowing an incision to be made with minimal blood loss. A high-fidelity micromanometer-conductance catheter (Millar Instruments) was inserted via the carotid artery and fed into the ascending aorta, through the aortic valve and finally into the left ventricle.

Recordings were taken once heart rate had stabilized using a PowerLab system (Millar). Pressure-volume (PV) changes allowed the measurement of maximal systolic pressure ($P_{\text{max}}$) and rate of pressure change ($\text{dP/dt}_{\text{max}}$). This was used to assess contractility in combination with the end systolic pressure volume relationship (ESPVR) and its slope the end systolic elastance ($E_{\text{es}}$), obtained upon transient occlusion of the inferior vena cava. Determination of the end systolic pressure ($P_{\text{es}}$) and end diastolic volume (EDV) enabled the calculation of the arterial elastance ($E_a$), allowing assessment of arterial-ventricular coupling when compared to $E_{\text{es}}$. Lusitropic function was assessed via minimum dP/dt values and the time constant of relaxation, tau. Analysis was performed using Millar’s PVAN™ software. These measurements are illustrated in the PV loops in figure 2.3.
Figure 2.3 **Measurements derived from PV loop analysis** A) Pressure and volume changes inside the left ventricle during the cardiac cycle. Illustrated are the stroke volume (SV), along with the end diastolic volume (EDV) and end systolic pressure (P_{es}), from which the arterial elastance (E_{a}) can be derived. B) An example of the change in PV relationship upon transient occlusion of the inferior vena cava, from which the end systolic pressure volume relationship (ESPVR) and its slope end systolic elastance (E_{es}) can be derived.

### 2.4.3 ECG analysis

The ECG provides an image of conduction through the heart during the cardiac cycle. 3-lead ECG recordings were taken over a period of 5 minutes using a PowerLab ECG system (AD Instruments). Mice were placed on a heat pad to maintain body temperature and electrodes inserted into the muscle of the right and left forelimb, and right hindlimb. Recordings were analysed using LabChart 7 (AD Instruments) to compare heart rate and a number of segment intervals between groups. These are illustrated on the diagram of a typical ECG trace in figure 2.4. The QT duration corrected for heart rate (QT_c) was calculated using the most common formula, that of Bazett (QT_c = QT/√(60/HR)).
2.4.4 Tissue harvesting

Following *in vivo* analysis mice were euthanized by cervical dislocation. Hearts were excised and drained of blood before determining the mass. Similarly, the lungs were removed and weighed, and the tibia length was measured for normalisation of heart and lung weights.

2.5 Histological analysis

Following excision hearts were dissected and a transverse section was cut through the ventricles. This was placed in 4% paraformaldehyde (PFA) and incubated for 3 days at 4°C to allow fixation to occur. Sections were then removed to histology cartridges (Raymond A. Lamb) and dehydrated overnight in a Shandon Citadel 2000 tissue processor before embedding in paraffin wax. 5μm sections were cut using a microtome after which they were mounted onto poly-l-lysine coated slides (VWR) and dried in a 37°C oven. Wax was removed by heating the base of the slides on a heat block, followed by placing them in
xylene. Slides were subsequently rehydrated by placing them in decreasing concentrations (100%, 90% and 70%) of industrial methylated spirit (IMS) for 2 minutes/concentration. Finally slides were rinsed under running tap water for 5 minutes before staining.

2.5.1 Haematoxylin & eosin staining for cell size analysis

Slides were immersed in Harris’ haematoxylin (Sigma Aldrich) to stain nuclei for 5 minutes and then rinsed under running tap water for 5 minutes. They were subsequently dipped in a weakly acidic alcohol solution (1% HCL in 70% ethanol) for approximately 5 seconds to differentiate, and then rinsed for a further 5 minutes. Sections were counterstained with eosin (Sigma) for 2 minutes and then rinsed in tap water before dehydrating in ascending concentrations of IMS (90%, 95%, 100%) for 2 minutes at a time. Alcohol was cleared through 3 immersions of 5 minutes in xylene and then mounted with DPX (Distyrene, plasticizer and xylene - Sigma) under a coverslip. Slides were left to dry overnight under a fume hood before imaging on an Axiovision light microscope. Mean cellular cross sectional area was determined using ImageJ software, measuring approximately 100 cells per heart to obtain a mean value for each mouse. All measurements were conducted after blinding to mouse genotype and treatment.

2.5.2 Masson’s trichrome stain for fibrosis

Following rehydration slides were re-fixed in Bouin’s solution for 2 hours to improve stain quality. They were then rinsed for 10 minutes before staining with haematoxylin for 5 minutes. After rinsing for 5 minutes they were differentiated in acid-alcohol, rinsed for a further 5 minutes and subsequently placed in ‘Red’ solution (GCC diagnostics) containing 0.9% biebrich scarlet, 0.1% ponceau fuchsin in 1% acetic acid for 5 minutes. After washing in distilled water, slides were differentiated again, this time in 5% phosphomolybdic acid (GCC diagnostics) for 15 minutes, to remove the Red solution from fibrous tissue. After rinsing slides were stained with aniline blue (GCC diagnostics) for 10 minutes to stain collagen fibres, rinsed once more and differentiated in 1% acetic acid for 1 minute. Finally slides were dehydrated, cleared and mounted as per H&E staining. Fibrosis was quantified using Leica Qwin software (Leica corporation) following blinding to mouse genotype and treatment.
2.6 Intracellular calcium handling analysis

All calcium handling analysis was performed using the ratiometric calcium indicator indo-1 (Gryniewicz et al., 1985). Ratiometric indicators are a popular choice in calcium imaging as they reduce the effects of uneven dye loading, leakage, and photobleaching, as well as being unaffected by changes in cell volume (Paredes et al., 2008). In all experiments mice were aged between 10 and 12 weeks.

2.6.1 Isolation of adult ventricular myocytes

The basic solution used throughout this process was a nominally Ca\(^{2+}\)-free Hanks’/HEPES buffer solution containing in mM: NaCl, 130; KCl, 5.4; MgCl\(_2\), 1.4; NaH\(_2\)PO\(_4\), 0.4; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5; creatine, 10; taurine, 20; glucose, 10. pH was adjusted to 7.4 at 37°C with NaOH. This solution will be referred to as isolation solution from this point.

Hearts were swiftly excised following sacrifice and cannulated via the aorta with a blunted 21 gauge needle, whereupon they were immediately anticoagulated and flushed clear of blood through retrograde perfusion with isolation solution containing 750μM CaCl\(_2\) and 125units/ml of heparin. The hearts were then transferred to Langendorff apparatus and secured tightly. They were perfused at 37°C, at a rate of 2ml/min, firstly for 5 minutes with isolation solution containing 0.1mM ethylene glycol tetraacetic acid (EGTA) to remove any Ca\(^{2+}\) present. This was followed by enzymatic digestion through perfusion with isolation solution supplemented with 150units/ml of collagenase, type 2 (Worthington) and 0.36units/ml of protease, type XIV (Sigma). Ventricles were then removed from the heart into fresh isolation solution and cut into small pieces, whereupon gentle agitation freed the cardiomyocytes into the solution which was transferred to a fresh tube. The remaining tissue was subjected to a second digestion (as this would occasionally produce cells of greater quality) through the addition of the collagenase/protease solution and shaking at 37°C for 5 minutes, after which tubes were spun down (500rpm for 1 minute). The supernatant was discarded and tissue resuspended in fresh isolation solution, agitated and the cardiomyocytes pipetted off to a fresh tube. All cardiomyocytes were then washed.
twice by centrifugation (1 minute at 500rpm), removal of the supernatant and resuspension in isolation solution.

### 2.6.2 Loading of myocytes with indo-1/AM

Suspended cardiomyocytes were incubated for 15 minutes in the dark with a 5μM cell permeant preparation of indo-1/acetoxyethyl ester (indo-1/AM, Invitrogen) dissolved in dimethylsulfoxide (DMSO). The dye was then deesterified to prevent further uptake into or leakage from cells via incubation in the dark for 30 minutes with 4ml of a solution containing in mM: NaCl, 135; HEPES, 10; glucose, 11; MgCl₂, 1.2; CaCl₂, 1; KCl, 4; probenecid, 2 (pH adjusted to 7.34 at 37°C with NaOH), referred to hereafter as perfusion solution.

### 2.6.3 Systolic transient measurements

0.5ml of loaded cell suspension was placed in a recording chamber mounted on a stage beneath an epifluorescent microscope (inverted Olympus IX70) connected to a camera (Olympus) and visualized using a x40 oil immersion objective lens with a numerical aperture of 1.30. Cells were left for 5 minutes to adhere, after which the chamber was superfused steadily at one end with perfusion solution warmed to 37°C and field stimulated at a frequency of 1Hz via two silver wire electrodes (IonOptix MyoPacer), whilst at the opposite end of the chamber solution was removed at the same rate via a peristaltic suction pump. A single, rhythmically contracting rod-shaped cardiomyocyte with well-defined T-tubular structure was then selected, from which fluorescence was measured when excited at 340nm (filter bandwidth ± 5 nm) with an arc. lamp (Cairn). Emissions of both free (at 485nm) and bound (at 405nm) indo-1 were measured using filters of bandwidths of 25 and 30 nm, respectively, for a period of 2-3 minutes using two photomultiplier tubes (Cairn) attached to a digital converter (IonOptix DSI-200). The background emissions were then recorded from an area of the chamber containing no cells.
2.6.4 Measurement of caffeine-evoked transients

Caffeine causes calcium to be released from the SR by binding to RyRs and increasing their open probability (Varro et al., 1993). This bypasses calcium uptake via SERCA meaning that cytosolic calcium clearance will be achieved almost exclusively via NCX and PMCA at the sarcolemma.

Prior to caffeine experiments, coverslips were coated overnight in a solution containing 10mg/ml of laminin (Sigma). This was to ensure that myocytes adhered to the chamber upon the addition of caffeine. Cells were loaded in the same manner as per previous experiments. A single coverslip was placed in the chamber and 0.5 ml of suspended myocytes added, after which they were left to adhere for 5 minutes. Systolic transients were recorded for a period of one minute at a frequency of 1Hz as before, following which the myopacer was stopped and superfusion and suction ceased. 0.5ml perfusion solution containing 20mM caffeine (Sigma) was then rapidly applied to the chamber via a pipette, hence bringing the total chamber volume to 1ml and caffeine concentration to 10mM. This produced a rapid rise in indo-1 ratio, which was allowed to decay to its full extent. Background measurements were recorded for each cell.

2.6.5 Measurement of caffeine-evoked transients under 0Na\(^+\)/0Ca\(^{2+}\) conditions

To assess the PMCA contribution to calcium clearance caffeine-evoked transients were recorded following the removal of extracellular sodium and calcium ions. This prevents calcium from leaving the cell via the NCX through eliminating its electrochemical gradient. Under these conditions the only routes for cytosolic clearance are the slow systems, PMCA and mitochondria.

Experiments proceeded as per the previous section. Following the recording of steady state transients for one minute stimulation was stopped and the perfusate switched to a modified solution containing no calcium with additional EGTA, and in which all sodium salts were replaced with lithium (0Na\(^+\)/0Ca\(^{2+}\) solution containing in mM: LiCl, 131; HEPES, 10; glucose, 10; MgCl\(_2\), 1; KCl, 4; probenecid, 2; EGTA, 1 with pH adjusted to 7.34 at 37°C.
with LiOH). Cells were superfused for a period of two minutes, following which caffeine was applied in the same manner as per before, except this time it was dissolved in 0Na\(^+\)/0Ca\(^{2+}\) solution. Transients were allowed to decay for around two minutes before a background reading was taken.

### 2.6.6 Calcium transient analysis

Calcium transient analysis was performed using Ionwizard software (IonOptix). For systolic transients, a period spanning roughly 20 twitches was selected once steady had been reached (approximately beats 30-50). These beats were averaged and decay rates were determined by the single exponential time constant (\(\tau\)) and time to return to 50% baseline. The \(\tau\) was also determined for caffeine-evoked transients under both sets of conditions (with and without extracellular sodium). All traces were then exported to Excel, whereby the background \(F_{405}\) and \(F_{485}\) measurements were subtracted from their respective channels in the transient recordings. The ratio over the course of each transient was then determined following subtraction of background fluorescence. The diastolic indo-1 ratio was determined by averaging recordings obtained during the first 0.02s of each transient. For ease of comparison all transients are presented following normalisation of the resting indo-1 ratio to 1. Rate constants of decay were calculated from the \(\tau\) as \(s^{-1}\), and the relative contributions of the various calcium clearance systems calculated from these for each cell. Example exponentials of decay displayed on mean transients were fitted using GraphPad Prism 5 software.
2.7 Statistical analysis

All data are presented as mean ± the standard error of \( n \) independent experiments. \( n \) numbers are displayed within each column in all histograms, and in figure legends for traces. In experiments where measurements were recorded from multiple cells per animal, namely calcium handling analysis and cellular cross sectional area, mean values were determined for each mouse and then used to calculate the mean and standard error for each group. \( n \) therefore refers to the number of mice rather than the number of cells. Statistical analysis was performed using Microsoft Excel and SPSS v16.0. Data was found to be normally distributed as determined by Shapiro-Wilk test for normality. Unless otherwise stated students unpaired t-test was used to assess significance in experiments where only two groups were present. For comparisons between four groups a 2-way ANOVA was used. In all experiments \( p<0.05 \) was taken to constitute significance.
Chapter 3
3. THE ROLE OF PMCA1 AND 4 IN THE UNSTRESSED HEART

3.1 Introduction

Tightly regulated calcium homeostasis is essential to the normal function and survival of every cell type in the body. The level of ionised calcium within the cell governs a multitude of processes, from muscle contraction and neurotransmitter release, to the regulation of gene transcription and cell death (Brini et al., 2013). The requirements for maintenance of adequate calcium levels vary greatly dependent on cell type, and can range from being highly dynamic in excitable tissues such as cardiac muscle to fairly constant in non-excitatable cells. As such, a variety of calcium channels, pumps and exchangers are expressed in a specific spatial pattern designed to best suit the needs of each cell type.

3.1.1 The PMCA in excitable and non-excitatable cells

In non-excitatable cells the PMCA is the main route via which calcium is exported from the cytosol. Their high affinity for calcium, typically exhibiting a dissociation constant of 0.2-0.5μM once activated, makes them an excellent candidate for fine tuning levels in the resting cell (Brini and Carafoli, 2009). In excitable cells however, large and rapid fluctuations in cytosolic calcium concentration require a more efficient means of extrusion, and thus the need for capacity exceeds that of affinity. In the brain, this may in part be achieved by sheer abundance of expression, with all four PMCA isoforms present and up to 10 times the total amount of PMCA when compared to non-excitatable cells (Guerini, 1998). However, the presence of the NCX also provides a high capacity system for extrusion in excitable tissue, and it is through this pathway that the majority of calcium leaves the cell during diastole in the heart.
3.1.2 The role of the PMCAs in cardiac calcium homeostasis and function

As described in section 1.4.2 the PMCA is one of four mechanisms through which calcium can be removed from the cytosol during diastole. However, the gross contribution of sarcolemmal ATPases to this process is relatively minor, estimated to be in the region of 1-5% dependent on species and experimental conditions, with higher capacity mechanisms predominating due to the need for rapid calcium clearance (Bers et al., 1996, Choi and Eisner, 1999b). Through pharmacological inhibition of the pumps in rat ventricular myocytes, Eisner and colleagues have demonstrated that modulation of PMCA activity is also able to influence calcium entry (via LTCC) and exit (via NCX) from the cell such that transient amplitude and resting calcium levels were altered (Choi and Eisner, 1999a, Choi and Eisner, 1999b, Choi et al., 2000).

Due to the absence of specific inhibitors however, none of these studies were able to deduce through which PMCA isoform each effect occurred. In recent years, through the use of transgenic animal models the specific roles of each isoform have begun to be elucidated. Of the four known isoforms of the PMCA, cardiac expression is mainly restricted to equivalent levels of PMCA1 and 4, although PMCA2 may also be expressed at very low abundance (Stauffer et al., 1993).

Targeted cardiac overexpression of PMCA4 in rats and mice has been found to have little effect on cardiac calcium handling, nor structure and function under basal conditions (Hammes et al., 1998, Oceandy et al., 2007, Wu et al., 2009). Similarly, global PMCA4 knockout mice do not display delayed extrusion of electrically stimulated or caffeine evoked transients indicating this isoform’s role in global calcium clearance to be negligible (Mohamed et al., 2011). However, PMCA4 deletion was shown to increase systolic calcium transient amplitude and basal contractility, through its interaction with nNOS and downstream modulation of PKA activity in dyadic regions in this study. These studies suggest that isoform 4 may indeed function to regulate localised calcium signalling in the heart, a theory which is supported by its known residence in signalling rich caveolae (Fujimoto, 1993, Hammes et al., 1998).
The significance of PMCA4’s interaction with nNOS may not be limited to modulating cardiac contractility. The two enzymes co-exist in a macromolecular complex at the plasma membrane with α1-syntrophin (Williams et al., 2006), and through this are linked to the cardiac sodium channel Na\textsubscript{v}1.5 which is responsible for the initial upstroke leading to membrane depolarisation during the action potential (Ueda et al., 2008). Mutations in the SNTA1 gene (encoding α1-syntrophin) capable of disrupting this complex have been identified as candidate susceptibility-loci for the potentially fatal rhythm disorder long QT syndrome and may be implicated in the causation of sudden infant death syndrome (Cheng et al., 2009).

Unlike PMCA4, knowledge of the role of PMCA1 in the heart is somewhat limited. In attempting to generate constitutive PMCA1 knockout mice, our group and others have found homozygous deletion to be embryonic lethal prompting authors to speculate that this isoform has a housekeeping function, contributing to the maintenance of low levels of resting calcium (Okunade et al., 2004). This occurrence has meant that a more targeted approach is required when studying PMCA1 function, and as such we have generated cardiomyocyte-specific knockout and overexpressing lines. Analysis of these has shown that, despite being capable of interacting with the α1-syntrophin-nNOS complex, isoform 1 does not significantly regulate nNOS activity suggesting a differential role for the pump compared to PMCA4 (Williams et al., 2006, Mohamed, 2008, Shaheen, 2010). Studies in smooth muscle tend to support the hypothesis that PMCA1 is the chief calcium pumping isoform of the pump. Vascular smooth muscle specific knockout of PMCA1 has been shown to induce hypertension in mice which was associated with an increase in basal calcium (Kobayashi et al., 2012), whilst studies in bladder smooth muscle have indicated that the deletion of a single copy of the pump is able to increase intracellular calcium and force of contraction (Liu et al., 2007).

In order to further elucidate the role of the two cardiac PMCA isoforms in normal calcium homeostasis and their influence on basal cardiac phenotype, this chapter describes the generation and characterisation of a novel mouse line with cardiomyocyte-specific deletion of both PMCA1 and 4 (PMCA1:4\textsuperscript{dcko} mice). Through comparison with PMCA1 and 4
single knockout mice, this would enable the identification of the specific function of each pump in the cardiomyocyte under normal physiological conditions.

3.1.3 Hypothesis

Isoforms 1 and 4 of the PMCA perform separate functions in the regulation of cardiac calcium homeostasis. PMCA1 provides the main bulk of extrusion via the ATPase at the sarcolemma, whilst PMCA4 mediates calcium signalling events capable of modulating cardiac contractility.

3.1.4 Aims

To generate a novel mouse line with cardiomyocyte-specific deletion of PMCA1 and 4, characterise the spontaneous cardiac phenotype in these mice as they develop throughout adulthood and compare basal intracellular calcium handling in double knockout and PMCA1 single knockout mice. This should enable the identification of the specific roles of the two isoforms in the resting heart, and allow assessment of the overall contribution of each isoform to diastolic calcium extrusion.
3.2 Results

3.2.1 Generation of PMCA1:4\textsuperscript{dcko} and control mice

In order to fully characterise the role of the PMCA in the heart, mice with cardiomyocyte-specific dual deletion of isoforms 1 and 4 were generated following the breeding strategy outlined in figure 3.1. This would provide the first \textit{in vivo} model in which to study the effect that complete absence of myocardial PMCA has on cardiac structure and function, and mimic the situation seen during end stage human heart failure in which expression of both isoform 1 and 4 is dramatically reduced (Borlak and Thum, 2003).

**Figure 3.1 Breeding strategy followed to generate PMCA1:4\textsuperscript{dcko} and control mouse colonies** Step 1 mated homozygous floxed mice for PMCA1 expressing the αMHC-Cre transgene with homozygous floxed PMCA4 mice. Double heterozygous offspring were mated in step 2 to produce 18 potential genotypes, including cardiomyocyte-specific double knockout mice and relevant controls, which were bred together as indicated in step 3 to establish colonies. tg, Cre-transgene; +, wild type allele; f, floxed allele.
3.2.1.1 Generation of desired genotypes and formation of breeding colonies

Having previously been generated in our laboratory (Shaheen, 2010), PMCA1<sup>cko</sup> mice homozygous for the floxed PMCA1 allele and expressing Cre-recombinase under the control of the αMHC promoter (αMHC-Cre<sup>tg/+</sup>:PMCA1<sup>f/f</sup>), were mated with homozygous PMCA4 floxed mice (PMCA4<sup>f/f</sup> – step 1, figure 3.1). All resultant offspring were therefore heterozygous for both the PMCA1 and 4 floxed alleles (PMCA1<sup>f/+</sup>:PMCA4<sup>f/+</sup>), with an expected 50% of these carrying the αMHC-Cre transgene. Individuals from this generation expressing Cre-recombinase were then mated with littermates not carrying the transgene (step 2, figure 3.1), producing progeny of 18 potential genotypes expected to be born according to Mendelian inheritance with frequencies ranging from 1/32 to 1/8. PCR reactions were performed on DNA extracted from ear snips of the pups to determine how many copies of the floxed PMCA1 and 4 alleles were carried, as well as the presence or absence of the αMHC-Cre transgene. Genotyping results from this step in the breeding strategy along with expected frequencies for each potential genotype are presented in table 3.1.

Of these genotypes, 4 would be used in this study. The presence of the αMHC-Cre transgene and two copies of the floxed PMCA1 and PMCA4 alleles (αMHC-Cre<sup>tg/+</sup>:PMCA1<sup>f/f</sup>:PMCA4<sup>f/f</sup>) would be predicted to produce a cardiomyocyte-specific PMCA1:4 double knockout mouse (PMCA1:4<sup>cko</sup>). A further 3 genotypes would be used as controls. Mice homozygous for both the floxed PMCA1 and 4 alleles but lacking the αMHC-Cre transgene and thus not undergoing recombination would act as littermate controls (PMCA1<sup>f/f</sup>:PMCA4<sup>f/f</sup> – referred to hereafter as PMCA1:4<sup>flox</sup>). By mating PMCA1:4<sup>cko</sup> and PMCA1:4<sup>flox</sup> progeny derived during step 2 of the breeding strategy, a colony producing these genotypes in a 50/50 ratio was established.

A number of reports have noted instances of Cre-recombinase expression causing cardiomyopathy when driven by the αMHC promoter (Buerger et al., 2006, Koitabashi et al., 2009), therefore a further control genotype carrying the αMHC-Cre transgene and wild type copies of the PMCA alleles was selected (αMHC-Cre<sup>tg/+</sup>:PMCA1<sup>+/+</sup>:PMCA4<sup>+/+</sup> - referred to hereafter as αMHC-Cre<sup>tg</sup> controls). These were bred with mice carrying no
transgene and wild type PMCA (PMCA1+/+;PMCA4+/+ - referred to hereafter as αMHC-Cre\textsuperscript{neg} controls) to form a second colony. αMHC-Cre\textsuperscript{neg} mice would act as littermate controls for the αMHC-Cre\textsuperscript{tg} mice and account for any genetic drift between the two breeding colonies.

<table>
<thead>
<tr>
<th>αMHC-Cre</th>
<th>PMCA1</th>
<th>PMCA4</th>
<th>Predicted %</th>
<th>No. of pups</th>
<th>Observed %</th>
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<td>neg</td>
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<td></td>
<td></td>
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</tr>
<tr>
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<tr>
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<td>5</td>
<td>4.35</td>
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<td>6</td>
<td>5.22</td>
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</tr>
<tr>
<td></td>
<td>f/f</td>
<td>3.125</td>
<td>3</td>
<td>2.61</td>
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<tr>
<td>Totals</td>
<td></td>
<td>100</td>
<td>115</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1 PCR genotyping results obtained during step 2 of the breeding strategy The 18 potential genotypes arising from mating PMCA1:4 double heterozygous mice with double heterozygotes also carrying the αMHC-Cre transgene, along with predicted and observed frequencies for each. The four genotypes required for this study are highlighted in light grey and were born at approximately the expected Mendelian ratio.
Confirmation of the generation of each of these is shown in figure 3.2 and table 3.2. Mice of all four genotypes were viable, born in approximately the ratio expected according to Mendelian inheritance and appeared outwardly indistinct from one another.

Figure 3.2 PCR results demonstrating the generation of the four genotypes to be used in this study A) αMHC-Cre PCR – samples 2 and 3 display the presence of a band at 350bp corresponding to the Cre-recombinase gene product. B) PMCA1\textsuperscript{flox} PCR – samples 1 and 2 contain a single band at 898bp, indicating that these mice are homozygous for the PMCA1\textsuperscript{flox} allele. Samples 3 and 4 display only a band at 754bp corresponding to the wild type allele. C) PMCA4\textsuperscript{flox} PCR – samples 1 and 2 display a single band of 832bp, expected in a mouse homozygous for the PMCA4\textsuperscript{flox} allele, whilst samples 3 and 4 contain a single band of 702bp suggesting that these mice are wild type for PMCA4. tg, Cre transgenic control; wt, wt control; ht, heterozygous control; f/f, homozygous floxed control
Table 3.2 Genotypes deduced from PCR results in figure 3.2

<table>
<thead>
<tr>
<th></th>
<th>Cre</th>
<th>PMCA1</th>
<th>PMCA4</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>neg</td>
<td>f/f</td>
<td>f/f</td>
<td>PMCA1:4&lt;sup&gt;fl&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>tg</td>
<td>f/f</td>
<td>f/f</td>
<td>PMCA1:4&lt;sup&gt;dk&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>tg</td>
<td>+/+</td>
<td>+/+</td>
<td>αMHC-Cre&lt;sup&gt;tg&lt;/sup&gt; control</td>
</tr>
<tr>
<td>4</td>
<td>neg</td>
<td>+/+</td>
<td>+/+</td>
<td>αMHC-Cre&lt;sup&gt;neg&lt;/sup&gt; control</td>
</tr>
</tbody>
</table>

3.2.1.2 Verification of the targeted deletion of PMCA1 and 4 in PMCA1:4<sup>dk</sup> mice

Once breeding colonies were established, it was first necessary to verify that (i) knockout of PMCA1 and 4 from the myocardium had been successful in PMCA1:4<sup>dk</sup> mice, (ii) the deletion was tissue-specific, and (iii) deletion had not occurred in PMCA1:4<sup>fl</sup> and αMHC-Cre<sup>tg</sup> controls.

DNA and protein were extracted from isolated cardiomyocytes and a variety of other tissues (brain, kidney, liver, lung, skeletal muscle and spleen) taken from 8 week old mice. Tissue-specific deletion was then ascertained by PCR and western blot. Using the primers and PCR conditions outlined in section 2.3.1.3, the regions spanning exon 2 of the PMCA1 gene or exons 2 and 3 of the PMCA4 gene were amplified to detect whether Cre-mediated recombination had occurred. In the case of PMCA1, the wild type allele generates a product of ~3.5kb compared to a 1.3kb amplicon seen upon Cre-excision. PMCA4 products for the wild type and Cre-excised alleles were expected to be ~5.5 and ~3kb, respectively. Figure 3.3 confirms that DNA extracted from PMCA1:4<sup>dk</sup> cardiomyocytes contained a band corresponding to the smaller knockout allele for both PMCA1 and 4, whilst only the wild type allele was expressed in all other tissues examined. In PMCA1:4<sup>fl</sup> and αMHC-Cre<sup>tg</sup> control samples the wild type band was present in all tissues including samples taken from cardiomyocytes, and no band corresponding to the Cre-excised allele could be detected. These results indicate that cardiomyocyte-specific deletion of the targeted region in both genes had been achieved in PMCA1:4<sup>dk</sup> mice, whilst control mice were not affected.
Figure 3.3 PCR confirmation of cardiomyocyte-specific Cre-mediated recombination in PMCA1:4^{dcko} mice A) PMCA1 and B) PMCA4 – The Cre-excised allele was present only in the sample extracted from PMCA1:4^{dcko} cardiomyocytes. All other tissues, in addition to cardiomyocytes isolated from control mice, contain exclusively the wild type allele. Figures on the DNA ladder represent kb; C, cardiomyocytes; B, brain; L, liver; K, kidney; S, spleen; H$_2$O, water control; +ve, positive control from known PMCA1^{cko} or PMCA4^{cko} cardiac tissue; wt, wild type control.
Having established the occurrence of targeted excision events in both genes at the DNA level, it was necessary to confirm that this correlated to a reduction in protein expression in PMCA1:4\textsuperscript{dcko} cardiomyocytes. Western blot analysis of protein extracts from PMCA1:4\textsuperscript{dcko}, floxed and αMHC-Cre\textsuperscript{tg} cardiomyocytes revealed the presence of a band at ~130kDa in samples from controls which was barely visible in knockout samples when probed with PMCA1 and PMCA4 antibodies (figure 3.4A-C). Quantification of band intensity relative to that of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (used as a loading control) revealed a 98% (p<0.01) and 97% (p<0.05) reduction in PMCA1 protein expression in PMCA1:4\textsuperscript{dcko} samples compared to floxed and αMHC-Cre\textsuperscript{tg} controls respectively. PMCA4 expression was also found to be diminished, by 97% and 96% (both p<0.05) when compared to the two control groups, suggesting that deletion of both isoforms from PMCA1:4\textsuperscript{dcko} cardiomyocytes had been successful.

PMCA1 and 4 protein expression was also assessed in a variety of non-cardiac tissues across the three mouse groups to verify that levels were unchanged (figure 3.4D). Bands at ~130kDa were present when probed with both PMCA1 and PMCA4 antibodies in all tissues examined, whilst appearing consistent among PMCA1:4\textsuperscript{dcko} and control groups. Taken together these results verify the cardiomyocyte-specific double deletion of PMCA1 and 4 in PMCA1:4\textsuperscript{dcko} mice, whilst ubiquitous expression of both isoforms remained in floxed and αMHC-Cre\textsuperscript{tg} controls.
Figure 3.4 PMCA1:4\textsuperscript{dcko} mice display cardiomyocyte-specific deletion of PMCA1 and PMCA4

A) Western blot analysis of PMCA1 and 4 protein expression in isolated cardiomyocytes. The Na\textsuperscript{+}/K\textsuperscript{+} ATPase was used as a loading control. B) PMCA1:4\textsuperscript{dcko} mice display a 98 (** p<0.01) and 97% (* p<0.05) reduction in PMCA1 expression compared to flox and tg controls respectively and C) a 97 (vs flox) and 96% (vs tg) reduction in PMCA4 expression (* both p<0.05) D) Non-cardiac tissues display consistent expression of both isoforms across the three groups. Where two bands are visible (eg. PMCA1, lung) this correlates to the presence of a and b splice variants.
3.2.2 Basal phenotype in PMCA1:4\textsuperscript{dcko} mice

Whilst PMCA1:4\textsuperscript{dcko} mice appeared outwardly indistinct from controls, in depth analysis of their basal cardiac phenotype was performed to determine the precise effect of PMCA ablation in cardiomyocytes. This encompassed \textit{in vivo} assessment of cardiac electrical activity, structure and function, \textit{ex vivo} examination of intracellular calcium dynamics and \textit{in vitro} molecular analysis in young adult mice (12 weeks old) and as they aged (24 and 32 weeks).

3.2.2.1 Cardiac structure in PMCA1:4\textsuperscript{dcko} mice

Changes in cardiac structure can be a key component of adverse remodelling. Increases in wall thickness underlie the development of cardiac hypertrophy whilst altered chamber size can be indicative of the left ventricular dilatation which accompanies many forms of cardiomyopathy. Given that isoform 4 of the PMCA in particular has been shown to interact physically with modulators of hypertrophy such as calcineurin and RASSF1A (Buch et al., 2005, Armesilla et al., 2004), it was important to assess whether PMCA deletion affected normal cardiac growth as PMCA1:4\textsuperscript{dcko} mice aged.

Table 3.3 provides a summary of gross normalised heart weight and \textit{in vivo} cardiac structure as measured by ultrasound in double knockout and floxed mice as they aged from 12 to 32 weeks. PMCA ablation had little effect on heart size or structure at any time point. Body weight increased similarly from 12 to 24 weeks in both groups, by which time it had plateaued with no further rise at 32 weeks. Total heart weight normalised to either this or tibia length revealed no significant differences at any age, suggesting PMCA1:4\textsuperscript{dcko} hearts were not hypertrophic.

The similarity in heart weight between PMCA1:4\textsuperscript{dcko} and floxed control mice was largely confirmed by echocardiographic imaging of the left ventricle, with no changes in either interventricular septal or posterior wall thickness at 12 weeks in knockout hearts compared to controls. Left ventricular posterior wall thickness in diastole was statistically increased in PMCA1:4\textsuperscript{dcko} hearts at 24 weeks (0.94 ± 0.02 vs 0.78 ± 0.03, p<0.01) which in turn led
to a greater relative wall thickness, however these differences were no longer apparent at 32 weeks. Left ventricular internal diameter rose as mice aged and did not differ between the two groups.

### Table 3.3 Cardiac structure in PMCA1:4^dcko^ mice aged 12, 24 and 32 weeks

<table>
<thead>
<tr>
<th></th>
<th>12 weeks</th>
<th>24 weeks</th>
<th>32 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>flox</td>
<td>dcko</td>
<td>p value</td>
</tr>
<tr>
<td><strong>BW (g)</strong></td>
<td>32.68 ± 1.06</td>
<td>33.55 ± 0.54</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Normalised heart weight</strong></td>
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</tr>
<tr>
<td>HW/TL (mg/mm)</td>
<td>7.00 ± 0.41</td>
<td>7.38 ± 0.36</td>
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</tr>
<tr>
<td>HW/BW (mg/g)</td>
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<td><strong>Chamber size</strong></td>
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<td>dLVD (mm)</td>
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<tr>
<td>sLVD (mm)</td>
<td>2.53 ± 0.17</td>
<td>2.47 ± 0.12</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Wall thickness</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dLVPW (mm)</td>
<td>0.69 ± 0.04</td>
<td>0.75 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>sLVPW (mm)</td>
<td>0.98 ± 0.04</td>
<td>1.08 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>dIVS (mm)</td>
<td>0.88 ± 0.04</td>
<td>0.97 ± 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>sIVS (mm)</td>
<td>1.30 ± 0.04</td>
<td>1.39 ± 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>RWT</td>
<td>0.45 ± 0.03</td>
<td>0.48 ± 0.03</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Table 3.3 Cardiac structure in PMCA1:4^dcko^ mice aged 12, 24 and 32 weeks**

BW, body weight; HW/TL, heart weight/tibia length; HW/BW, heart weight/body weight; d, diastolic; s, systolic; LVD, left ventricular internal diameter; LVPW, left ventricular posterior wall thickness; IVS, interventricular septal thickness; RWT, relative wall thickness.
3.2.2.2 Cardiac function in PMCA1:4\textsuperscript{dcko} mice

Having observed few structural abnormalities at any time point in knockout mice, inotropic and lusitropic performance was assessed \textit{in vivo} through echocardiography and haemodynamic analysis following left ventricular catheterisation to ascertain whether cardiac function was also unaffected.

The information provided in table 3.4 demonstrates that as PMCA1:4\textsuperscript{dcko} mice developed with age, systolic and diastolic function closely matched controls. Left ventricular fractional shortening and ejection fraction remained fairly constant from 12 to 32 weeks in each group, whilst both load-dependent and -independent parameters of contractility also showed no significant differences. Having deleted two calcium extrusion systems from cardiomyocytes, it might be expected that any alterations in cardiac performance would be witnessed during diastole rather than systole; however isovolumic relaxation was also unaffected following PMCA ablation.

![Table 3.4 Cardiac function in PMCA1:4\textsuperscript{dcko} mice aged 12, 24 and 32 weeks](image)

Table 3.4 Cardiac function in PMCA1:4\textsuperscript{dcko} mice aged 12, 24 and 32 weeks  FS, fractional shortening; EF, ejection fraction; dP/dt max/min, maximum/minimum rate of pressure change in left ventricle; ESPVR, end systolic pressure volume relationship; Tau, isovolumic relaxation time constant.
Basal cardiac phenotype in knockout mice was further characterised through examination of electrical activity by unconscious 3-lead ECG under avertin anaesthesia. Table 3.5 details that heart rate did not differ between PMCA1:4\textsuperscript{dcko} and PMCA1:4\textsuperscript{flox} mice at any examined time point from 12 to 32 weeks, nor were any changes observed in QRS or QT interval. A small but significant decrease in PR interval was detected following PMCA ablation at 12 weeks (39.42 ± 1.04 vs 42.75 ± 1.15, p<0.05), however this was no longer apparent at 24 or 32 weeks.

<table>
<thead>
<tr>
<th></th>
<th>12 weeks</th>
<th>24 weeks</th>
<th>32 weeks</th>
<th>12 weeks</th>
<th>24 weeks</th>
<th>32 weeks</th>
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<tr>
<td></td>
<td>flox (n=8)</td>
<td>dcko (n=8)</td>
<td>p value</td>
<td>flox (n=8)</td>
<td>dcko (n=7)</td>
<td>p value</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>582.64 ± 19.15</td>
<td>584.51 ± 16.01</td>
<td>NS</td>
<td>568.71 ± 13.19</td>
<td>578.35 ± 18.15</td>
<td>NS</td>
</tr>
<tr>
<td>RR (msec)</td>
<td>103.96 ± 3.67</td>
<td>103.25 ± 3.08</td>
<td>NS</td>
<td>105.89 ± 2.40</td>
<td>103.97 ± 1.89</td>
<td>NS</td>
</tr>
<tr>
<td>PR (msec)</td>
<td>42.75 ± 1.15</td>
<td>39.42 ± 1.04</td>
<td>p&lt;0.05</td>
<td>41.27 ± 1.67</td>
<td>37.42 ± 1.50</td>
<td>NS</td>
</tr>
<tr>
<td>QRS (msec)</td>
<td>11.58 ± 0.50</td>
<td>11.06 ± 0.69</td>
<td>NS</td>
<td>12.45 ± 0.30</td>
<td>13.47 ± 0.89</td>
<td>NS</td>
</tr>
<tr>
<td>QT (msec)</td>
<td>20.64 ± 0.78</td>
<td>19.46 ± 0.71</td>
<td>NS</td>
<td>21.68 ± 1.20</td>
<td>21.07 ± 1.21</td>
<td>NS</td>
</tr>
<tr>
<td>QT\textsubscript{c} (msec)</td>
<td>64.13 ± 2.27</td>
<td>60.58 ± 1.88</td>
<td>NS</td>
<td>66.68 ± 3.67</td>
<td>65.41 ± 3.77</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 3.5 ECG parameters in PMCA1:4\textsuperscript{dcko} mice aged 12, 24 and 32 weeks HR, heart rate; QT\textsubscript{c}, QT interval corrected for heart rate.
3.2.2.4 Intracellular calcium handling in PMCA1:4\textsuperscript{dcko} mice

In cardiomyocytes, the contribution of the PMCA to bulk calcium clearance is thought to be relatively minor compared to reuptake via SERCA into the sarcoplasmic reticulum and exchange for sodium at the plasma membrane via NCX (Bers, 2002), and the finding that diastolic function was unaffected in PMCA1:4\textsuperscript{dcko} mice \textit{in vivo} suggested this to indeed be the case. However, it was possible that altered expression or function of other calcium handling proteins could compensate for PMCA ablation and therefore further investigation of calcium handling at a cellular and molecular level was required.

3.2.2.4.1 Expression of diastolic calcium clearance systems in PMCA1:4\textsuperscript{dcko} mice

SERCA2a and NCX1 protein expression was examined by western blot in heart homogenates taken from 12 week old PMCA1:4\textsuperscript{dcko} and PMCA1:4\textsuperscript{flox} mice. Figure 3.5 illustrates that expression of both proteins remained unchanged in knockout hearts, which when taken with the \textit{in vivo} data suggests that no major compensatory changes were required to offset the loss of PMCA in order to maintain normal diastolic function.

![Figure 3.5 Cardiac NCX and SERCA expression in 3 month old PMCA1:4\textsuperscript{dcko} mice](image)

A) Representative western blots and B) quantification thereof NCX1 and SERCA2a protein expression normalised to the loading control α-Tubulin. Knockout mice exhibited similar expression to controls.
3.2.2.4.2 Systolic calcium transients in PMCA1:4\textsuperscript{dcko} mice

To investigate the precise effect of PMCA 1 and 4 deletion on intracellular calcium handling, isolated ventricular myocytes from 12 week old mice were loaded with the fluorescent calcium indicator indo-1 and stimulated electrically at a frequency of 1Hz. Resting indo-1 ratios did not differ amongst knockout and control mice (dcko 0.82 ± 0.05 vs flox 0.82 ± 0.05, p=0.97). The mean traces presented in figure 3.6 indicate the amplitude of the systolic transient to be higher in PMCA1:4\textsuperscript{dcko} myocytes compared to floxed controls, and analysis revealed a significant increase of around 17% (0.155 ± 0.011 vs 0.133 ± 0.005, p<0.05).

The rate of decay however was found to be similar amongst the two groups, clearly illustrated upon plotting the mean traces as percentage decay from the peak to account for the greater amplitude in knockout cells (figure 3.6C). Quantification of the rate of calcium decay confirmed that PMCA ablation did not delay extrusion of the systolic transient significantly (figure 3.6D, half-time of decay dcko 155 ± 7 vs flox 147 ± 8 ms, p=0.48).
Figure 3.6 Systolic transients in 12 week old PMCA1:4<sup>dcko</sup> mice  
A) Mean systolic transients in isolated ventricular myocytes. B) Amplitude of systolic transient was significantly increased in PMCA1:4<sup>dcko</sup> myocytes (0.155 ± 0.011 vs 0.133 ± 0.005, * p<0.05)  
C) Systolic transients expressed as percentage decay from peak. D) Half-life of systolic transients (the time taken to decay to 50% of the peak). n = 96 and 77 cells taken from 13 (flox) and 9 (dcko) mice respectively.
3.2.2.4.3 Calcium entry, release and reuptake in PMCA1:4^{dcko} mice

A higher peak in systolic calcium could be caused by a variety of factors, such as increased calcium entry through the L-type calcium channel, augmented release from the SR via the RyR or a rise in SR load. Amplitude of the systolic transient has also been seen to rise in PMCA4 knockout mice, attributed to greater PKA activity causing an increase in L-type current and phosphorylation of the RyR at serine-2808 (Mohamed et al., 2011). To give an indication as to the mechanism behind the increase in double knockout mice, expression and phosphorylation status of key proteins governing calcium entry, release and reuptake were examined by western blot in samples taken from 12 week old PMCA1:4^{dcko} and floxed control cardiomyocytes.

L-type calcium channel expression was unchanged following PMCA ablation (figure 3.7A), and RyR$_{Ser2808}$ phosphorylation when normalised to total RyR abundance was not significantly altered (flox 1.00 ± 0.09 vs dcko 1.58 ± 0.27, p=0.09 – figure 3.7B). A further substrate for PKA in the heart is the accessory protein phospholamban, whose inhibitory actions on SERCA are released upon its phosphorylation causing enhanced uptake into the SR. Serine-16 phosphorylation status did not differ significantly in PMCA1:4^{dcko} mice compared to controls (pPLN$_{Ser16}$/PLN ratio 1.88 ± 0.42 vs 1.00 ± 0.30, p=0.14 – figure 3.7C). These results do not therefore provide sufficient evidence to determine whether PKA activity is affected in PMCA1:4^{dcko} mice in a similar manner to PMCA4 knockout mice, nor is it possible to draw a conclusion as to the mechanism behind the increased amplitude of systolic calcium seen in PMCA1:4^{dcko} mice.
Figure 3.7 Routes for calcium entry, release and reuptake in 12 week old PMCA1:4^{dcko} mice Representative western blots performed on protein extracted from isolated cardiomyocytes demonstrating A) L-type calcium channel expression normalised to the loading control GAPDH was unchanged in PMCA1:4^{dcko} mice, B) PMCA ablation did not significantly increase RyR phosphorylation at serine-2808 or C) phospholamban phosphorylation at serine-16.
3.2.2.4 Systolic calcium transients in αMHC-Cre<sup>tg</sup> controls

To verify that the increased amplitude witnessed in PMCA1:4<sup>dcko</sup> mice was a consequence of PMCA deletion, intracellular calcium handling was also examined in myocytes isolated from 3 month old αMHC-Cre<sup>tg</sup> and αMHC-Cre<sup>neg</sup> controls. As figure 3.8 shows, mean systolic transients were similar among the two groups with no difference in either amplitude or rate of decay, indicating that calcium handling was not affected by the presence of Cre-recombinase.

**Figure 3.8 Systolic transients in 12 week old αMHC-Cre<sup>tg</sup> controls** A) Mean systolic transients in isolated αMHC-Cre<sup>tg</sup> and αMHC-Cre<sup>neg</sup> myocytes. There was no significant difference in either (B) amplitude or (C) rate of calcium clearance between αMHC-Cre<sup>tg</sup> mice and littermate controls. *n* = 27 and 17 cells taken from 3 αMHC-Cre<sup>neg</sup> and αMHC-Cre<sup>tg</sup> mice respectively.
3.2.3 Intracellular calcium handling in PMCA1<sup>cko</sup> mice

As stated above, PMCA4 deletion alone has been shown to lead to increased cytosolic calcium following electrical stimulation (Mohamed et al., 2011). To investigate whether PMCA1 deletion had any influence on the altered amplitude of systolic transient witnessed in PMCA1:4<sup>dcko</sup> mice, calcium handling was also examined in PMCA1<sup>cko</sup> myocytes.

3.2.3.1 Systolic calcium transients in PMCA1<sup>cko</sup> mice

Ventricular myocytes were isolated from 12 week old PMCA1<sup>cko</sup> and PMCA1<sup>f/f</sup> control mice and loaded with indo-1. As was the case in PMCA1:4<sup>dcko</sup> myocytes, diastolic indo-1 ratio was similar in PMCA1<sup>cko</sup> and floxed control cells (cko 0.93 ± 0.03 vs flox 0.88 ± 0.05, p=0.35). Field stimulation at 1Hz elicited no significant differences in either amplitude or decay of the systolic transient in knockout myocytes (figure 3.9). This indicates that increases in cytosolic calcium witnessed upon stimulation in double knockout cells were a consequence of PMCA4 deletion only.
Figure 3.9 Systolic transients in 12 week old PMCA1cko mice A) Mean systolic transients in isolated PMCA1cko and PMCA1f/f myocytes. PMCA1 ablation did not affect either (B) amplitude or (C) half-time for decay of the transient. n = 29 and 34 cells taken from 5 (PMCA1f/f) and 4 (PMCA1cko) mice.
3.2.4 Contributions of PMCA1 and 4 to cytosolic calcium clearance in cardiomyocytes

As previously mentioned, numerous studies assessing PMCA involvement in global calcium clearance during diastole have estimated varying contributions ranging from less than 1% to upwards of 5% (Bassani et al., 1994, Bassani et al., 1995b, Choi and Eisner, 1999b). The variance appears to be largely dependent upon species, however, there is surprisingly little information available on the PMCA contribution in mice and, more importantly, no studies have separated the PMCA1 and PMCA4 components. Our group has found the rate of calcium decay to be unaltered in PMCA4 knockout mice even after inhibition of the faster clearance systems SERCA and NCX (Mohamed et al., 2011), suggesting that the bulk of the PMCA contribution arises from PMCA1. To test this hypothesis, calcium decay was examined following caffeine application under external conditions of 0Na\(^+\) and 0Ca\(^{2+}\) (to prevent SR uptake and Na\(^+\)/Ca\(^{2+}\) exchange, respectively) in PMCA1\(^{cko}\) and PMCA1:4\(^{cko}\) myocytes.

3.2.4.1 Relative contributions to calcium clearance in PMCA1\(^{cko}\) mice

Isolated PMCA1\(^{cko}\) and PMCA1\(^{flt}\) myocytes from 12 week old mice were loaded with indo-1 and stimulated at 1Hz for 60 seconds to assess the rate of decay of the systolic transient. Stimulation was then stopped and the perfusate changed from the standard perfusion solution to a modified solution containing no Na\(^+\) or Ca\(^{2+}\) ions for 120s to inhibit calcium extrusion via NCX upon subsequent rapid application of 10mM caffeine. This opens the ryanodine receptors, emptying SR calcium stores and negating reuptake via SERCA. Under these conditions the only routes remaining for calcium to leave the cytosol are at the sarcolemma via PMCA or through mitochondrial uptake. Any differences in decay between knockout and control cells can therefore be attributed to the deletion of PMCA1.
Figure 3.10 Calcium extrusion upon caffeine application under 0Na+/0Ca^{2+} conditions in 12 week old PMCA1^{cko} mice

A) Mean normalised caffeine evoked transients following removal of external Na^+ and Ca^{2+} in isolated PMCA1^{cko} and PMCA1^{f/f} myocytes. Black lines are single exponentials fitted to the mean traces. B) Time constant of calcium decay was significantly prolonged in PMCA1^{cko} myocytes (** p<0.01) n = 9 and 12 cells taken from 3 (flox) and 4 (cko) mice respectively.

The mean caffeine evoked transients recorded under 0Na+/0Ca^{2+} conditions presented in figure 3.10 clearly show a shallower slope of decay in PMCA1^{cko} cells, with a significantly increased decay time (\( \tau = 26.74 \pm 2.49 \) vs 12.22 \( \pm 1.83 \) s, p<0.01). The mean rate constants of decay for both systolic and caffeine evoked transients upon removal of external Na^+ and Ca^{2+} in knockout and control mice are shown in table 3.6. The residual decay in PMCA1^{cko} mice after SERCA and NCX inhibition can be assumed to equate to clearance via PMCA4 and the mitochondrial uniporter (z). Similarly, decay of the caffeine-evoked transient under 0Na+/0Ca^{2+} conditions in PMCA1^{f/f} mice can be attributed to these two transporters working in combination with PMCA1 (y). By performing the calculations in equations 3.1 and 3.2, the maximal percentage contributions of the remaining active clearance systems towards the decay of the systolic transient can be determined for each cell studied in PMCA1^{f/f} and PMCA1^{cko} mice respectively. Mean ‘slow system’ contributions to global calcium clearance were found to equate to 1.79 \( \pm 0.26\)% and 0.83 \( \pm 0.12\)% in control and knockout cells respectively (table 3.6, p<0.01). By subtracting the residual slow contribution in PMCA1^{cko} cells from that in control cells, this estimates the PMCA1 contribution to global calcium clearance to be 0.97 \( \pm 0.38\)% (equation 3.3).
Table 3.6 Contributions to calcium clearance in 12 week old PMCA1^{cko} and control mice

Rate constants of decay of systolic and caffeine evoked transients under 0Na^{+}/0Ca^{2+} conditions in PMCA1^{ff} and PMCA1^{cko} mice, along with the active extrusion systems under each experimental condition and their calculated contributions to clearance of the systolic transient. ** p<0.01 vs PMCA1^{ff}. n = 9 and 12 cells taken from 3 (flox) and 4 (cko) mice respectively mito, mitochondrial uptake.

<table>
<thead>
<tr>
<th></th>
<th>Systolic</th>
<th>Caffeine / 0Na^{+} / 0Ca^{2+}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PMCA1^{ff}</td>
<td>PMCA1^{cko}</td>
</tr>
<tr>
<td>Rate constant of decay (s^{-1})</td>
<td>5.57 ± 0.36</td>
<td>5.59 ± 0.14</td>
</tr>
<tr>
<td>Active extrusion systems</td>
<td>SERCA NCX PMCA1 mito</td>
<td>SERCA NCX PMCA4 mito</td>
</tr>
<tr>
<td>% contribution</td>
<td>1.79 ± 0.26 %</td>
<td>0.83 ± 0.12 % **</td>
</tr>
</tbody>
</table>

**Equation 3.1 Combined PMCA1, PMCA4 and mitochondrial contribution to calcium clearance**

\[ \text{Slow contribution in PMCA1}^{ff} \text{ mice} = \frac{X}{W} \times 100\% \]

**Equation 3.2 Combined PMCA4 and mitochondrial contribution to calcium clearance**

\[ \text{Slow contribution in PMCA1}^{cko} \text{ mice} = \frac{Z}{X} \times 100\% \]

**Equation 3.3 PMCA1 contribution to global calcium clearance**

\[
\text{PMCA1 contribution} = \text{PMCA1}^{ff}_{\text{slow}} - \text{PMCA1}^{cko}_{\text{slow}} = (1.79 \pm 0.26\%) - (0.83 \pm 0.12\%) = 0.97 \pm 0.38\%
\]
3.2.4.2 Relative contributions to calcium clearance in PMCA1:4\textsuperscript{dcko} mice

To ascertain whether PMCA4 deletion further slowed the rate of calcium decay in the absence of SERCA and NCX activity, the experiments above were repeated in myocytes isolated from 12 week old PMCA1:4\textsuperscript{dcko} and PMCA1:4\textsuperscript{flox} mice. This would provide an evaluation of the combined PMCA1 and 4 contributions to calcium clearance in cardiomyocytes.

Mean caffeine-evoked transients recorded under 0Na\textsuperscript{+}/0Ca\textsuperscript{2+} conditions are presented in figure 3.11. The time constant of decay was again significantly prolonged in double knockout myocytes compared to controls (32.36 ± 4.30 vs 12.73 ± 1.78 s, p<0.01). Mean rate constants of decay are described in table 3.7. The same calculations outlined in section 3.2.4.1 were performed in all cells to determine the mean contribution of the active slow calcium clearance systems in PMCA1:4\textsuperscript{flox} and PMCA1:4\textsuperscript{dcko} mice, and these were found to equate to 1.75 ± 0.35% and 0.69 ± 0.08% respectively (table 3.7, p<0.01). The combined PMCA1 and 4 contribution to global calcium clearance was then calculated using equation 3.4 to be 1.06 ± 0.42%. This suggests that the role of PMCA4 in global calcium extrusion is largely negligible, and that isoform 1 accounts for the vast majority of the PMCA contribution.

![Figure 3.11](image_url)

**Figure 3.11 Calcium extrusion upon caffeine application under 0Na\textsuperscript{+}/0Ca\textsuperscript{2+} conditions in 12 week old PMCA1:4\textsuperscript{dcko} mice**  
A) Mean normalised caffeine-evoked transients transients fitted with exponentials following removal of external Na\textsuperscript{+} and Ca\textsuperscript{2+} in isolated PMCA1:4\textsuperscript{dcko} and PMCA1:4\textsuperscript{flox} myocytes. B) Time constant of calcium decay was higher in PMCA1:4\textsuperscript{dcko} myocytes (**) p<0.01). n = 8 and 9 cells taken from 5 (flox) and 4 (dcko) mice respectively.
Table 3.7 Contributions to calcium clearance in 12 week old PMCA1:4^{dcko} and control mice

Rate constants of decay of systolic and caffeine evoked transients under 0Na+/0Ca^{2+} conditions in PMCA1:4^{flox} and PMCA1^{cko} mice, along with the active extrusion systems under each experimental condition and their calculated contributions to clearance of the systolic transient. * p<0.05, ** p<0.01 vs flox. n = 8 and 9 cells taken from 5 (flox) and 4 (dcko) mice respectively. mito, mitochondrial uptake.

<table>
<thead>
<tr>
<th>Active extrusion systems</th>
<th>Systolic</th>
<th>Caffeine / 0Na^+ / 0Ca^{2+}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PMCA1:4^{flox}</td>
<td>PMCA1:4^{flox}</td>
</tr>
<tr>
<td></td>
<td>PMCA1:4^{dcko}</td>
<td>y</td>
</tr>
<tr>
<td>Rate constant of decay (s^{-1})</td>
<td>5.63 ± 0.36</td>
<td>5.65 ± 0.54</td>
</tr>
</tbody>
</table>
| Active extrusion systems | SERCA
NCX
PMCA1
mito | SERCA
NCX | mito |
| % contribution | 1.75 ± 0.35 % | 0.69 ± 0.08 % * |

Combined PMCA contribution = PMCA1:4^{flox}_{slow} − PMCA1:4^{dcko}_{slow}

= (1.75 ± 0.35%) − (0.69 ± 0.08%)

= 1.06 ± 0.42 %

Equation 3.4 Total PMCA contribution to global calcium clearance
3.3 Discussion

The tissue and cell-specific distribution of the various PMCA isoforms and splice variants suggests specialized roles adapted to suit the requirements of each particular cell type. In the heart expression is mainly restricted to isoforms 1 and 4, with each thought to contribute around 50% of total cardiac PMCA abundance (Stauffer et al., 1993, Stauffer et al., 1995). This chapter describes the generation of a novel mouse line in which both isoforms are deleted from the myocardium, enabling the first in vivo analysis of the cardiac phenotype following dual ablation of PMCA1 and 4. In addition, by comparing calcium handling in isolated double knockout myocytes to PMCA1 single knockout cells this study is the first to distinguish the specific roles of the two isoforms in the regulation and clearance of cytosolic calcium in cardiomyocytes.

3.3.1 Generation of mice with cardiomyocyte-specific deletion of PMCA1 and 4

Through the use of Cre/loxP technology driven by the αMHC promoter, a viable mouse with cardiomyocyte-specific deletion of both PMCA1 and 4 was successfully bred. PMCA1:4\(^{dcko}\) mice were born in a ratio in accordance with that predicted by Mendelian inheritance, and visually indistinguishable from floxed littermates, as well as αMHC-Cre\(^g\) and αMHC-Cre\(^n\) controls bred on the same background strain and descended from the same lineage. Double knockouts survived into adulthood and were capable of continued germline transmission. By using this system of targeted gene silencing two major obstacles were avoided, (i) the global deletion of PMCA1 is known to be embryonic lethal (Okunade et al., 2004), and (ii) constitutive knockout of PMCA4 leads to male infertility as a result of a defect in the sperm resulting in the inability to achieve the hypermotility required for fertilization (Schuh et al., 2004).

PCR analysis of DNA extracted from PMCA1:4\(^{dcko}\), flox and αMHC-Cre\(^g\) cardiomyocytes, in addition to a number of other organs indicated that excision of the targeted region of the PMCA1 and 4 alleles had occurred exclusively in PMCA1:4\(^{dcko}\) myocytes. Confirmation that this correlated with a reduction in protein was achieved through western blot. Whilst PMCA levels in non-cardiac tissues were consistent amongst knockouts and controls, PMCA1 and 4 protein expression was found to be reduced by over 95% in extracts from
PMCA1:4<sup>dcko</sup> cardiomyocytes compared to both floxed and αMHC-Cre<sup>tg</sup> cells. This extent of downregulation is within the expected range, Cre having been shown to catalyse excision events in 80-100% of cells in which it is introduced with the use of a reliable promoter (Sauer, 1998).

3.3.2 Dual deletion of PMCA1 and 4 does not impact on cardiac growth or function under normal physiological conditions

Assessment of the *in vivo* cardiac phenotype in PMCA1:4<sup>dcko</sup> mice as they aged from 12 to 32 weeks revealed no major abnormalities in structure, function or electrical activity. A small increase in thickness of the left ventricular free wall was noted in diastole in 24 week old PMCA1:4<sup>dcko</sup> mice, but as no such increase was found in systole nor a correlation to increased heart weight, and as thickening was no longer apparent at 32 weeks it is likely that this is simply a statistical anomaly that one might expect when measuring multiple parameters at multiple time points. Similarly, a statistically significant shortening of the PR interval was measured in double knockout mice at 12 weeks but no longer apparent in 24 week old mice, and this is likely a consequence of the 5% chance of encountering a false positive when considering significance to begin at the 95% confidence interval.

These results suggest that neither isoform of the PMCA plays a significant role in regulating normal cardiac growth during adulthood, and since no sign of compensatory upregulation was witnessed in other calcium handling proteins, the PMCAs may be dispensable for the maintenance of normal cardiac function under physiological conditions. This is in agreement with the current literature as to the basal phenotype of mice in which PMCA4 has either been overexpressed or silenced specifically in the myocardium, where no changes are observed in echocardiographic parameters or relaxation rates with respect to controls in the absence of a pathological stimulus (Oceandy et al., 2007, Wu et al., 2009).

Global knockout of PMCA4 has recently been shown to increase basal contractility through modulation of local nitric oxide signalling and consequently phosphodiesterase
activity at the plasma membrane; however, contractility appeared unaffected in PMCA1:4^dcko mice. One possible explanation for this could be due to the added ablation of PMCA1, although basal contractility in young PMCA1^cko mice is not compromised making this unlikely (Shaheen, 2010). Alternatively, in the constitutive PMCA4 knockout there could be an additive effect of PMCA4 deletion in cardiac cell types other than cardiomyocytes, for example cardiac fibroblasts or endothelial cells, which enhances contractility. A third possibility may be a technical issue with regards to the measurement of cardiac contractility in anaesthetised mice. Although tribromoethanol is the anaesthetic of choice when studying cardiac function in small rodents due to reportedly limited cardiodepressive effects (Chu et al., 2006), in our hands we witness a substantial difference in heart rate between conscious (typically ranging from 600 to 800 bpm) and anaesthetised mice (in the range of 400 and 600 bpm), and this may mask a potential functional difference.

With regards to the role of PMCA1 in the heart, the finding that basal cardiac performance was not compromised in PMCA1^cko and PMCA1:4^dcko mice suggests that this isoform does not perform a major housekeeping function in all tissues, contrary to some authors’ speculation (Liu et al., 2007, Okunade et al., 2004). Rather, it may suggest that akin to PMCA4, PMCA1 may take on a signalling role in the heart, or alternatively have reserve capabilities to be called upon during times of stress. This second hypothesis would be supported by evidence of compensatory PMCA upregulation (no isoforms specified) in SERCA knockout mice and isolated rat myocytes transfected with NCX shRNA, as well as the finding that the PMCA is able to maintain adequate calcium efflux for normal homeostasis in heart tubes from NCX knockout embryos at low frequency stimulation (Louch et al., 2010, Hurtado et al., 2005, Reuter et al., 2003).

### 3.3.3 Specific roles for PMCA1 and 4 in intracellular calcium handling

The *in vivo* data suggested that, in accordance with the current understanding of PMCA involvement in calcium handling in the heart, the sarcolemmal ATPases have only a minor role to play in the diastolic clearance of cytosolic calcium compared to SERCA and NCX. In order to dissect the specific roles of the two cardiac PMCA isoforms, basal calcium
handling was studied in isolated PMCA1^cko and PMCA1:4^cko myocytes. Importantly, no change in SERCA or NCX expression was noted in PMCA1:4^cko mice, and as this has also been shown to be the case following PMCA1 ablation (Shaheen, 2010) any resultant phenotype in the two lines could likely be attributed to be a direct consequence of PMCA silencing.

3.3.3.1 Global calcium extrusion via the PMCA is mainly dependent upon PMCA1

The rate of decay of systolic transients following electrical stimulation at 1Hz as measured by the half-life or time constant was not significantly altered in myocytes with deletion of PMCA1, nor dual deletion of isoforms 1 and 4. This indicates their overall contributions to be slight, and presumably undetectable in the presence of SERCA and NCX activity using the technique of field stimulation employed here given the greater potential for background noise when compared to more sensitive patch-clamp techniques.

To circumvent this, calcium extrusion following the inhibition of the faster systems through depletion of extracellular sodium and caffeine application was examined in each mouse line. The time constant of exponential decay was significantly prolonged in PMCA1:4^cko myocytes under these conditions, and by calculating the rate constant of this decay and comparing it to the rates in control myocytes under 0Na^+/0Ca^{2+}/caffeine and steady state conditions an estimation as to the maximal PMCA contribution to global calcium clearance was made at around 1%.

With regards to the current literature concerning the proportion of calcium extruded beat to beat via the PMCA in cardiomyocytes, this is largely in agreement. Studies by Don Bers’ group in a variety of mammalian species have found the ‘slow’ systems (combined PMCA extrusion and mitochondrial uptake) to remove between 1 and 4% of cytosolic calcium during a contraction dependent on species, with the greatest contribution in ferret and smallest in rat (Bers et al., 1996). This study would place the PMCA contribution at the lower end of this spectrum, as might be expected in a small rodent such as the mouse. However, investigations isolating extrusion via the PMCA using the general inhibitor
carboxyeosin in rat ventricular myocytes have estimated a slightly higher contribution of between 3 and 5% (Mackiewicz and Lewartowski, 2006, Choi and Eisner, 1999b). The former of these studies was performed under similar experimental conditions to those employed here, evoking systolic transients at a frequency of 1Hz at 37°C, whilst the latter stimulated at 0.5Hz at room temperature which may partly account for the discrepancies. Furthermore, the lower estimation deduced during the current study may be a reflection of interspecies variation, for despite the similarities between the two rodent species, resting heart rate is in the order of 2 to 3 magnitudes higher in the mouse (Noujaim et al., 2004).

The data obtained from PMCA1:4^{cko} mice confirmed the results of previous studies ascribing a small role to the PMCAs in cytosolic calcium clearance. Something that earlier experiments have been unable to achieve, however, is a distinction between extrusion via PMCA1 and PMCA4, and by repeating the experiments in PMCA1^{cko} myocytes it was possible to estimate the contribution of this isoform in isolation. Interestingly, upon deletion of PMCA1 a similar slowing of the rate constant compared to controls was observed as was witnessed in the double knockout, and the PMCA1 contribution was also approximated at 1%.

This suggests PMCA1 to be largely responsible for diastolic extrusion via sarcolemmal calcium ATPases, and is consistent with the current opinion of PMCA4’s function as a signalling molecule in the heart. Studies in transgenic mice and rats have shown neither overexpression nor knockout of PMCA4 to alter the rate of systolic calcium transient decay, and moreover have detected no significant changes in decay following SERCA and NCX inhibition compared to controls (Hammes et al., 1998, Oceandy et al., 2007, Wu et al., 2009, Mohamed et al., 2011). Furthermore, PMCA4 has been shown to associate with caveolin 3 in signalling microdomains in the plasma membrane (Hammes et al., 1998).

The notion that PMCA-mediated calcium extrusion is mainly reliant upon PMCA1, whilst PMCA4 has specialised signalling roles is not limited to cardiac cells. Studies in bladder smooth muscle found the deletion of one copy of PMCA1 to raise intracellular calcium levels when stimulated with KCl, whereas PMCA4 ablated bladders were found to have
altered responses to carbachol stimulation, suggesting this isoform to regulate contraction through an acetylcholinergic signal transduction pathway (Liu et al., 2007). Similarly, in a breast cancer cell line PMCA1 was found to be the chief calcium pumping isoform whereas knockdown of PMCA4 had little effect on the rate of calcium extrusion following stimulation. Consequently, knockdown of PMCA1 caused cells to be susceptible to ionomycin-mediated cell death via necrosis, whilst PMCA4 silencing led to caspase-mediated cell death via apoptosis (Curry et al., 2012).

Whilst the loss of PMCA1’s calcium pumping capabilities was found to be of little consequence here in the resting murine heart where the bulk of cytosolic calcium clearance is achieved through reuptake into SR stores, this is certainly not the case in all tissues. This can be highlighted no better than by the recent wealth of studies reporting associations between mutations in the ATP2B1 gene and hypertension (Hirawa et al., 2013), and the findings that mice lacking the gene in vascular smooth muscle exhibit raised blood pressure associated with increased intracellular calcium (Kobayashi et al., 2012).

Hence, the isoform-specific roles (or lack thereof in the case of PMCA4) of the two ubiquitous PMCA isoforms in global calcium clearance may well be reflected in their relative expression patterns during embryonic development and in other cell types. PMCA1 is widely expressed from an early stage in mouse embryos, and continues to be far more abundant than PMCA4 throughout the gestational period (Zacharias and Kappen, 1999). This may reflect the need for robust calcium regulation given the critical importance of calcium-dependent processes during embryonic development (Webb and Miller, 2003), and may also account for the occurrence of embryonic lethality in constitutive PMCA1 knockout mice. Conversely, in certain cell types in which fluctuations in intracellular calcium concentration are relatively small it appears that PMCA4 expression predominates. For example, PMCA4 is the main isoform found in erythrocytes (Stauffer et al., 1995), where total calcium content is unusually low and resting concentrations of ionised calcium are estimated to be in the region of 20-60nM (Engelmann, 1991).

One might conclude therefore that each cell type is equipped with a calcium clearance system designed to best suit its particular needs. In cells such as cardiac muscle, which
constantly have to cope with large fluctuations in calcium concentration, the demand for high capacity clearance dictates removal via the faster systems, whereas in cell types where the main requirement of calcium homeostasis is the maintenance of a certain level of intracellular calcium the PMCA may dominate. The presence of multiple PMCA isoforms may afford an extra level of control, with the relative expression of each isoform being tailored to provide optimal calcium homeostasis in each cell type.

3.3.3.2 Regulation of systolic calcium transient amplitude by PMCA4

Over the past decade an emergent role for PMCA4 in the regulation of calcium signalling has been identified in the heart and vasculature, through protein-protein interactions with its many binding partners (Oceandy et al., 2011, Cartwright et al., 2009). The data presented in this chapter indicate that the deletion of this isoform did not affect the rate cytosolic calcium decay, even in the absence of SERCA and NCX activity. However, PMCA1:4\textsuperscript{dcko} myocytes did exhibit a greater level of peak intracellular calcium compared to controls following electrical stimulation, which was not reciprocated in mice carrying ablation of PMCA1 only. This suggests that the loss of PMCA4, but not PMCA1, contributed to the increase in amplitude of the systolic transient.

Transgenic mice with global deletion of PMCA4 have recently been shown to have a similar phenotype, supporting the notion that this isoform is responsible for the increased amplitude (Mohamed et al., 2011). The mechanism behind this was found to involve the delocalisation of the PMCA4 interacting molecule neuronal nitric oxide synthase (nNOS) from the plasma membrane, and its subsequent modulation of PKA activity in cytoplasmic regions resulting in increased phosphorylation of RyR\textsubscript{Ser2808} and LTCC activity. Western blot analysis in PMCA1:4\textsuperscript{dcko} myocytes did not find RyR\textsubscript{Ser2808} phosphorylation to be significantly increased, with a large degree of variability within groups and relatively small sample size (n=4). Performing a power calculation suggests that increasing this to n=6 should be sufficient to determine whether RyR\textsubscript{Ser2808} phosphorylation is significantly affected in PMCA1:4\textsuperscript{dcko} myocytes. LTCC expression was unaltered; however, as current density was not examined it is not possible to comment on whether channel activity was affected following PMCA ablation. Phosphorylation of PLN\textsubscript{Ser16}, a further PKA substrate
situated in a similar locale to the dyadic RyR and LTCC, was also examined and found no significant difference between PMCA1:4\textsuperscript{flox} and knockout myocytes, although sample size may again have prevented a firm conclusion being drawn on this matter, with a power calculation suggesting that an $n$ of 8 may give a better indication as to the phosphorylation status of this serine residue. During the current study it was therefore not possible to determine whether the observed increase in systolic calcium in PMCA1:4\textsuperscript{dcko} myocytes was mediated through a similar mechanism to that witnessed in PMCA4 single knockout mice.

In PMCA4 global knockout mice higher systolic calcium correlated to an increase in basal contractility in vivo (Mohamed et al., 2011), which was not witnessed in double knockouts. Some possible explanations for this such as the potential involvement of non-myocyte cardiac cell populations in a global knockout or cardiosuppression due to anaesthesia are discussed in section 3.3.2 and further investigation is warranted. Measurement of cell shortening would avoid these two issues and may provide clarification as to whether basal contractility is affected in PMCA1:4\textsuperscript{dcko} mice. One might hypothesise from the current literature that RyR phosphorylation by PKA at Ser2808 may well provide beneficial effects in terms of contractility as it has been shown that – unlike CaMKII phosphorylation sites such as Ser2814 – it is not involved in disease progression following insult (Zhang et al., 2012, Respress et al., 2012, Fischer et al., 2013).

In support of the data from PMCA4 knockout mice, acute PMCA inhibition with carboxyeosin has also been shown to increase systolic transient amplitude in cardiomyocytes (Choi and Eisner, 1999b, Mackiewicz and Lewartowski, 2006), which correlated with increased cell shortening in the latter study. PMCA4 overexpression however appears to have no influence on transient amplitude in the heart in the majority of studies (Hammes et al., 1998, Oceandy et al., 2007, Wu et al., 2009), which one might expect as the presence of the calcium pump will attract nNOS to the sarcolemma, thus not affecting signalling at the SR.
3.3.4 Study limitations

The experiments conducted in this chapter aimed to investigate the roles of PMCA1 and 4 in the resting heart. To achieve this aim a mouse line was generated with cardiomyocyte-specific deletion of both isoforms, however cardiac expression will still remain in non-myocyte cell lineages, therefore these results can only actually attest to the role of the PMCAs in cardiac muscle.

Nevertheless, this is still the first model to allow the in vivo study of the concomitant ablation of both PMCA1 and 4 in cardiomyocytes, which should essentially account for a total absence of PMCA. However, a small amount of PMCA2 mRNA has been detected in the adult human and rat heart, although this has been estimated to account for less than 2% of total PMCA expression and not been verified at the protein level (Hammes et al., 1994, Santiago-García et al., 1996, Stauffer et al., 1993, Stauffer et al., 1995). As PMCA2 expression was not examined in this study, it is possible that a small amount of PMCA remains in PMCA1:4\textsuperscript{dko} myocytes in the form of isoform 2, and therefore the potential for compensatory upregulation cannot be completely ruled out. Similarly, whilst western blotting by chemiluminescence provides a reasonable degree of sensitivity when examining protein expression and no change was witnessed in NCX or LTCC levels, it was not possible to comment on the activity of these ion transport systems using the methods employed here. Therefore, there is the possibility that compensatory mechanisms may be activated following PMCA ablation which went undetected, as has been witnessed in rat myocytes following PMCA inhibition (Choi and Eisner, 1999a, Choi and Eisner, 1999b).

In the same way that expression analysis of NCX and LTCC does not give a measure of their activity, using the phosphorylation status of residues on RyR and PLN which act as PKA substrates as a readout for PKA activity in PMCA1:4\textsuperscript{dko} mice provides an indirect and insensitive measure, and even more so as a measure of calcium release. A more accurate representation of these could be achieved by performing a PKA activity assay, and examining fractional release from the SR using patch clamp.

One of the original aims of this study was to assess whether any spontaneous phenotype developed in PMCA1:4\textsuperscript{dko} mice with age, as diseases associated with cardiac remodelling
tend to manifest themselves in later life; however for reasons described in chapter 5 surrounding the potential for Cre-toxicity, the spontaneous phenotype could not be studied beyond the age of 8 months. Given this issue the majority of experiments were performed alongside control mice expressing the αMHC-Cre transgene. Calcium handling was found to be unaffected in electrically stimulated cardiomyocytes, however due to time constraints experiments assessing the rate of decay of the caffeine evoked transient under $0\text{Na}^+/0\text{Ca}^{2+}$ conditions were not performed in αMHC-Cre$^{\text{tg}}$ mice. Therefore it is not possible to say conclusively that the presence of Cre had no effect when assessing the PMCA contribution in PMCA1:4$^{\text{dko}}$ and PMCA1$^{\text{cko}}$ mice.

### 3.3.5 Conclusions

Through the generation and basal phenotyping of a novel conditional knockout mouse with ablation of isoforms 1 and 4 of the PMCA in cardiomyocytes, this chapter provides evidence that the calcium pumps do not play a major housekeeping role in the resting heart. Rather, each isoform appears to have a specific role in terms of calcium homeostasis. PMCA4 is able to influence the amplitude of the systolic transient, most likely through indirect regulation of SR calcium cycling via nNOS-dependent PKA activity, whereas the small amount of calcium extruded from the cell during diastole through the PMCA occurs almost exclusively via PMCA1. Whilst this equates to only a small proportion of total calcium in cardiomyocytes, it may be of relevance in determining the cell specific distribution of the calcium pumps throughout the rest of the body.
Chapter 4
4. THE ROLE OF PMCA1 AND 4 DURING PATHOLOGICAL AND PHYSIOLOGICAL STRESS

4.1 Introduction

In response to stresses which increase workload, the myocardium hypertrophies in an attempt to maintain adequate cardiac output. The nature of the ensuing hypertrophy varies greatly dependent on the type of stimulus, and myocyte growth can often be accompanied by ventricular remodelling, cardiac fibrosis and even cell death. Calcium-dependent signalling processes are central to the regulation of this growth, and perturbations in these processes can eventually result in cardiac dysfunction and failure (Frey et al., 2000). Understanding the mechanisms which govern the transition from adaptive cardiac growth into decompensation is key to the development of new strategies to combat the progression into heart failure.

4.1.1 The potential role of the PMCA during cardiac hypertrophy

The results discussed in the previous chapter suggest that in cardiomyocytes isoforms 1 and 4 of the PMCA act independently from one another to perform specific roles in calcium homeostasis, with PMCA1 accounting for the small amount of calcium extruded via the ATPases at the sarcolemma and PMCA4 capable of influencing calcium dynamics, likely through the regulation of signalling pathways. However, under normal physiological conditions it appears that these roles do not contribute significantly to the maintenance of cardiac function or regulation of cardiac growth.

That is not to say that the PMCAs are necessarily redundant in the heart. The identification of functional interactions between isoform 4 and molecules such as calcineurin (Buch et al., 2005) and RASSF1A (Armesilla et al., 2004), both modulators of cardiac growth and hypertrophic signalling via the NFAT and ERK1/2 pathways respectively (Molkentin et al., 1998, Oceandy et al., 2009), prompted researchers to hypothesise that PMCA4 may be involved in the regulation of pathological hypertrophy.
Transgenic overexpression of this isoform in the myocardium has produced somewhat conflicting results, with mice displaying an increased concentric hypertrophic response to 7 days treatment with isoprenaline (Oceandy et al., 2007), but an attenuated response to pressure overload and combined angiotensin/phenylephrine infusion, likely through inhibition of calcineurin-NFAT signalling (Wu et al., 2009). Wu and colleagues also investigated the effect of PMCA4 silencing during pressure overload in this study and found a mild increase in heart weight and a small decrease in systolic function compared to control mice. Conversely, recent work in our group has found that a novel specific PMCA4 inhibitor was able to both prevent pressure overload induced growth and treat pre-existing hypertrophy (Abou-Leisa, 2013).

In support of the notion that PMCA4 overexpression enhances growth, cultured transgenic rat myocytes have been shown to have increased rates of protein synthesis following α- and β-adrenergic stimulation (Hammes et al., 1998), however the same transgenic line also displayed reduced expression of the hypertrophic marker BNP when stimulated with endothelin-1 which would support an anti-hypertrophic role (Piuhola et al., 2001). Despite the seemingly contradictory results of these studies, one conclusion that it might be safe to draw is that PMCA4 is capable of modulating pathological hypertrophy. In contrast, modulation of PMCA4 expression has been shown to not affect the hypertrophic response to exercise (Wu et al., 2009).

To date no studies have examined the role of PMCA1 in response to pathological stimuli. However, an interaction with calcineurin has been witnessed in endothelial cells and heart tissue (Holton et al., 2010, Shaheen, 2010) indicating the possibility for its regulation by this isoform. Furthermore, sarcolemmal vesicle preparations prepared from spontaneously hypertrophic and aortic banded rat hearts have demonstrated increased calcium ATPase activity (Nakanishi et al., 1989), which given the preferential role of PMCA1 in calcium extrusion when compared to PMCA4, might suggest the possibility for an enhanced role in calcium clearance in the hypertrophic heart. In contrast, the transition to failure appears to negatively regulate the PMCA in both human heart tissue and following MI in the rat (Borlak and Thum, 2003, Mackiewicz et al., 2009).
4.1.2 *In vivo* rodent models of left ventricular hypertrophy and failure

Many experimental models are available to the researcher wishing to study stress responses to hypertrophic stimuli in the heart. Ideally, these should provide a reliable and reproducible insult amongst subjects, be associated with minimal mortality rates and costs, and perhaps most importantly be of clinical and physiological relevance.

4.1.2.1 Rodent models for the study of physiological hypertrophy

Exercise models used to induce physiological left ventricular hypertrophy in small rodents generally involve either a period of running or swimming. Both induce left ventricular hypertrophy, although swimming appears to produce more pronounced bradycardia, greater contractility and is associated with higher levels of circulating catecholamines (Schaible and Scheuer, 1979, Geenen et al., 1988).

Swimming has the advantage of being cheap and easy to set up, with minimal equipment required. The degree of hypertrophy achieved is variable dependent on the protocol, but optimal conditions have been determined for use in mice (Evangelista et al., 2003). The potential disadvantage to the use of swimming is the inability to quantify exercise intensity.

Running can be either voluntary or forced. A drawback of voluntary wheel running is the issue of motivation, which may not be consistent amongst subjects and hence exercise intensity levels may vary (Perrino et al., 2011). Therefore the preference is for forced treadmill running, achieved using electric shock. However, this has higher associated costs when compared to swimming and may also introduce a significant stress response which is not replicated in sedentary controls.
4.1.2.2 Rodent models of pathological hypertrophy and heart failure

The quest to study the mechanisms involved in the development and progression of left ventricular hypertrophy has generated numerous strategies for its study. These include surgical, chemical and genetic interventions allowing the model to be tailored to a particular aetiology.

Pressure overload, for example, is typically induced in mice through surgical constriction of the transverse aorta (TAC) between the brachiocephalic trunk and left common carotid arteries (Rockman et al., 1991), or alternatively through suprarenal constriction of the abdominal aorta (Ohkusa et al., 1997), more common in the rat. Each of these models has several advantages, in that they are associated with relatively low mortality, are highly reproducible and induce a significant and predictable hypertrophic response which eventually progresses to failure (Muders and Elsner, 2000). However, whilst aortic constriction certainly bears a resemblance to aortic stenosis and to a lesser extent hypertension, the sudden induction of pressure overload still means there are substantial differences from these diseases in the clinical setting. An alternative model for the study of hypertensive hypertrophy can be achieved through chronic administration of vasoactive drugs such as angiotensin II or phenylephrine via osmotic minipumps (Wu et al., 2009). Whilst these approaches may provide valuable insight upon activation of their respective signalling pathways, hypertrophy will generally be associated with the involvement of many additional pathways. Genetically modified rat models, such as the spontaneously hypertensive and Dahl salt sensitive rat, have also been used in the study of hypertrophy. Similar models are not available in the mouse however, and it takes many weeks for the progression to decompensation to occur (Balakumar et al., 2007).

Even the most clinically relevant models of pathological insult have their disadvantages. Myocardial infarction is typically induced via ligation of the left anterior descending artery, thus mimicking the human form of the disease. This model is associated with a much higher mortality rate compared to hypertrophic models such as TAC, there is often considerable variation in infarct size and the issue of sudden initiation of insult remains (Balakumar et al., 2007).
Whilst each model has its drawbacks, there is no doubt that their use has increased our understanding of disease mechanisms. In the current project, we wished to study the effect of PMCA ablation during the development of pathological and physiological hypertrophy, and therefore selected the well-established and reliable models of TAC and swimming. Pressure overload was induced by TAC for a period of 2 weeks in the PMCA1\(^{\text{cko}}\) and PMCA1:4\(^{\text{dcko}}\) mouse lines, whilst double knockout mice also underwent a programme of 4 weeks of swimming.

4.1.3 Hypothesis

PMCA4 significantly regulates the pathological hypertrophic response to pressure overload, but not the physiological response to swimming. PMCA1 may take on a greater role in calcium clearance during the early compensated stage of hypertrophy.

4.1.4 Aims

To examine the cardiac phenotype in response to pathological and physiological stress induced by pressure overload and chronic exercise respectively following PMCA ablation. This should verify whether the PMCA contributes significantly to the development of either form of hypertrophy.
4.2 Methods

4.2.1 In vivo models of hypertrophy

In order to study the roles of PMCA1 and 4 in the development of pathological and physiological hypertrophy, the well-established methods of pressure overload by transverse aortic constriction (TAC) and chronic swimming were chosen. First described by Rockman et al., TAC has since become one of the most widely used disease models in cardiac research, especially in the rodent (Rockman et al., 1991). It offers several advantages in that it should provide a consistent insult amongst subjects, and produces a consistent pattern of compensated concentric hypertrophy over a predictable time course which can later progress to decompensation (Muders and Elsner, 2000). Swimming similarly has a number of pluses given its ease to set up and slightly heightened cardiac response when compared to other forms of exercise induced hypertrophy (Schaible and Scheuer, 1979).

4.2.1.1 Transverse aortic constriction

Eight week old PMCA1\(^{cko}\) and PMCA1:4\(^{dcko}\) mice, alongside their respective floxed and transgenic controls were subjected to two weeks pressure overload by TAC as previously described (Rockman et al., 1991). Typically this procedure will produce a 35-45mmHg pressure gradient between the right and left carotid arteries, although in some hands this appears to be much higher (Hill et al., 2000, Wu et al., 2009). All surgical procedures were performed by Min Zi.

Mice were anaesthetised using a combination of ketamine and xylazine (100 & 5 mg/kg IP respectively) and then intubated allowing for artificial ventilation with oxygen during the procedure. The chest cavity was opened and a 7-0 silk suture was tied around a blunted 27-gauge needle placed parallel to the aorta between the brachiocephalic trunk and left common carotid artery. Once secure the needle was withdrawn leaving a constriction in place. The chest cavity was sutured shut and mice were administered 0.1mg/kg buprenorphine IP at the first sign of recovery. During the recovery period mice were placed in an incubator set to 37°C and monitored closely for signs of ill health. Sham
operated controls underwent the same anaesthesia, procedure and analgesia except the suture was passed around the back of the aorta and subsequently withdrawn without tying, thus not leaving a constriction. *In vivo* analysis was performed following two weeks following which mice were sacrificed. For calcium analysis, mice were euthanized on day 8 and experiments were performed as described in chapter 2.

4.2.1.2 Chronic swimming

These experiments were performed alongside Donna Page and formed the basis of her MRes thesis in tissue engineering. 8 week old PMCA1:4^dcko and floxed control mice were subjected to a program of chronic swimming twice a day for 90 minutes for a period of four weeks. The protocol selected had been previously described to induce the greatest degree of hypertrophy when compared to a number of other swimming regimens (Evangelista et al., 2003).

A tank was filled with water and the temperature maintained between 29 and 31°C. A low pressure pump was placed at either end of the tank to create bubbles and encourage swimming. On day 1 mice were swum for 10 minutes, whilst sedentary controls were dipped into the tank to account for the possible stress induced by being introduced to water. Any mice considered to be weak swimmers were moved to the sedentary group. Following the swim mice were dried briefly by hand and then placed in an incubator set at 37°C. After 4 hours of rest, a second 10 minute period of swimming was performed. Swim time was increased by 10 minutes per day up to day 9 in order to ensure that the mice could cope with the full 90 minute swim period. Thereafter mice were swum twice a day for 90 minutes with 4 hours rest in between. This continued up until day 28 whereupon cardiac analysis was performed and mice sacrificed.
4.2.2 In vivo analysis in conscious mice or involving recovery

Heart rate was assessed by conscious ECG at three time points over the course of the swimming experiment. ECG was performed on day 0, day 14 and day 28 using an ECGenie (Mouse specifics) connected to a Powerlab system (AD instruments). Mice were placed on a platform where they stood on three electrodes. Recordings were measured for a period of 5 minutes to allow for acclimatisation. Analysis was carried out as per unconscious ECG. Recovery echo was also performed during the swimming experiment. The procedure was the same as that described in chapter 2, except that 3% isofluorane was used to induce anaesthesia. Conscious ECG was also performed in double knockout TAC experiments.

4.2.3 In vitro analysis of NFκB activity following knockdown of PMCA4

Primary neonatal rat cardiomyocytes (NRCM) provide a good means in which to study signalling pathways in vitro as they remain stable in culture for a number of days allowing their manipulation by gene transfer (Louch et al., 2011). NRCMs were isolated from 1-3 day old Sprague Dawley rats and prepared for culture using methods as previously described (Mohamed et al., 2011). The cultured cardiomyocytes were kindly prepared for me by colleagues and all adenoviruses were kindly provided to me by Dr. Delvac Oceandy.

4.2.3.1 NFκB localisation by immunocytochemistry

NRCM were plated on laminin coated cover slips in 24 well plates at 6x10^5 cells per well and transfected with adenovirus containing PMCA4 and control shRNA (C2 shRNA containing a scrambled sequence) for a period of 4 days. 20ng of TNF-α was added to wells to induce nuclear translocation of NFκB (Higuchi et al., 2002) for 4 hours before cells were washed with PBS and fixed with 3.7% formalin for 20 minutes. Cells were then washed before permeabilisation with 0.1% Triton-X for 15 minutes, after which they were washed and blocked overnight in 1% BSA. The following day primary antibodies for NFκB (Santa Cruz) and sarcomeric α-actinin (Sigma) were added to each well at a dilution of 1:100 for 2 hours before washing and further incubation for 2 hours with anti-rabbit Texas Red and anti-mouse FITC-conjugated secondary antibodies (1:200 dilution - Jackson Laboratories) targeted against NFκB and α-actinin respectively. Nuclei were
stained with DAPI (Invitrogen) before the coverslips were placed onto slides. Images were acquired using an Olympus BX51 fluorescent microscope at 20X magnification.

4.2.3.2 NFκB luciferase activity

24 well plates containing 7x10^5 NRCM were transfected with PMCA4 or control shRNA and incubated at 37°C for 3 days following which an NFκB luciferase reporter vector was transfected for 1 day. 20ng of TNF-α was added the following day for 4 hours before cells were washed in PBS and subsequently lysed with luciferase lysis buffer (Promega) for a period of 20 minutes. Luciferase enzymes can report transcriptional activity of the promoter to which they are fused by emitting light upon the expression of a suitable substrate. 10μl of lysate was added to a tube and placed in a luminometer (Berthold Technologies Lumat LB 9507). This was programmed to dispense lysis buffer along with 100μl of the luciferase substrate luciferin, upon which light emission was measured for 2 seconds following a 2 second delay.
4.3 Results

The results described in the previous chapter suggest isoform-specific roles for PMCA1 and 4 in finely regulating calcium homeostasis under basal conditions. These subtle changes witnessed upon PMCA ablation did not produce any overt spontaneous phenotype in vivo however, and so experiments were designed to establish whether the pumps took on a greater role during cardiac stress. Altering expression or activity of isoform 4 has been shown to influence the hypertrophic response to transverse aortic constriction via regulation of the calcineurin-NFAT pathway (Wu et al., 2009, Abou-Leisa, 2013), therefore this model of pressure overload was selected to compare the response in PMCA1\textsuperscript{cko} and PMCA1:4\textsuperscript{dcko} lines. This would provide insight into the role of each isoform at times of pathological stress; however, the mechanisms governing cardiac hypertrophy are thought to vary greatly depending on whether the stimulus is of a pathological or physiological nature, and therefore PMCA1:4\textsuperscript{dcko} mice also underwent a course of intense athletic training to examine the phenotype following physiological stress.

4.3.1 The role of PMCA1 during pressure overload-induced hypertrophy

At 8 weeks of age, PMCA1\textsuperscript{cko} and floxed control mice underwent surgical aortic banding or a sham operation. After two weeks, in vivo cardiac structure and function was examined, as well as histological phenotype following sacrifice.

4.3.1.1 The hypertrophic response in PMCA1\textsuperscript{cko} mice following TAC

Transverse aortic constriction (TAC) is perhaps the most commonly used experimental model of hypertrophy in small rodents, with the theoretical advantage of inducing left ventricular pressure overload to the same extent in each subject by controlling the degree of constriction. To give an indication that this was in fact the case, the maximal pressure inside the left ventricle (P\textsubscript{max}) was recorded via invasive catheterisation prior to sacrifice. Compared to sham operated controls, TAC induced an expected rise in systolic pressure in both PMCA1\textsuperscript{flo} and knockout mice, of ~70mmHg and ~60mmHg respectively (figure 4.1, both p<0.05). Importantly however, P\textsubscript{max} did not differ significantly between the two TAC
genotypes (159.68 ± 9.73 vs 146.36 ± 12.63 mmHg, p=0.43), suggesting the severity of constriction to be comparable amongst groups.

![Graph showing maximal left ventricular pressure in PMCA1^cko and control mice following two weeks TAC.](image)

**Figure 4.1** Maximal left ventricular pressure in PMCA1^cko and control mice following two weeks TAC. \( P_{\text{max}} \) increased similarly in PMCA1^f/f and PMCA1^cko mice following two weeks TAC. *** p<0.001 flox TAC vs sham; * p<0.05 cko TAC vs sham.

Having established that knockout and control hearts had been subjected to similar increases in workload following TAC, the nature of the hypertrophic response was subsequently examined. Compared to their respective sham operated controls, pressure overload induced similar rises in normalised heart weight in PMCA1^f/f and PMCA1^cko mice of 51 and 48% (figure 4.2A, both p<0.01). M-mode imaging of the left ventricle by echocardiography revealed this increase in heart weight to be accompanied by comparable thickening of the interventricular septum in the two TAC cohorts (figure 4.2B, PMCA1^f/f 0.77 ± 0.04 vs 1.13 ± 0.05 mm, p<0.001; PMCA1^cko 0.87 ± 0.03 vs 1.07 ± 0.04 mm, p<0.01).
Figure 4.2 The hypertrophic response in PMCA1<sup>cko</sup> mice following two weeks TAC A) Heart weight normalised to tibia length rose similarly in PMCA1<sup>cko</sup> and floxed controls following TAC (cko sham vs TAC 6.14 ± 0.23 vs 9.06 ± 0.16 mg/mm, *** p<0.001; flox sham vs TAC 5.67 ± 0.47 vs 8.57 ± 0.61, ** p<0.01) B) Diastolic interventricular septal thickness increased similarly in knockout and control mice after TAC (flox sham vs TAC 0.77 ± 0.04 vs 1.13 ± 0.05 mm, *** p<0.001; cko sham vs TAC 0.87 ± 0.03 vs 1.07 ± 0.04 mm, ** p<0.01) C) Internal diameter of the left ventricle during diastole was larger in PMCA1<sup>cko</sup> TAC hearts compared to sham and flox TAC controls (* p<0.05 vs PMCA1<sup>cko</sup> sham, † p<0.05 vs PMCA1<sup>f/f</sup> TAC) D) PMCA1<sup>f/f</sup> TAC hearts experienced a rise in relative wall thickness compared to PMCA1<sup>cko</sup> TAC and sham operated controls ( ** p<0.01 vs PMCA1<sup>f/f</sup> sham, † p<0.05 vs PMCA1<sup>cko</sup> TAC). HW/TL, heart weight/tibia length ratio; d, diastolic; LVD, internal left ventricular diameter; IVS, interventricular septal thickness; RWT, relative wall thickness.
These results suggested that PMCA1 deletion did not affect the extent of cardiac growth following TAC. Further analysis of cardiac structure, however, revealed a significantly greater internal diameter of the left ventricle during diastole in PMCA1<sup>cko</sup> TAC hearts (4.31 ± 0.27 mm) compared to both sham operated (3.61 ± 0.09 mm, p<0.05) and PMCA1<sup>f/f</sup> TAC controls (3.59 ± 0.11, p<0.05, figure 4.2C), which when considered alongside the similar increases in wall thickness between the two TAC groups suggests that the nature of the hypertrophic response differed. Increased wall thickness without enlargement of chamber size, as witnessed in PMCA1<sup>f/f</sup> hearts, is characteristic of concentric cardiomyocyte hypertrophy, whilst the paralleled increase in septal thickness and ventricular diameter observed in knockout hearts is more indicative of eccentric hypertrophy. This was reflected in the relative wall thickness (figure 4.2D), which rose significantly after TAC in floxed controls (0.53 ± 0.01 vs 0.44 ± 0.02, p<0.01 vs sham) whilst remaining unaltered in PMCA1<sup>cko</sup> mice (0.45 ± 0.04 vs 0.46 ± 0.02 TAC vs sham).

A prominent underlying characteristic of cardiac hypertrophy is the enlargement of individual myocytes, and therefore mean cellular cross sectional area (CSA) was examined in H&E stained histological sections to further compare the degree of hypertrophy following TAC. Figure 4.3 shows that PMCA1<sup>cko</sup> and floxed control myocytes experienced a similar expansion in width in response to TAC (mean CSA flox sham vs TAC 251.00 ± 4.27 vs 327.23 ± 21.60 μm<sup>2</sup>, p<0.05; cko sham vs TAC 261.17 ± 12.61 vs 327.98 ± 15.18 μm<sup>2</sup>, p<0.05). This supports the in vivo data, confirming a similar extent of hypertrophic growth in the two TAC groups.
Figure 4.3 Cardiomyocyte cross sectional area in PMCA1\(^{cko}\) mice following two weeks TAC A) Representative H&E stained transverse histological sections from which mean myocyte CSA was calculated. Scale bars = 20\(\mu\)m B) Quantification of mean CSA. TAC induced a similar degree of growth in PMCA1\(^{cko}\) and PMCA1\(^{ff}\) myocytes (* both p<0.05 vs respective sham operated controls)
4.3.1.2 Cardiac function in PMCA1\textsuperscript{cko} mice following TAC

The normal adaptive response to TAC during the first 2 weeks is one of concentric cardiac hypertrophy, with preservation or even slight improvement in systolic left ventricular function (Nakamura et al., 2001). Interestingly though, PMCA1\textsuperscript{cko} mice appeared to display characteristics of an eccentric hypertrophic response, with evidence of left ventricular dilation. Whilst this pattern of hypertrophy is more commonly associated with models of volume overload, it could also be indicative of a transition to decompensation (Sciarretta and Sadoshima, 2010) and thus systolic and diastolic function were examined.

Further analysis of chamber size by M-mode echocardiography revealed that in addition to left ventricular dilation during diastole (see figure 4.2C), PMCA1\textsuperscript{cko} TAC hearts also displayed chamber enlargement at maximal contraction (figure 4.4A & B; systolic left ventricular diameter (in mm) 3.43 ± 0.27 vs 2.36 ± 0.12 cko sham, p<0.05 and 2.43 ± 0.17 flox TAC, p<0.01). Furthermore, hearts displayed a reduction in fractional shortening indicating that systolic function was compromised (figure 4.4C; cko TAC 20.56 ± 1.62\% vs 34.83 ± 1.88 cko sham, p<0.001 and 32.44 ± 3.26 flox TAC, p<0.05). Impaired contractility will generally equate to insufficient blood being ejected during systole, and \textit{in vivo} haemodynamic assessment by left ventricular catheterisation confirmed a 59 and 49\% reduction in stroke volume in PMCA1\textsuperscript{cko} TAC mice compared to sham and PMCA1\textsuperscript{ff} TAC controls respectively (figure 4.4D, both p<0.01).
Figure 4.4 Systolic function in PMCA1<sup>cko</sup> mice following two weeks TAC A) Example M-mode echo traces taken from PMCA1<sup>f/f</sup> and PMCA1<sup>cko</sup> TAC hearts B) Left ventricular dilation during systole in PMCA1<sup>cko</sup> TAC hearts (* p<0.05 vs PMCA1<sup>cko</sup> sham, †† p<0.01 vs PMCA1<sup>f/f</sup> TAC) C) Reduced fractional shortening in PMCA1<sup>cko</sup> TAC hearts (*** p<0.001 vs PMCA1<sup>cko</sup> sham, † p<0.05 vs PMCA1<sup>f/f</sup> TAC) D) Reduced stroke volume in PMCA1<sup>cko</sup> TAC hearts (8.21 ± 1.50 μl vs 20.22 ± 2.06 cko sham, ** p<0.01 and 15.99 ± 1.54 flox TAC, †† p<0.01). d, diastolic; s, systolic; LVD, internal left ventricular diameter; FS, fractional shortening; SV, stroke volume

Further insight into cardiac performance can be gained from pressure-volume loop analysis which can be used to examine the interaction between the left ventricle and arterial system, commonly altered during decompensated heart failure. Left ventricular or end systolic elastance (E<sub>es</sub>), derived from the slope of the ESPVR, is considered a gold standard measure of left ventricular contractility being relatively insensitive to cardiac loading (Burkhoff, 2013). Figure 4.5A shows this to be increased in PMCA1<sup>f/f</sup> TAC hearts by 81% compared to sham operated controls indicating improved contractility (p<0.05), whilst E<sub>es</sub> remained unchanged in knockout hearts post-TAC (7.70 ± 1.19 vs 7.22 ± 1.55 mmHg/μl PMCA1<sup>cko</sup> sham). Arterial elastance (E<sub>a</sub>) is derived from the ratio of end systolic pressure
and stroke volume, and provides a measure of afterload placed on the ventricle (Kelly et al., 1992). This was found to be elevated in both control and knockout hearts following TAC (both p<0.01 vs respective sham controls), however to a far greater extent in PMCA1<sup>cko</sup> hearts (20.64 ± 3.57 vs 9.98 ± 0.93 mmHg/μl flox TAC, p<0.05 figure 4.5B). From these measures of ventricular and arterial elastance an evaluation of arterial-ventricular coupling can be made (E<sub>a</sub>/E<sub>es</sub>). Figure 4.5C indicates that whilst this ratio remained similar to sham controls in PMCA1<sup>f/f</sup> TAC mice, E<sub>a</sub>/E<sub>es</sub> was raised significantly in PMCA1<sup>cko</sup> TAC mice (3.29 ± 0.65 vs 0.86 ± 0.26 cko sham, p<0.05 and 0.87 ± 0.18 flox TAC, p<0.01). These results indicate that whilst pumping efficiency was maintained in control TAC hearts, PMCA1<sup>cko</sup> hearts failed to compensate for the increased load inflicted by aortic constriction.
Figure 4.5 Arterial-ventricular coupling in PMCA1^cko mice following two weeks TAC

A) Left ventricular end systolic elastance (E_{es}). This index of contractility was increased significantly in controls compared to PMCA1^cko hearts following TAC, as well as sham operated controls (12.69 ± 1.63 vs 7.70 ± 1.19 cko TAC, † p<0.05 and 7.02 ± 1.11 mmHg/μl flox sham, * p<0.05)  

B) TAC induced a roughly 2- and 4-fold increase in effective arterial elastance (E_{a}) in PMCA1^ff and PMCA1^cko mice respectively (** both p<0.01 vs sham controls). This was significantly higher in knockout animals († p<0.05)  

C) PMCA1^cko TAC mice had a greater E_{a} to E_{es} ratio compared to controls (* p<0.05 vs cko sham, ‡‡ p<0.01 vs flox TAC)
The data presented in figures 4.2-5 indicate that at the end of the 2 week period of pressure overload, PMCA1\(^{ff}\) mice displayed a phenotype of compensated cardiac hypertrophy, whilst PMCA1\(^{cko}\) hearts experienced a dramatic reduction in systolic function and exhibited signs of dilated cardiomyopathy. This phenotype can often be accompanied by diastolic dysfunction, and therefore lusitropic haemodynamic analysis was also performed.

The minimum rate of pressure change in the left ventricle (dP/dt\(_{\text{min}}\)) acts as a measure of the force of relaxation, and this was found to be stronger in PMCA1\(^{ff}\) mice following TAC compared to sham controls (-7174 ± 546 vs -4849 ± 721 mmHg/s, p<0.05, figure 4.6A). This suggests an improvement in diastolic function, which was not witnessed in PMCA1\(^{cko}\) mice (-5581 ± 666 vs cko sham -4912 ± 644 mmHg/s, p=0.53). The time constant of isovolumic relaxation (\(\tau\)) was prolonged significantly in knockout TAC hearts compared to floxed TAC controls (8.83 ± 0.80 vs 6.64 ± 0.32, p<0.05, figure 4.6B) and suggests that in addition to experiencing systolic dysfunction, PMCA1\(^{cko}\) mice also suffered from impaired myocardial relaxation.

![Figure 4.6](image_url)

Figure 4.6 Diastolic function in PMCA1\(^{cko}\) mice following two weeks TAC A) dP/dt\(_{\text{min}}\) was significantly enhanced in PMCA1\(^{ff}\) mice following TAC (* p<0.05 vs flox sham), but not in PMCA1\(^{cko}\) hearts B) Isovolumic relaxation time constant was increased in PMCA1\(^{cko}\) hearts after TAC compared to banded controls († p<0.05).
The *in vivo* data presented above indicates that PMCA1\(^{cko}\) mice rapidly progress into a state of decompensated hypertrophy when challenged with two weeks pressure overload, displaying greatly impaired systolic performance, left ventricular dilation and diastolic dysfunction. The elevated $E_a/E_{es}$ ratio (figure 4.5C) suggests that the left ventricle was unable to cope with the increased demands placed on the heart, indicative of heart failure. Pulmonary oedema is a classical presenting symptom of left ventricular failure as incomplete emptying during systole leads to a build-up of fluid in the regions returning blood to the left side, and therefore lung weight was examined. Figure 4.7 shows that PMCA1\(^{cko}\) mice exhibited significantly increased normalised lung weight compared to PMCA1\(^{ff}\) controls following TAC (lung weight/body weight ratio 11.45 ± 1.10 vs 7.81 ± 0.85 mg/g, *p*<0.05), and more than a 2-fold increase from sham operated controls (p<0.01) confirming the failing phenotype witnessed *in vivo*.

**Figure 4.7 Normalised lung weight in PMCA1\(^{cko}\) mice following two weeks TAC**

PMCA1\(^{cko}\) TAC mice displayed signs of pulmonary oedema compared to sham controls (LW/BW 11.45 ± 1.10 vs 5.43 ± 0.21 mg/g, ** *p*<0.01) as well as an increase in lung weight compared to PMCA1\(^{ff}\) TAC mice († *p*<0.05) LW/BW, lung weight / body weight ratio.
4.3.1.3 Cardiac structure and function in αMHC-Cre^tg control mice following TAC

It was important to confirm that the dilated failing phenotype witnessed in PMCA1^cko mice following pressure overload was not an artefact caused by the presence of Cre-recombinase. 8 week old αMHC-Cre^neg and αMHC-Cre^tg controls were therefore subjected to the same 2 week TAC procedure and the hypertrophic and functional responses assessed.

Figure 4.8A indicates that the two control groups experienced a similar rise in heart weight normalised to tibia length in response to TAC (αMHC-Cre^neg sham vs TAC 5.64 ± 0.39 vs 7.58 ± 0.48 mg/mm, p<0.05; αMHC-Cre^tg sham vs TAC 4.79 ± 0.34 vs 7.18 ± 0.28 mg/mm, p<0.001, figure 4.2A). Crucially however, both αMHC-Cre^neg and αMHC-Cre^tg TAC groups displayed evidence of a compensated concentric hypertrophic response, with no overt signs of left ventricular dilation or dysfunction (figure 4.8B-D). This suggests that the altered response to TAC in PMCA1^cko mice was a resultant effect of PMCA1 deletion.
Figure 4.8 The cardiac response in αMHC-Cre\textsuperscript{neg} and αMHC-Cre\textsuperscript{tg} mice following two weeks TAC A) Heart weight normalised to tibia length rose similarly in Cre negative and transgenic controls following TAC (* \(p<0.05\) Cre\textsuperscript{neg}, *** \(p<0.001\) Cre\textsuperscript{tg}) B) Left ventricular internal diameter in diastole was not affected by TAC in either cohort C) Fractional shortening was preserved in both αMHC-Cre\textsuperscript{neg} and αMHC-Cre\textsuperscript{tg} mice following TAC D) TAC did not induce a significant rise in normalised lung weight in either control genotype. HW/TL, heart weight/tibia length ratio; dLVD, diastolic internal left ventricular diameter; FS, fractional shortening; LW/BW, lung weight/body weight ratio.
4.3.1.4 Intracellular calcium dynamics in PMCA1$^{cko}$ mice during the early response to pressure overload

The progression into decompensated hypertrophy witnessed in PMCA1$^{cko}$ mice following just 2 weeks TAC was surprising given a comparable increase in wall thickness to controls. The in vivo data suggested that this adaptation did not produce the improvement in inotropic or lusitropic performance required to meet the increase in workload imposed upon the heart during pressure overload following PMCA1 ablation. Since intracellular calcium dynamics govern contraction and relaxation, and given that previous experiments had found PMCA1 to be the chief calcium pumping isoform of the sarcolemmal ATPase, it was hypothesised that altered calcium handling could be behind the development of the failing phenotype. In order to investigate this, systolic and caffeine-evoked transients were examined in ventricular myocytes isolated from PMCA1$^{ff}$ and PMCA1$^{cko}$ hearts 1 week post-surgery when physical signs of heart failure were not evident in knockout mice, with a particular view to testing the hypothesis that PMCA1 may take on a greater responsibility in calcium extrusion under stress conditions.

4.3.1.4.1 Systolic transients in PMCA1$^{cko}$ mice following 1 week TAC

Sham and TAC myocytes isolated from PMCA1$^{ff}$ and PMCA1$^{cko}$ mice were stimulated at a frequency of 1Hz and steady-state transients recorded. TAC had no effect on diastolic calcium levels in either knockout or control myocytes (Indo-1 ratio flox sham 0.92 ± 0.04 vs 0.94 ± 0.03 TAC, p=0.67; cko sham 0.88 ± 0.04 vs 0.90 ± 0.05 TAC, p=0.81). Mean normalised traces depicted an increase in calcium transient amplitude in control cells following TAC, which was not witnessed in PMCA1$^{cko}$ TAC cells (PMCA1$^{ff}$ TAC 0.190 ± 0.014 vs sham 0.130 ± 0.004 and PMCA1$^{cko}$ TAC 0.137 ± 0.013, figure 4.9A & B both p<0.05). In contrast, the rate of calcium decay was unaltered in either knockout or control cells 1 week post-surgery (figure 4.9C & D).
Figure 4.9 Systolic calcium transients in PMCA1^{cko} mice following one week of TAC
A) Mean normalised transients after electrical stimulation at 1Hz in sham (solid lines) and TAC (dotted lines) myocytes. B) Transient amplitude rose in PMCA1^{ff} cells following TAC (** p<0.01), and was significantly higher than in PMCA1^{cko} TAC myocytes († p<0.05) which exhibited similar peak levels to sham controls. C) Mean traces displayed as % of peak to account for the altered amplitude. D) Single exponential time constant was not affected by pressure overload in either knockout or flox cells). n = 29-38 cells taken from 4-5 mice as indicated.
4.3.1.4.2 Caffeine-evoked transients in PMCA1\textsuperscript{cko} mice following 1 week TAC

The experiments in the previous section indicate an increase in amplitude of the systolic transient in control TAC cells which was not reciprocated following PMCA1 ablation. The source of this extra calcium could be accounted for by increased calcium entry, SR load, SR release or a combination of these factors. To investigate this myocytes were stimulated with 10mM caffeine, which would empty the SR and give an indication of SR load. In addition, it would allow assessment of the rate of calcium extrusion from the cell via NCX and PMCA with the effects of SR uptake being negated.

Figure 4.10 presents mean normalised caffeine-evoked transients recorded in myocytes from each of the four experimental groups. PMCA1\textsuperscript{fl} cells again displayed a greater amplitude of peak intracellular calcium following TAC compared to knockout myocytes as well as sham-operated controls (figure 4.10B, both p<0.05), indicative of an increase in SR calcium content during pressure overload-induced hypertrophy in wild-type cells. Examination of the rate of removal of the caffeine-evoked transient revealed no difference in decay time constant (\(\tau\)) between sham and TAC myocytes, suggesting that pressure overload did not alter the relative contribution of calcium extrusion from the cell to overall clearance from the cytosol (figure 4.10C). A comparison of the rate of decay in control and knockout myocytes found that deletion of PMCA1 did not prolong the \(\tau\) significantly under sham or TAC conditions indicating PMCA1 does not play a critical role in diastolic calcium extrusion under basal or pressure overloaded conditions (\(\tau\) – flox vs cko sham 2.03 ± 0.22 vs 2.47 ± 0.29 s; flox vs cko TAC 1.96 ± 0.26 vs 2.51 ± 0.32 s).
Figure 4.10 Caffeine-evoked calcium transients in PMCA1\textsuperscript{cko} mice following one week of TAC A) Mean normalised traces upon rapid application of 10mM caffeine for the indicated period in PMCA1\textsuperscript{f/f} and PMCA1\textsuperscript{cko} sham (darker lines) and TAC (paler lines) myocytes. Examples of exponentials of decay fitted to the traces are shown in red (flox) and blue (cko) under sham (solid lines) and TAC (dotted lines) conditions. B) Quantification of the peak rise in Indo-1 in response to caffeine application, indicating an increase in amplitude following pressure overload in PMCA1\textsuperscript{f/f} cells compared to sham controls (0.46 ± 0.05 vs 0.33 ± 0.03, * p<0.05) and PMCA1\textsuperscript{cko} TAC myocytes (0.33 ± 0.02, † p<0.05). C) Single exponential time constant of decay was not altered following pressure overload. n = 4-11 cells taken from 3-5 mice as indicated.
4.3.1.4.3 Caffeine-evoked transients after removal of extracellular \( \text{Na}^+ \) and \( \text{Ca}^{2+} \) in PMCA1\(^{\text{cko}}\) mice following 1 week TAC

Systolic and caffeine-evoked transients appeared to indicate that pressure overload induced an increase in peak intracellular calcium during contraction, which may in part be due to a greater SR load, in control cells but not in PMCA1\(^{\text{cko}}\) myocytes. Data also indicated that the rate of calcium decay was unaltered following TAC, and to confirm that PMCA1-mediated efflux was similarly unaffected during pressure overload-induced hypertrophy the rate of decay of the caffeine-evoked transient was analysed after the removal of extracellular \( \text{Na}^+ \) and \( \text{Ca}^{2+} \).

The mean normalised traces presented in figure 4.11A indicate that transients recorded in PMCA1\(^{\text{ft}}\) TAC myocytes closely matched those in sham-operated controls. By examining transients in PMCA1\(^{\text{cko}}\) myocytes, it was also possible to ascertain that extrusion via PMCA4 and mitochondrial uptake also remained unchanged following TAC. These findings were confirmed upon examination of the \( \tau \), which whilst clearly exhibiting a difference between flox and knockout cells under sham and TAC conditions (figure 4.11B, both \( p<0.01 \)), did not reveal a change following pressure overload. Taken together with the data in the previous two sections this suggests that diastolic extrusion was not altered by TAC in either PMCA1\(^{\text{cko}}\) or PMCA1\(^{\text{ft}}\) myocytes.
Figure 4.11 Caffeine-evoked calcium transients after removal of extracellular Na\(^+\) and Ca\(^{2+}\) following one week of TAC

A) Mean normalised traces upon rapid application of 10mM caffeine for the indicated period after removal of external sodium and calcium ions in PMCA1\(^{ff}\) and PMCA1\(^{cko}\) sham (darker lines) and TAC (paler lines) myocytes. Examples of exponentials of decay fitted to the traces are shown in red (flox) and blue (cko) under sham (solid lines) and TAC (dotted lines) conditions. B) Time constant of decay was significantly higher in PMCA1\(^{cko}\) myocytes compared to PMCA1\(^{ff}\) cells under both sham (27.77 \(\pm\) 2.58 vs 12.32 \(\pm\) 2.75, ** p<0.01) and TAC conditions (31.07 \(\pm\) 2.21 vs 13.69 \(\pm\) 1.52, +++ p<0.001), but not altered by pressure overload. \(n = 9-11\) cells taken from 3-4 mice as indicated.
4.3.1.4.4 Contributions of extrusion systems to calcium clearance following 1 week TAC

From the data described in sections 4.3.1.4.1-3 it was possible to make an estimation as to the overall contribution of each calcium clearance system at rest and after pressure overload. Mean rate constants of decay are shown in table 4.1, derived from the τ under each set of experimental conditions. The proportion of global calcium extrusion remaining following caffeine application in the presence and absence of external Na\(^+\) and Ca\(^{2+}\) was determined for all cells using equations 4.1-4.4, and the mean data are presented in table 4.1. By subtracting the appropriate contributions as per the calculations in equation 4.5, an estimate was made as to the percentage of global calcium removed from the cytosol by SERCA, NCX, PMCA1 and PMCA4/mitochondrial uptake in PMCA1\(^{ff}\) and PMCA1\(^{cko}\) sham and TAC mice.

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<td>SERCA, NCX, PMCA1, PMCA4 mito</td>
<td>NCX, PMCA1, PMCA4 mito</td>
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Table 4.1 Calcium clearance in PMCA1\(^{ff}\) and PMCA1\(^{cko}\) mice following 1 week TAC. Mean rate constants of decay in s\(^{-1}\) of systolic and caffeine-evoked transients in PMCA1\(^{ff}\) and PMCA1\(^{cko}\) TAC mice along with sham operated controls. The active extrusion systems under each experimental condition and their calculated mean contributions to clearance of the systolic transient are also shown. n = 3-5 mice per group. mito, mitochondrial uptake.
Caffeine-evoked decay in PMCA1<sup>f/f</sup> mice (PMCA1<sub>caffeine</sub><sup>f/f</sup>) = \( \frac{w}{u} \times 100\% \)

Equation 4.1 Combined NCX, PMCA1, PMCA4 and mitochondrial contributions to calcium clearance in PMCA1<sup>f/f</sup> mice

Caffeine-evoked decay in PMCA1<sup>cko</sup> mice (PMCA1<sub>caffeine</sub><sup>cko</sup>) = \( \frac{x}{v} \times 100\% \)

Equation 4.2 Combined NCX, PMCA4 and mitochondrial contributions to calcium clearance in PMCA1<sup>cko</sup> mice

Slow contribution in PMCA1<sup>f/f</sup> mice (PMCA1<sub>slow</sub><sup>f/f</sup>) = \( \frac{y}{u} \times 100\% \)

Equation 4.3 Combined PMCA1, PMCA4 and mitochondrial contribution to calcium clearance in PMCA1<sup>f/f</sup> mice

Slow contribution in PMCA1<sup>cko</sup> mice (PMCA1<sub>slow</sub><sup>cko</sup>) = \( \frac{z}{v} \times 100\% \)

Equation 4.4 Combined PMCA4 and mitochondrial contribution to calcium clearance in PMCA1<sup>cko</sup> mice

PMCA1<sup>f/f</sup>

\[
SERCA = 100 - \text{PMCA1}_{\text{caffeine}}^{f/f} \\
NCX = \text{PMCA1}_{\text{caffeine}}^{f/f} - \text{PMCA1}_{\text{slow}}^{f/f} \\
\text{PMCA1} = \text{PMCA1}_{\text{slow}}^{f/f} - \text{PMCA1}_{\text{slow}}^{cko} \\
\text{PMCA4 + mito} = \text{PMCA1}_{\text{slow}}^{cko}
\]

PMCA1<sup>cko</sup>

\[
SERCA = 100 - \text{PMCA1}_{\text{caffeine}}^{cko} \\
NCX = \text{PMCA1}_{\text{caffeine}}^{cko} - \text{PMCA1}_{\text{slow}}^{cko} \\
\text{PMCA1} \text{ assumed as 0} \\
\text{PMCA4 + mito} = \text{PMCA1}_{\text{slow}}^{cko}
\]

Equation 4.5 Calculations performed to assess individual contributions of calcium clearance systems in PMCA1<sup>f/f</sup> and PMCA1<sup>cko</sup> sham and TAC mice
The results of these calculations are shown in table 4.2. TAC appeared to have very little effect on the relative contributions of each system to global calcium clearance. In all cell genotypes the vast majority (>90%) of calcium was taken back up into the SR via SERCA. Extrusion from the cell via NCX accounted for 7-8% of the remaining calcium clearance, whilst the combined contributions of PMCA4 and mitochondrial uptake totalled less than 1% in all cell genotypes. The PMCA1 contribution to calcium decay did not differ significantly after TAC. These results confirm that diastolic calcium clearance was unaffected in knockout and control myocytes following one week of pressure overload.

<table>
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</tbody>
</table>

Table 4.2 The percentage contribution of calcium extrusion systems to global calcium clearance in PMCA1<sup>野生</sup> and PMCA1<sup>cko</sup> mice following 1 week TAC.

### 4.3.1.4.5 Expression of calcium clearance systems in PMCA1<sup>cko</sup> mice following 1 week TAC

Data suggested the relative contributions of SERCA and NCX to calcium clearance to be largely unaltered during the early response to pressure overload in either PMCA1<sup>cko</sup> or floxed control mice. However, several rodent models of hypertrophy have noted increased NCX expression (Studer et al., 1997, Zwadlo and Borlak, 2005), whilst reduced SERCA abundance is thought to play a key role during the transition from compensated hypertrophy into cardiac failure (Feldman et al., 1993). Protein levels of these key players in cardiac calcium cycling were therefore examined by western blot in cardiomyocytes isolated from each experimental group 1 week post-surgery (figure 4.12).
TAC induced a 60% increase from baseline NCX expression in PMCA1\textsuperscript{ff} mice, which differed significantly from levels seen in corresponding knockout TAC myocytes (1.61 ± 0.00 vs 0.82 ± 0.15, p<0.05 – figure 4.12B). Conversely, SERCA expression was unaffected 1 week after the onset of pressure overload in either PMCA1\textsuperscript{cko} or control myocytes (figure 4.12C).

Figure 4.12 NCX and SERCA protein abundance in PMCA1\textsuperscript{cko} and control mice following one week of TAC A) Western blots detailing NCX and SERCA expression in myocytes isolated from PMCA1\textsuperscript{ff} and PMCA1\textsuperscript{cko} mice 1 week after the onset of pressure overload. B) Quantification of NCX abundance after normalisation to the loading control β-actin. PMCA1\textsuperscript{ff} TAC mice displayed higher levels of expression compared to sham controls, as well as knockout TAC myocytes († p<0.05) which did not display an increase compared to sham controls. C) Normalised SERCA expression did not differ amongst experimental groups.
4.3.2 The combined effect of PMCA1 and 4 deletion during pressure overload-induced hypertrophy

PMCA1 ablation seemingly prevented the improvement in ventricular performance required to meet the increased demands placed on the heart by aortic constriction despite a comparable extent of growth to controls, and PMCA1\(^{cko}\) hearts subsequently progressed into a decompensated state within two weeks of the onset of pressure overload. PMCA4 disruption on the other hand has produced conflicting results in terms of regulating the response to TAC; pharmacological inhibition attenuated pathological hypertrophy whilst a functional gene knockout displayed a slight increase in heart size (Abou-Leisa, 2013, Wu et al., 2009). In order to further investigate the role of PMCA4 during the development and progression of pressure overload-induced hypertrophy, PMCA1:4\(^{dko}\) mice were also subjected to 2 weeks TAC, to see whether the additional deletion of PMCA4 would either temper or exacerbate the phenotype witnessed in PMCA1\(^{cko}\) mice.

TAC or sham operations were performed on PMCA1:4\(^{dko}\) mice and littermate PMCA1:4\(^{flo\!x}\) controls at 8 weeks of age, as per the experiments in PMCA1\(^{cko}\) mice. After two weeks of pressure overload, \textit{in vivo} cardiac structure, function and electrical activity were examined before subsequent sacrifice and tissue harvesting for morphometric, histological and molecular analysis.
4.3.2.1 The hypertrophic response in PMCA1:4\textsuperscript{dcko} mice following TAC

As was the case in the PMCA1\textsuperscript{cko} TAC experiments, pressure overload induced a comparable increase in maximal left ventricular pressure in PMCA1:4\textsuperscript{flox} and PMCA1:4\textsuperscript{dcko} mice of ~60mmHg and ~55mmHg, respectively as measured by ventricular catheterisation (flox sham vs TAC 100.97 ± 3.96 vs 159.67 ± 5.55 mmHg, p<0.001; dcko sham vs TAC 101.66 ± 3.56 vs 154.34 ± 6.24 mmHg, p<0.001 – figure 4.13).

![Figure 4.13 Maximal left ventricular pressure in PMCA1:4\textsuperscript{dcko} and control mice following two weeks TAC](image)

**Figure 4.13** Maximal left ventricular pressure in PMCA1:4\textsuperscript{dcko} and control mice following two weeks TAC $P_{\text{max}}$ increased similarly in PMCA1:4\textsuperscript{flox} and PMCA1:4\textsuperscript{dcko} mice following two weeks TAC, *** both p<0.001 vs sham operated controls
As demonstrated in figure 4.14A, this resulted in a 34% increase in normalised heart weight in PMCA1:4\(^{\text{flox}}\) controls. Surprisingly, PMCA1:4\(^{\text{dcko}}\) TAC hearts exhibited a lesser rise in heart weight of just 23% compared to sham operated controls, and were significantly smaller than floxed TAC hearts (7.03 ± 0.30 vs 8.50 ± 0.58 mg/mm, p< 0.05). Echocardiographic imaging of the left ventricle confirmed this diminished hypertrophic response, with double knockout TAC hearts undergoing less thickening of the interventricular septum compared to PMCA1:4\(^{\text{flox}}\) TAC controls (1.06 ± 0.03 vs 1.27 ± 0.09 mm, p<0.05 – figure 4.14B).

These results were in contrast to the hypertrophic response witnessed in PMCA1\(^{\text{cko}}\) mice following pressure overload, where heart weight increased similarly to controls and wall thickening was accompanied by chamber dilation. Figure 4.14C indicates that this was not the case in double knockout TAC hearts, whose internal left ventricular diameter in diastole did not differ from either sham or flox TAC controls. Consequently, rather than producing the eccentric pattern of hypertrophy seen in PMCA1 single knockouts, TAC induced an attenuated concentric response upon dual PMCA1 and 4 ablation, reflected by a small rise in relative wall thickness compared to sham controls (0.60 ± 0.01 vs 0.43 ± 0.02, p<0.001), but significantly less than in PMCA1:4\(^{\text{flox}}\) TAC controls (0.70 ± 0.04, p<0.01 – figure 4.14D).
Figure 4.14 The hypertrophic response in PMCA1:4^{dcko} mice following two weeks TAC A) PMCA1:4^{dcko} mice experienced an attenuated increase in heart weight normalised to tibia length († p<0.05) following TAC (TAC 7.03 ± 0.30 vs sham 5.70 ± 0.13 mg/mm, ** p< 0.01) compared to PMCA1:4^{flox} controls (TAC 8.50 ± 0.58 vs sham 6.30 ± 0.37 mg/mm, ** p<0.01) B) TAC induced an increase in diastolic interventricular septal thickness in control and knockout hearts (flox sham vs TAC 0.86 ± 0.04 vs 1.27 ± 0.09 mm, *** p<0.001; dcko sham vs TAC 0.80 ± 0.01 vs 1.06 ± 0.03 mm, *** p<0.01), however to a lesser extent in PMCA1:4^{dcko} mice († p<0.05) C) No signs of left ventricular dilation in flox or dcko mice following TAC. D) PMCA1:4^{flox} TAC hearts experienced a large rise in relative wall thickness compared to sham controls (0.70 ± 0.04 vs 0.47 ± 0.03, *** p<0.001) which was attenuated († p<0.05) but still apparent in PMCA1:4^{dcko} TAC hearts (0.60 ± 0.01 vs 0.43 ± 0.02 sham, *** p<0.001). HW/TL, heart weight/tibia length ratio; d, diastolic; LVD, internal left ventricular diameter; IVS, interventricular septal thickness; RWT, relative wall thickness.
Concentric cardiac remodelling is typically characterised by an increase in cardiomyocyte width, and so H & E stained transverse histological sections were examined to see whether the attenuated hypertrophic response in PMCA1:4\textsuperscript{dcko} mice following TAC translated to a reduction in mean CSA. Representative images of whole heart sections and magnified areas used for measurement are presented in figure 4.15A. Mean CSA was increased by 27% and 17% following TAC in PMCA1:4\textsuperscript{flox} and PMCA1:4\textsuperscript{dcko} mice, respectively (figure 4.15B, both p<0.001). In support of the \textit{in vivo} data, this rise was significantly smaller in the double knockout TAC group (275.43 ± 7.42 vs 299.72 ± 8.00 μm\textsuperscript{2} flox TAC, p<0.05).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.15.png}
\caption{Histological analysis of the hypertrophic response to 2 weeks TAC in PMCA1:4\textsuperscript{dcko} mice A) Representative H&E stained transverse histological sections from which mean myocyte CSA was calculated. Scale bars; low magnification = 2mm, higher magnification = 50μm B) Quantification of mean CSA. PMCA1:4\textsuperscript{dcko} cell size was significantly smaller († p<0.05) following TAC compared to floxed controls (flox sham vs TAC 236.61 ± 6.78 vs 299.72 ± 8.00 μm\textsuperscript{2}, *** p<0.001; dcko sham vs TAC 235.27 ± 4.50 vs 275.43 ± 7.42 μm\textsuperscript{2}, *** p<0.001) }
\end{figure}
4.3.2.2 Cardiac function in PMCA1:4<sup>dcko</sup> mice following TAC

The data presented in section 4.3.2.1 suggested that PMCA4 ablation attenuated the hypertrophic response to TAC, and prevented the development of left ventricular dilation witnessed in PMCA1<sup>cko</sup> mice. PMCA1 deletion had also led to deterioration of ventricular performance upon pressure overload (see section 4.3.1.2), and therefore cardiac contractility and lusitropy were examined in PMCA1:4<sup>dcko</sup> TAC mice to investigate whether PMCA4 deletion was able to impact positively on the cardiac dysfunction seen in PMCA1<sup>cko</sup> mice.

In stark contrast to PMCA1<sup>cko</sup> mice, systolic performance was fully preserved in PMCA1:4<sup>dcko</sup> mice following TAC; mice displayed no signs of deterioration in fractional shortening or a significant reduction in contractility as measured by the maximal rate of pressure change upon left ventricular catheterisation (figures 4.16A & B). Similarly, diastolic performance was not compromised after pressure overload, with no prolongation of the isovolumic relaxation time constant (figure 4.16C). Consequently, unlike in PMCA1 single knockout mice, PMCA1:4<sup>dcko</sup> mice displayed no signs of pulmonary congestion following TAC (figure 4.16D), suggesting that the attenuation in cardiac hypertrophy resulting from the additional deletion of PMCA4 was able to prevent the transition into decompensation.
Figure 4.16 Systolic and diastolic function in PMCA1:4 \textsuperscript{dko} mice following two weeks TAC. No deterioration in systolic performance was witnessed following TAC in PMCA1:4 \textsuperscript{dko} mice as measured by A) fractional shortening or B) maximal rate of pressure change during systole. C) Lusitropic function was not compromised in double knockout mice following pressure overload as measured by the $\tau$. D) Normalised lung weight was not increased significantly in flox or dcko mice following TAC. FS, fractional shortening; dP/dt max, maximum rate of pressure change in left ventricle; Tau, isovolumic relaxation time constant; LW/TL, lung weight tibia/length ratio.
4.3.2.3 The hypertrophic response in αMHC-Cre<sup>tg</sup> control mice following TAC

Cre-recombinase had been shown to not affect cardiac structure or function following TAC in controls bred through the same lineage as PMCA1<sup>cko</sup> mice (see section 4.3.1.3), and to ensure that this was also the case for the PMCA1:4<sup>dcKO</sup> lineage TAC experiments were performed on αMHC-Cre<sup>neg</sup> and αMHC-Cre<sup>tg</sup> controls. The main phenotype witnessed in double knockout mice was that of a reduction in left ventricular hypertrophy 2 weeks post-surgery. Figure 4.17 demonstrates that the hypertrophic response was not affected by the presence of the Cre transgene, as measured by both gross normalised heart weight and echocardiographic estimation of left ventricular mass. Heart weight/tibia length ratio increased by 52% and 56% in αMHC-Cre<sup>neg</sup> and αMHC-Cre<sup>tg</sup> control mice after TAC (both p<0.01 – figure 4.17A), whilst a calculation of the left ventricular mass following echocardiographic measurement of left ventricular wall thickness and chamber size revealed a 38% and 40% rise in the two TAC cohorts after normalisation (both p<0.05 – figure 4.17B). This data suggested that the attenuated hypertrophic response to pressure overload in PMCA1:4<sup>dcKO</sup> mice resulted from PMCA ablation.

**Figure 4.17 The hypertrophic response in αMHC-Cre<sup>neg</sup> and αMHC-Cre<sup>tg</sup> mice following two weeks TAC**

A) Heart weight normalised to tibia length rose similarly in Cre negative and transgenic controls following TAC (αMHC-Cre<sup>neg</sup> sham vs TAC 6.68 ± 0.33 vs 10.15 ± 1.11 mg/mm, ** p<0.01; αMHC-Cre<sup>tg</sup> sham vs TAC 6.08 ± 0.24 vs 9.48 ± 0.64 mg/mm, ** p<0.01) B) Echocardiographic estimation of left ventricular mass also found the hypertrophic response to TAC to be comparable amongst αMHC-Cre<sup>neg</sup> and αMHC-Cre<sup>tg</sup> controls (αMHC-Cre<sup>neg</sup> sham vs TAC 4.18 ± 0.29 vs 5.75 ± 0.46 mg/g, * p<0.01; αMHC-Cre<sup>tg</sup> sham vs TAC 4.30 ± 0.35 vs 6.01 ± 0.44 mg/g, * p<0.01). HW/TL, heart weight/tibia length ratio; LVM, left ventricular mass.
4.3.2.4 ECG analysis in PMCA1:4\textsuperscript{dcko} mice following TAC

Examination of cardiac electrical activity by conscious ECG revealed no significant differences in heart rate, nor PR, QRS or QT intervals, indicating that transmission through the cardiac conduction system was not compromised during pressure overload in either PMCA1:4\textsuperscript{dcko} or control mice (table 4.3).

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<tr>
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<tr>
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<tr>
<td>QT\textsubscript{c} (msec)</td>
<td>67.72 ± 4.89</td>
<td>68.38 ± 6.52</td>
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Table 4.3 Cardiac electrical activity in PMCA1:4\textsuperscript{dcko} mice following 2 weeks TAC HR, heart rate; QT\textsubscript{c}, QT interval corrected for heart rate.
4.3.2.5 Cardiac fibrosis in PMCA1:4<sup>dcko</sup> mice following TAC

Pathological hypertrophy is often associated with increased interstitial fibrosis, which contributes to disease progression by increasing myocardial stiffness. Masson’s trichrome staining of transverse histological sections revealed negligible levels of collagen fibres (stained blue) in sham hearts, whilst TAC induced a significant rise in the fibrotic area in PMCA1:4<sup>flox</sup> mice (6.30 ± 0.55 vs sham 0.89 ± 0.17 %, p<0.001 – figure 4.18A & B). Fibrosis levels were increased to a lesser extent in PMCA ablated TAC hearts (3.31 ± 0.72 vs sham 1.08 ± 0.42 %, p = 0.06), and were significantly lower than in floxed TAC controls (p<0.05).

For further validation of the reduction in cardiac fibrosis in PMCA1:4<sup>dcko</sup> hearts following pressure overload, transcript levels of the COL1A1 and COL3A1 genes encoding precursor components of collagen types I and III, respectively, were examined by qPCR (figure 4.18C & D). Pressure overload led to a 5.9 and 4.6-fold increase in COL1A1 and COL3A1 expression in floxed controls (both p<0.001 vs sham controls), compared to a smaller 3.3 and 3.5-fold upregulation in PMCA1:4<sup>dcko</sup> samples (both p<0.05 vs sham controls). Whilst neither procollagen transcript level was significantly reduced in knockout samples compared to floxed TAC controls, the pattern of expression generally supported the notion of a reduction in interstitial fibrosis garnered from histological staining.
Figure 4.18 Histological and molecular analysis of the fibrotic response to 2 weeks TAC in PMCA1:4\textsuperscript{dcko} mice

A) Representative Masson’s trichrome stained transverse histological sections from which the % interstitial fibrosis was calculated. Collagen fibres (blue), muscle fibres (red). B) Quantification of cardiac fibrosis. PMCA1:4\textsuperscript{dcko} TAC hearts displayed a significant reduction in fibrotic area compared to floxed controls (\textdagger p<0.05). C) \textit{COL1A1} and D) \textit{COL3A1} mRNA expressions were increased after pressure overload in PMCA1:4\textsuperscript{flox} hearts (*** p<0.001), and to a lesser extent in PMCA1:4\textsuperscript{dcko} hearts (\textit{COL1A1}, * p<0.05 vs dcko sham; \textit{COL3A1}, *** p<0.001 vs dcko sham).
4.3.2.6 A preliminary investigation into the mechanism behind the attenuation of pathological remodelling upon PMCA4 deletion

When compared to the phenotype witnessed in PMCA1\textsuperscript{cko} mice following TAC, the data presented in sections 4.3.2.1-5 suggest that PMCA4 ablation was able to ameliorate the induction of pathological left ventricular remodelling during pressure overload through an attenuation of the extent of hypertrophy and fibrosis, with no loss of systolic or diastolic function. By what means this was achieved mechanistically was not clear, however current knowledge of PMCA4 function suggested this would likely be through regulation of a calcium-dependent signalling pathway.

A recent study found PMCA4 gene silencing to inhibit the nuclear translocation, and hence activation, of NF-κB in breast cancer cells (Curry et al., 2012), and it was hypothesised that a similar mechanism in cardiomyocytes may contribute to the diminished hypertrophic response in PMCA1:4\textsuperscript{dcko} mice given the critical role of NF-κB activation during cardiac hypertrophy (Li et al., 2004). In order to test this hypothesis, preliminary experiments were conducted in cultured neonatal rat cardiomyocytes (NRCM) to see whether knockdown of PMCA4 using shRNA was able to prevent the nuclear translocation of NF-κB upon stimulation.
4.3.2.6.1 The effect of PMCA4 silencing on TNFα-induced nuclear translocation and activation of NF-κB in NRCM in vitro

Stimulation via the inflammatory cytokine tumour necrosis factor alpha (TNF-α) is a common pathway for NF-κB activation, and one known to lead to cardiomyocyte hypertrophy (Higuchi et al., 2002). TNF-α was therefore chosen to induce nuclear translocation of NF-κB in NRCM in the presence and absence of PMCA4. NF-κB localisation was examined by immunocytological staining and activity using a luciferase reporter assay under the control of the NF-κB promoter.

Figure 4.19A presents representative merged images taken from control and PMCA4 knockdown NRCM with and without TNF-α stimulation. In the absence of TNF-α, cells transfected with PMCA4 shRNA displayed mainly perinuclear staining for NF-κB (red), whilst control cells exhibited a certain amount of nuclear staining (purple when merged with blue DAPI). Following TNF-α stimulation there appeared to be a greater degree of nuclear NF-κB expression in control cells, whilst PMCA4 knockdown appeared to reduce translocation to a large extent with cells still displaying perinuclear expression.

To further explore the hypothesis that PMCA4 silencing could attenuate the nuclear translocation of NF-κB, the effect of PMCA4 knockdown on NF-κB luciferase activity following stimulation with TNF-α was investigated. Figure 4.19B shows that TNF-α induced a 62% increase in luciferase activity in control cells. In the absence of TNF-α PMCA4 knockdown cells displayed a 75% reduction in luciferase activity compared to controls, and even upon TNF-α stimulation this increased to only 70% of unstimulated controls. Taken together these two preliminary experiments suggest that PMCA4 may be capable of regulating NF-κB activity in cardiomyocytes, and that this could be a promising avenue to explore the mechanisms behind the attenuated hypertrophic response to pressure overload witnessed upon PMCA4 deletion in vivo.
Figure 4.19 The effect of PMCA4 silencing on TNFα-induced nuclear translocation and activation of NF-κB in NRCM  

A) Representative immunocytological images of NRCM infected with control (left) and PMCA4 (right) shRNA, in the presence (bottom) or absence (top) of TNF-α stimulation, stained with anti-NF-κB p65 (red), anti-α-actinin (green) and DAPI (blue). B) NF-κB luciferase activity was reduced to 25% of controls in NRCM infected with PMCA4 shRNA, and remained lower than basal levels in control cells even after TNF-α stimulation. n = 1 independent experiment, with 6 well replicates per group (SEM reflected in the error bars)
4.3.3 The role of the PMCA during exercise-induced hypertrophy

The experiments in sections 4.3.1 and 4.3.2 suggest that PMCA1 and 4 play key, yet completely independent roles in regulating the hypertrophic and functional response to pressure overload. The processes involved in regulating cardiac growth in response to physiological stimuli are thought to be very different from those of a pathological nature however, and therefore the response to chronic exercise was also examined in 8 week old PMCA1:4^dcko and floxed control mice. These experiments were carried out in conjunction with Donna Page and used for the publication of her master’s thesis.

4.3.3.1 Chronic swimming as a model of physiological hypertrophy

Forced swimming for a period of 4 weeks was used as a model of exercise-induced hypertrophy. Days 1-9 comprised of a training schedule, increasing swim time from 2 periods of 10 minutes on day 1 by 10 minutes per day until the full 90 minute period was reached on day 9. This acclimatised swimmers to the protocol and ensured that they would be able to maintain a good level of exercise once the full swim time was reached, whilst also enabling identification of any mice considered weak swimmers, which were moved to the rest group. Thereafter mice were swum for a period of 90 minutes twice a day for 6 days out of 7 for the remainder of the 4 weeks. When compared to a number of different schedules, this time-course has been previously shown to produce the best hypertrophic response (Evangelista et al., 2003). The rest groups were dipped into the water twice a day to account for any stress induced by the introduction of the mice to water.

Cardiac structure and electrical activity were monitored by echocardiography and conscious ECG prior to the commencement of the swim period, following 2 weeks swimming and at the end of the swim period whereupon haemodynamic analysis, sacrifice and tissue harvesting were performed. Figure 4.20 shows how heart rate and left ventricular mass were affected in swim groups over the course of the experiment. After 2 weeks swimming, both PMCA1:4^dcko and control mice experienced a significant drop in resting heart rate compared to their initial rates at the start of the experiment (flox swim 0 vs 2 weeks 669.83 ± 48.85 vs 518.91 ± 34.41 bpm; dcko swim 0 vs 2 weeks 724.40 ± 40.12 vs 448.15 ± 22.44 bpm, both p<0.05 – figure 4.20A). In controls this equated to a
23% reduction, whilst a 38% lowering of resting heart rate was witnessed in PMCA1:4\textsuperscript{dcko} swimmers. There was little change in resting heart rate during the remaining 2 weeks of exercise, with levels reduced by 26 and 32% in PMCA1:4\textsuperscript{flox} and double knockout swimmers respectively compared to initial rates (both p<0.05 – figures 4.20A, C & D). No significant changes were witnessed in resting heart rate in either rest group over the course of the experiment.

Estimation of left ventricular mass by echocardiography over the course of the swimming period revealed no change in PMCA1:4\textsuperscript{flox} swimmers following 2 weeks exercise, and an increase of 17% in PMCA1:4\textsuperscript{dcko} mice although this did not reach significance (figure 4.20B). By the end of the 4 week period both swim groups did exhibit signs of left ventricular hypertrophy however, with LVM rising by 63% and 44% in PMCA1:4\textsuperscript{flox} and double knockout swimmers respectively compared to prior to commencement of exercise (flox swim 0 vs 4 weeks 148.49 ± 28.44 vs 241.82 ± 33.69 mg; dcko swim 0 vs 4 weeks 159.57 ± 14.57 vs 229.35 ± 13.00 mg, both p<0.05). Again, resting mice experienced no change in LVM during the 4 week period. These results suggest that the period of exercise induced a good degree of hypertrophy in swimmers, and produced a physiological response which could be taken forward for analysis.
Figure 4.20 The effects of 4 weeks swimming on resting heart rate and left ventricular mass in PMCA1:4\textsuperscript{dcko} and floxed control mice

A) Resting heart rate as measured by conscious ECG dropped in both swim groups after 2 weeks of exercise, and this reduction was sustained for the remainder of the swim period († p<0.05 paired t-test vs 0 weeks swim) B) Left ventricular mass as measured by echocardiography was increased significantly after 4 weeks of exercise in both swim groups (flox † p<0.05 paired t-test vs 0 weeks swim, dcko ♠ p<0.01 paired t-test vs 0 weeks swim). Representative ECG traces recorded in one PMCA1:4\textsuperscript{dcko} subject C) prior to commencement of and D) following 4 weeks of swimming demonstrating a clear reduction in resting heart rate. n = 3–4 mice per group.
4.3.3.2 The hypertrophic response to chronic swimming in PMCA1:4^{dcko} mice

The data presented above suggested the extent of hypertrophy to be similar amongst knockout and floxed swimmers. To further characterise the nature of the hypertrophic response heart size and cardiac structure were analysed at the end of the swim period. Figure 4.21A demonstrates that exercise induced a similar 30% rise in normalised heart weight in the two swim groups (flox rest vs swim 4.10 ± 0.09 vs 5.35 ± 0.16 mg/g, p<0.05; dcko rest vs swim 4.35 ± 0.07 vs 5.66 ± 0.18 mg/g, p<0.01). This was accompanied by comparable thickening of the interventricular septum (flox rest vs swim 0.84 ± 0.04 vs 1.03 ± 0.05 mm, p<0.05; dcko rest vs swim 0.87 ± 0.02 vs 0.99 ± 0.03 mm, p<0.05 – figure 4.21B) and a small increase in left ventricular chamber size of 8% and 13% in PMCA1:4^{flox} and PMCA1:4^{dcko} (p<0.05 vs rest) swim groups, respectively (figure 4.21C). This led to no overall change in relative wall thickness in either swim group (figure 4.21D), suggesting that chronic exercise induced a classical eccentric pattern of hypertrophy which did not differ upon PMCA ablation.
Figure 4.21 The hypertrophic response in PMCA1:4 dcko mice following 4 weeks swimming

A) Both PMCA1:4 dcko (** p<0.01) and floxed control (* p<0.05) mice experienced a 30% increase in heart weight normalised to body weight following 4 weeks of exercise compared to their respective rested controls

B) Swimming induced a similar increase in diastolic interventricular septal thickness in control and knockout hearts (* both p<0.05 vs rest)

C) A small increase in diastolic left ventricular diameter was witnessed in both flox (4.99 ± 0.16 vs 4.64 ± 0.05) and dcko (5.05 ± 0.10 vs 4.47 ± 0.23, * p<0.05) swimmers compared to rested controls.

D) Relative wall thickness did not change after exercise in either swim group. HW/BW, heart weight/body weight ratio; d, diastolic; LVD, internal left ventricular diameter; IVS, interventricular septal thickness; RWT, relative wall thickness.
4.3.3.3 Cardiac function following chronic swimming in PMCA1:4\textsuperscript{dcko} mice

Physiological hypertrophy is typically associated with preserved or even heightened left ventricular function. To verify that PMCA ablation did not alter this, systolic and diastolic performance were examined by echocardiography and left ventricular catheterisation. Exercise did not lead to any significant changes in resting cardiac contractility as measured by fractional shortening or the maximum rate of pressure change in the left ventricle in either PMCA1:4\textsuperscript{dcko} or floxed control mice (figures 4.22A &B), nor lusitropy as measured by the time constant of relaxation and minimum rate of pressure change (figures 4.22C &D). These results confirm that PMCA deletion had no effect on resting cardiac performance following chronic exercise.

![Graphs showing systolic and diastolic function in PMCA1:4\textsuperscript{dcko} mice following 4 weeks swimming](image)

**Figure 4.22** Systolic and diastolic function in PMCA1:4\textsuperscript{dcko} mice following 4 weeks swimming Resting systolic performance was not altered following 4 weeks of exercise in either PMCA1:4\textsuperscript{dcko} mice or floxed controls as measured by A) fractional shortening or B) maximal rate of pressure change during systole. Lusitropic function was also unaffected in double knockout mice following the swim period as measured by C) the τ or D) minimum rate of pressure change during diastole. FS, fractional shortening; dP/dt\textsubscript{max/min}, maximum/minimum rate of pressure change in left ventricle; Tau, isovolumic relaxation time constant.
4.3.3.4 ECG analysis following chronic swimming in PMCA1:4\textsuperscript{dcko} mice

Athletic training is commonly associated with a lowering of resting heart rate, and it appeared that this was indeed the case in swim groups as early as 2 weeks after commencement of exercise (see section 4.3.3.1). To assess whether electrical activity was altered in any other way in PMCA1:4\textsuperscript{dcko} mice following 4 weeks exercise, ECG traces were examined and interval times recorded (table 4.4). Resting heart rate was significantly lower in both swim groups compared to their respective sedentary controls (flox swim vs rest 497.97 ± 18.55 vs 734.73 ± 47.17 bpm, p<0.01; dcko swim vs rest 494.77 ± 18.54 vs 771.77 ± 21.25 bpm, p<0.001). Consequently RR intervals were prolonged in swim groups (both p<0.01), however, no significant differences were noted in any other interval times following exercise in either swim group. Taken together with the results described in sections 4.3.3.2 & 4.3.3.3, this suggests that neither isoform 1 or 4 of the PMCA regulate the hypertrophic response during physiological hypertrophy.

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<tr>
<td>QRS (msec)</td>
<td>11.12 ± 0.79</td>
<td>11.81 ± 0.48</td>
</tr>
<tr>
<td>QT (msec)</td>
<td>18.07 ± 1.70</td>
<td>18.09 ± 1.60</td>
</tr>
<tr>
<td>QT\textsubscript{c} (msec)</td>
<td>62.82 ± 6.01</td>
<td>61.81 ± 3.38</td>
</tr>
</tbody>
</table>

Table 4.4 Cardiac electrical activity in PMCA1:4\textsuperscript{dcko} mice following 4 weeks swimming
Swimming led to a reduction in resting heart rate and prolonged RR interval in both flox (** p<0.01) and dcko mice (*** p<0.001). HR, heart rate; QT\textsubscript{c}, QT interval corrected for heart rate.
4.4 Discussion

It is believed that adapting to the demands imposed by pressure or volume overload requires a carefully coordinated growth response in the myocardium accompanied by crucial alterations in calcium handling in order to maintain adequate cardiac output. As well as being central to the improvement in contractility through excitation-contraction coupling, calcium also plays a key role in the regulation of signalling pathways which dictate the growth response. Here we show that manipulation of the PMCAs profoundly influences the hypertrophic and functional response to pressure overload, with PMCA1 ablation preventing necessary improvements in inotropy, whilst the additional silencing of PMCA4 attenuated pathological growth and remodelling. In contrast, PMCA ablation did not affect the development of hypertrophy in response to endurance training. This discussion will explore the possible mechanisms behind these surprising results, and what they may indicate of the role of PMCA1 and 4 in cardiomyocytes.

4.4.1 Pressure overload induces acute decompensation in PMCA1 ablated hearts

Despite experiencing a comparable increase in cardiac mass following 2 weeks TAC, PMCA1cko mice developed acute dilated cardiomyopathy whilst controls remained in the compensated state. Analysis of intracellular calcium handling suggested that this was preceded by a failure to initiate adaptive changes designed to enhance SR calcium cycling during the compensatory stages of left ventricular hypertrophy.

4.4.1.1 TAC exerted a similar increase in workload in PMCA1cko and floxed control mice

One of the advantages of the TAC model of pressure overload is that by controlling the degree of constriction, the workload exerted on the ventricle should be fairly constant amongst subjects. To give an indication that this was the case, the maximal pressure during systole ($P_{max}$) was measured by left ventricular catheterisation prior to termination, and no significant difference between the two TAC cohorts was found. $P_{max}$ should be approximately equal to the maximal systolic pressure in the aorta proximal to the constriction, and therefore this suggests that each group was subjected to a comparable
increase in workload. Hence any subsequent phenotype witnessed in PMCA1cko mice can be assumed to not be influenced by experimental differences.

4.4.1.2 PMCA1 ablation does not affect the extent of hypertrophic growth following TAC

The onset of left ventricular hypertrophy typically begins in the first 3 days following TAC, and has reached its full extent by day 10 (Nakamura et al., 2001). Following 14 days of pressure overload, PMCA1cko and floxed control mice both exhibited signs of left ventricular hypertrophy as indicated by an increase in normalised heart weight, wall thickness and mean myocyte cross sectional area. However, no significant difference was noted amongst the two TAC groups in any of these parameters indicating that the extent of hypertrophy was not affected by PMCA1 deletion. Importantly, this would suggest that PMCA1, at least in its absence, does not regulate calcineurin-NFAT signalling.

4.4.1.3 PMCA1cko mice show signs of left ventricular dilation and decompensation following pressure overload

Subsequent to the development of concentric left ventricular hypertrophy in the week or so immediately following TAC, a stable period of compensation will typically ensue before a progression to decompensation approximately 4-6 weeks after surgery (Esposito et al., 2002, Ling et al., 2009, Toischer et al., 2013). 2 weeks after TAC PMCA1Cre mice, as one would expect, displayed a classical pattern of concentric hypertrophy as evidenced by an increase in relative wall thickness, and displayed no signs of systolic or diastolic dysfunction indicating that they were indeed in the compensated state. In stark contrast, PMCA1cko mice exhibited signs of left ventricular dilation, impaired systolic function and pulmonary congestion typical of the progression into decompensation and failure. Importantly, these signs were absent in αMHC-CreTAC controls indicating that they resulted from PMCA1 ablation. Haemodynamic analysis revealed that contractility improved in control hearts following TAC as evidenced by an increase in left ventricular elastance (Ees), which ensured that

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efficient arterial-ventricular coupling ($E_a/E_{es}$) was maintained in the face of increased arterial load, a pattern typical to that seen clinically in hypertensive patients (Chantler et al., 2008). Interestingly, this increase in contractility was not reciprocated in PMCA1$^{cko}$ mice, and consequently the rise in arterial elastance ($E_a$) induced by TAC caused a mismatch in the interaction between ventricle and aorta as evidenced by an elevated $E_a/E_{es}$ ratio, characteristic of systolic heart failure (Chantler et al., 2008). Alterations in arterial-ventricular coupling affect cardiac energetics, requiring higher myocardial oxygen consumption for a given stroke work, and $E_a/E_{es}$ has been found to correlate strongly with both prognosis and severity in heart failure patients (Ky et al., 2013). It is of note that the optimal range for resting $E_a/E_{es}$ of 0.6-1.2 is conserved amongst species (Chantler et al., 2008), and therefore it might be possible to draw comparison between human and mouse for this parameter. Strikingly, whilst both sham and TAC controls fell within the optimal range, the value of 3.3 witnessed in PMCA1$^{cko}$ mice would place them in the range of NYHA class IV heart failure patients as measured by Ky et al., indicating the severity of the phenotype.

Although the phenotype observed in PMCA1$^{cko}$ mice was primarily one of systolic heart failure, differences were also observed in diastolic function. After TAC PMCA1$^{flo}$ mice exhibited an increased rate of pressure change during diastole indicative of enhanced lusitropy which, similar to the improved contractility was not witnessed in PMCA1$^{cko}$ mice. This resulted in a slight prolongation of the time constant of relaxation in knockouts compared to controls. An improvement in lusitropic function during the compensated stage of hypertrophy is consistent with the findings of a number of other studies (Ohkusa et al., 1997, Berni et al., 2009, Li et al., 2009), and these results again demonstrate a lack of adaptation to pressure overload in PMCA1$^{cko}$ mice.
4.4.1.4 Calcium handling following pressure overload

The \textit{in vivo} data indicated that after two weeks of pressure overload PMCA1\textsuperscript{cko} hearts developed severe systolic dysfunction with associated left ventricular dilation and some impairment in relaxation, in contrast to controls which remained in the expected compensated state. This was surprising given the similar increase in cardiac mass and ventricular pressure between the two groups. Haemodynamic analysis suggested that mechanisms were activated in control mice following TAC which led to enhanced contractility and lusitropy, but not in knockouts. Considering that each of these processes is essentially governed by cytosolic calcium during EC coupling, together with the fact that PMCA1 is a calcium pump, the most logical hypothesis as to the mechanism behind the dysfunction was one of altered calcium handling. However, if one were to examine calcium handling in PMCA1\textsuperscript{cko} mice after 2 weeks of TAC once they had already progressed into decompensation little mechanistic insight into its development would be gained. Therefore, an intermediate time point of 1 week post-TAC was chosen, when physical signs of heart failure had not manifested but hypertrophy should already be established (Nakamura et al., 2001).

4.4.1.4.1 PMCA1\textsuperscript{cko} mice do not exhibit adaptive changes in EC coupling during systole following TAC

In accordance with the literature regarding calcium handling in mice following 7 days of TAC, PMCA1\textsuperscript{ff} mice demonstrated an increase in peak intracellular calcium during systole (Toischer et al., 2010), which assuming that calcium handling remains fairly constant during compensated hypertrophy would correlate with the increase in contractility witnessed \textit{in vivo} at 2 weeks. Interestingly, PMCA1\textsuperscript{cko} myocytes did not exhibit the same elevation in systolic calcium following TAC as was witnessed in controls, which may provide an explanation as to why contractility did not improve in these mice. Indeed, reports of increases in systolic transient amplitude during compensated hypertrophy are consistent amongst both transverse and abdominal aortic constriction models of pressure overload, as well as hypertensive hypertrophic models such as the Dahl salt sensitive and spontaneously hypertensive rat suggesting this to be an important adaptation (Toischer et al., 2010, Sumida et al., 1998, Nagata et al., 1998, Fowler et al., 2005).
From experiments in the pressure overloaded rat heart, it appears that elevations in systolic transient amplitude occur primarily through increased SR fractional release, possibly in combination with an increase in load (Carvalho et al., 2006, Ohkusa et al., 1997, Toischer et al., 2010). Compatible with the reports of Carvalho et al in the rat 7 days post-TAC, PMCA1<sup>+/+</sup> mice exhibited an increase in amplitude of the caffeine-evoked transient which would be consistent with greater SR loading, whilst the amplitude in knockout cells again remained unchanged. However, caffeine-evoked transients following removal of extracellular sodium and calcium ions appeared to be of a similar magnitude in wild type sham and TAC myocytes, which would be consistent with the findings of Toischer et al. where no change in SR content was noted in mice following 7 days of TAC (Toischer et al., 2010). Therefore further investigation into SR load would be warranted before drawing a firm conclusion on this matter. One possible explanation for the discrepancy in the present study could be the saturation of the signal at higher calcium concentrations under 0Na<sup>+</sup>/0Ca<sup>2+</sup> conditions. It should be noted that the simple application of caffeine only provides an indication as to the SR content, whereas a more accurate estimation can be derived from the additional assessment of the integral of the inward current via NCX (Varro et al., 1993).

4.4.1.4.2 Diastolic calcium clearance is not altered following TAC

In contrast to the adaptations in calcium handling witnessed during systole following pressure overload, surgery did not have a significant effect on calcium decay rates in either control or knockout myocytes. This is in agreement with the literature, where global calcium decay rates have been reported to be unaltered in various models of compensated hypertrophy (Carvalho et al., 2006, Chorvatova et al., 2004, Nagata et al., 1998, Berni et al., 2009). This would suggest that diastolic function was not compromised in PMCA1<sup>cKO</sup> mice 1 week after TAC, and that the progression into decompensation primarily resulted from systolic dysfunction.

At first glance, it would appear that an unchanged rate of calcium decay in PMCA1<sup>+/+</sup> mice following TAC would conflict with the <i>in vivo</i> data (again assuming that the phenotype remained constant from days 8 to 14), which found enhanced lusitropic function as evidenced by an increase in dP/dt<sub>min</sub>. A similar situation has been reported following abdominal aortic banding in the rat, where the authors hypothesised that altered calcium-
myofilament interactions may be accountable (Berni et al., 2009). However, a simpler explanation for the discrepancy here may be that whereas calcium decay was assessed by the \( \tau \), which is derived from decay throughout the exponential period, \( \frac{dP}{dt_{min}} \) is a measure of the maximal rate of pressure change during diastole. Given that exponential decay dictates that calcium will decrease at a rate proportional to its current value, the higher amplitude in control TAC myocytes will lead to a faster initial rate of decay which could therefore generate a greater maximal force of relaxation.

4.4.1.4.3 Relative contributions to calcium clearance are not altered following TAC

Similar to the decay rate of systolic transients, clearance of caffeine-evoked transients in the presence or absence of extracellular sodium was also not affected significantly in hypertrophic myocytes. From the rate constants of exponential decay in control and knockout myocytes, the relative contributions of SERCA, NCX, PMCA1 and PMCA4/mitochondrial uptake to global calcium clearance in sham and TAC mice were estimated. The results indicated that the relative contribution of each system was not altered following pressure overload, which with regards to SERCA and NCX is in accordance with previous reports (Carvalho et al., 2006). Importantly, these results disproved the hypothesis that PMCA1 took on a greater role in calcium clearance following TAC.

4.4.1.4.4 TAC-induced upregulation of NCX expression in control but not PMCA1\(^{cko}\) myocytes

Reports of increased NCX expression during hypertrophy are fairly widespread, and have been shown to occur within the first few hours of the onset of pressure overload (Studer et al., 1997, Chorvatova et al., 2004, Kent et al., 1993). However, given that the relative contribution of NCX to calcium extrusion was not altered following pressure overload, it was still somewhat surprising to find evidence of its upregulation by western blot in PMCA1\(^{fr}\) TAC myocytes. Moreover, similar to the increase in systolic calcium and \textit{in vivo} inotropic function, this did not appear to be reciprocated PMCA1\(^{cko}\) myocytes following TAC. This raises the question as to whether there could be a link between these phenomena.
Contrary to the effects of raised NCX levels in heart failure, when SERCA expression is concomitantly reduced and it is believed that this results in depletion of SR stores and hence lesser calcium release during systole (Houser et al., 2000), an increased NCX current during compensated hypertrophy has been shown to correlate with increases in both SR load and systolic transient amplitude (Chorvatova et al., 2004). Furthermore, overexpression of the exchanger in the mouse myocardium was found to lead to no worse outcome following long term TAC when compared to wild type controls, and even corrected for reductions in systolic calcium and myocyte shortening (Wang et al., 2013).

Chorvatova et al. hypothesised that their observations could be explained by an increase in calcium entry via NCX operating in the reverse mode, which becomes activated following a rise in intracellular sodium (Chorvatova et al., 2004). Authors have demonstrated that calcium influx through the exchanger is capable of triggering calcium release from the SR in much the same way as the LTCC (Vites and Wasserstrom, 1996), and NCX overexpressing cells can maintain adequate CICR for contractions upon electrical stimulation when the LTCC is blocked with nifedipine (Yao et al., 1998). Moreover, in a canine model of hypertrophy, calcium influx via reverse NCX and not LTCC has been found to generate an increase in cell shortening, peak calcium and SR content (Sipido et al., 2002).

One might hypothesise therefore, that NCX upregulation is a necessary adaptation during the early stages of hypertrophy, required to enhance SR calcium cycling, release and ultimately contractility, and that the failure to initiate this pathway in PMCA1$^{cko}$ TAC myocytes during the present study resulted in the early transition into systolic dysfunction and decompensation.
4.4.1.4.5 The potential role of PMCA1 in the transition to decompensation

The data discussed in this chapter suggests that PMCA1 ablation results in systolic dysfunction due to a failure to increase calcium cycling and consequently contractility in response to the greater demand placed on the heart during pressure overload. However, the question as to how the loss of a diastolic calcium extrusion pump which removes only a fraction of cytosolic calcium during normal EC coupling may cause this remains unanswered. The finding that the relative contribution of the ‘slow’ systems to calcium extrusion did not increase following TAC ruled out the hypothesis that PMCA1 may take on a greater role in this process under stress conditions. Instead it may suggest that, like PMCA4, in the heart isoform 1 has a role to play in the regulation of calcium-dependent signalling.

First, let us consider the hypothesis put forth in the preceding section with regards to NCX expression. Whereas there is strong evidence that PMCA4 resides, at least to some extent, in caveolae at the sarcolemma (Hammes et al., 1998), the subcellular localisation of PMCA1 has thus far remained elusive. However, recent work in detubulated myocytes has found carboxy eosin-sensitive (and hence PMCA-mediated) calcium extrusion to be completely abolished under these conditions, suggesting this function to only be performed at the T-tubule (Chase and Orchard, 2011). Given the results of the present study which found PMCA-mediated calcium efflux to occur almost exclusively via PMCA1, this would be compatible with PMCA1 being localised primarily at the T-tubule. Similarly, a large proportion (over 60%) of NCX-mediated efflux has also been found to occur at the T-tubule suggesting that the two proteins are localised within the same subcellular domain (Despa et al., 2003). This introduces the possibility that PMCA1 could potentially modulate NCX expression through local signalling in this region, for example through the regulation of calcium-dependent transcriptional pathways via CaMKII or HDACs (Menick et al., 2013), and that PMCA1 ablation prevents initiation of the signal to induce NCX transcription at the onset of hypertrophy. It is important to note of course that upregulation of NCX is thought to contribute to the pathological progression of disease in the long term, not only through the depletion of intracellular stores in heart failure, but also due to calcium entry via reverse NCX leaving cells more susceptible to arrhythmia (Reuter et al.,
2005, Sipido et al., 2002). Therefore gaining further insight into its regulation would be highly interesting as a strategy to combat these phenomena.

Of course this is just one possible mechanism through which PMCA1 silencing may influence the progression into decompensation. Before the negative effects of receptor desensitisation and downregulation take effect, increased levels of circulating catecholamines following pressure overload may initially lead to the enhancement of inotropy and lusitropy via activation of PKA and its substrates at the SR and plasma membrane (Barki-Harrington et al., 2004). This could play a role in increasing systolic calcium as was witnessed in PMCA1^{−/−} myocytes, and whilst PMCA1 is not currently known to regulate sympathetic signalling, a blunted response to β-adrenergic stimulation has been witnessed in transgenic and knockout PMCA4 mice (Mohamed et al., 2009, Mohamed et al., 2011).

A further contributing factor to the progression to failure in PMCA1^{cko} TAC hearts could arise from the onset of dilation. According to the Laplace relationship, an increase in chamber size without proportional thickening of the wall leads to increased wall stress, which prompts further induction of pathological stimuli eventually resulting in cell death and replacement with fibrous tissue (Diwan and Dorn, 2007). However, it seems unlikely that this would be the initial trigger for the progression into decompensation, and rather it would just accelerate adverse remodelling once it had begun.

Regardless of the mechanism, the results clearly suggest that the loss of PMCA1 may increase the susceptibility to HF under the conditions of pressure overload. This could be of the utmost importance given the associations between mutations affecting ATP2B1 and hypertension (Hirawa et al., 2013), and may provide a genetic link as to how lifetime risk for the development of heart failure increases two fold in patients with systolic blood pressure >160mmHg compared to normotensives (Go et al., 2013).
4.4.2 PMCA4 ablation attenuates the hypertrophic response to pressure overload and prevents cardiac dysfunction in PMCA1:4<sup>dcko</sup> hearts

The ability of PMCA4 to regulate pathological hypertrophic growth <i>in vivo</i> has begun to be documented in recent years; however there is some controversy as to whether its silencing has a positive or negative effect on outcome (Abou-Leisa, 2013, Wu et al., 2009). The data presented in the current study indicates that even in combination with PMCA1 deletion, pathological remodelling was attenuated following 2 weeks of TAC in hearts lacking PMCA4. Surprisingly, the deleterious effects of PMCA1 ablation on systolic function witnessed in single knockout mice (discussed in the previous section) were not apparent in PMCA1:4<sup>dcko</sup> mice following pressure overload, suggesting that upon the additional ablation of PMCA4, this issue was somehow circumvented.

4.4.2.1 PMCA1:4<sup>dcko</sup> mice display an attenuated response to pressure-overload induced hypertrophy

As was the case in PMCA1<sup>cko</sup> mice, TAC induced a comparable rise in P<sub>max</sub> of 55-60 mmHg in control and double knockout hearts compared to sham operated controls suggesting the increase in workload to be similar between the two groups. Importantly, this was also equivalent to the increase witnessed in PMCA1<sup>cko</sup> and control mice and thus it could be considered reasonable to compare the results from the two datasets.

In contrast to the findings in PMCA1 ablated hearts, the extent of hypertrophy was significantly diminished in PMCA1:4<sup>dcko</sup> mice compared to controls following 2 weeks of pressure overload, as evidenced by a reduction in normalised heart weight, wall thickness and myocyte cross-sectional area. The presence of a normal hypertrophic response in αMHC-Cre<sup>tg</sup> controls discounted any influence from the Cre-transgene. Assuming that the effect of PMCA1 deletion remained constant between single and double knockouts (ie. it does not regulate the growth response to pressure overload), this would suggest that PMCA4 ablation accounted for the attenuation in hypertrophy.
As stated in the previous section, this would not be the first report of PMCA4’s involvement in the regulation of the hypertrophic response to pathological stimuli. More than a decade ago ventricular myocytes isolated from PMCA4 overexpressing rats were found to have increased rates of protein synthesis upon catecholamine stimulation in culture, which would be sympathetic to a pro-hypertrophic response (Hammes et al., 1998). In contrast, when challenged with ET-1 infusion, intact perfused rat hearts from the same line demonstrated attenuation of hypertrophic signalling (Piuhola et al., 2001). Presumably differences in the experimental model, agonists and readouts used during these studies may account for the conflicting results, however what they do demonstrate is a potential for PMCA4 to regulate hypertrophic signalling.

Consistent with the reports of Hammes et al., transgenic mice overexpressing PMCA4 were later found to exhibit a greater hypertrophic response to chronic β-adrenergic stimulation \textit{in vivo} (Oceandy et al., 2007), whilst more recently our group has shown specific pharmacological inhibition of PMCA4 to impede the induction and progression of pressure overload induced hypertrophy (Abou-Leisa, 2013). From the results of these two studies it would appear reasonable to deduce the general concept that PMCA4 positively regulates hypertrophy whilst PMCA4 ablation or inhibition attenuates it, and the findings in PMCA1:4\textsuperscript{dcko} mice would certainly corroborate the second part of this.

However, this concept is in conflict with the results of a study conducted by Molkentin and colleagues in which PMCA4 overexpressing mice demonstrated a blunted hypertrophic response to a variety of pathological stimuli including pressure overload, whilst inactivating PMCA4 using a gene targeting approach led to a very small, but significant rise in normalised heart weight following 2 weeks of TAC with evidence of decompensation at 4 weeks (Wu et al., 2009). Indeed, the authors presented compelling evidence that PMCA4 overexpression negatively regulates calcineurin-NFAT signalling \textit{in vivo} and \textit{in vitro} and that this attenuates hypertrophic growth. This does not necessarily contradict the findings of the present study, however, as knockout and overexpression models should not automatically be considered as opposites. PMCA4 interacts physically with the catalytic domain of calcineurin to inhibit its activity through lowering the calcium concentration in its microdomain (Buch et al., 2005); hence more PMCA4, more
recruitment of calcineurin to the sarcolemmal microdomain, more inhibition of calcineurin activity. In contrast, when PMCA4 is knocked out, rather than serving to unduly activate calcineurin, there simply won’t be an interaction between the molecules.

However, Wu et al. also found PMCA4 null mutants to have an increased hypertrophic response to TAC, which on the face of it would appear to be in direct contradiction with the findings presented here. This discrepancy may be due to differences in the gene targeting approaches used to generate the two models. Whereas in PMCA1:4\textsuperscript{dcko} mice the ATG start codon located in exon 2 of the PMCA4 allele was targeted and thus transcription prevented, the model developed by Shull and colleagues utilised in Molkentin’s study targets only amino acids 448-474 in exon 11 which contain the catalytic phosphorylation site of PMCA4 (Okunade et al., 2004). As a consequence, it is possible that protein is still expressed (indeed the authors do not assess PMCA4 protein expression in their knockout) and that this is just a functional knockout. If so then the calcineurin interaction domain which has been mapped to amino acids 501-575 of PMCA4 (Buch et al., 2005) would likely be unaffected. This would then serve to attract calcineurin to an inactive calcium pump in a region where calcium levels are presumably high due to its accumulation, hence positively regulating calcineurin signalling.

### 4.4.2.2 Cardiac function is preserved in PMCA1:4\textsuperscript{dcko} mice following TAC

Having witnessed a progression into acute decompensation in PMCA1\textsuperscript{cko} myocytes following 2 weeks of pressure overload, it was surprising to find that cardiac function appeared in no way compromised in PMCA1:4\textsuperscript{dcko} mice following TAC. Whereas PMCA1 ablated hearts were dilated and hypotonicile, internal ventricular diameter in double knockouts remained similar to sham and TAC controls and contractility was preserved. As a consequence, pulmonary congestion which was elevated in PMCA1\textsuperscript{cko} mice was not evident in the double knockout.

So, again making the supposition that PMCA1 ablation serves the same function in PMCA1:4\textsuperscript{dcko} and PMCA1\textsuperscript{cko} mice following TAC, how might the additional deletion of
PMCA4 from the myocardium rescue the failing phenotype? In order to answer this question we must first consider what instigated the progression into decompensation in PMCA1\(^{cko}\) mice. The data indicate that this was primarily caused by two factors – a failure to increase calcium cycling and hence contractility, in addition to accelerated adverse remodelling.

Since the increase in workload induced by pressure overload was similar in the two experiments, this would necessitate that in order to maintain contractility in PMCA1:4\(^{dcko}\) mice the deficit in calcium cycling would need to be corrected. Given that the deletion of PMCA4 has been shown to increase the amplitude of the systolic transient as well as basal contractility \textit{in vivo} (Mohamed et al., 2011), it is quite plausible that the additional ablation of PMCA4 in double knockout mice could achieve this. Indeed, experiments in the previous chapter demonstrated increased levels of peak intracellular calcium during systole under basal conditions in PMCA1:4\(^{dcko}\) myocytes. However, due to time constraints an assessment of calcium handling in double knockouts following TAC has yet to be performed. In contradiction to the theory outlined above is the finding that contractility as assessed by dP/dt\(_{\text{max}}\) was unchanged in pressure overloaded PMCA1:4\(^{dcko}\) mice. However, this was also the case in floxed controls, contrary to what one would expect during compensated hypertrophy (Ohkusa et al., 1997, Berni et al., 2009, Li et al., 2009), and further assessment of \textit{in vivo} contractility would be warranted before drawing any conclusions on this matter. As an index of contractility the ESPVR and its slope E\(_{\text{es}}\) (as was found to be elevated in PMCA1\(^{f/f}\) mice) are considered to provide a more accurate assessment due to the dependence of dP/dt\(_{\text{max}}\) on load (Burkhoff, 2013), and indeed studies have shown that E\(_{\text{es}}\) can correlate with left ventricular function when dP/dt\(_{\text{max}}\) does not (Sagawa et al., 1977). Unfortunately analysis of these parameters in PMCA1:4\(^{dcko}\) and control mice during this experiment could not be performed due to technical issues during their collection.

The second contributing factor to the adverse response to pressure overload in PMCA1\(^{cko}\) mice was one of left ventricular dilation, well known to be associated with myocyte death and myocardial fibrosis (Diwan and Dorn, 2007). Upregulation of these two processes correlates significantly with a decline in systolic function in the pressure overloaded heart.
(Hein et al., 2003), and their inhibition can substantially impede disease progression (Wencker et al., 2003, Matsusaka et al., 2006). In PMCA1:4^dcko hearts, compared to controls not only was the extent of hypertrophy reduced after TAC, but also the onset of dilation was prevented and interstitial fibrosis was less evident. This suggests that in attenuating pathological growth, PMCA4 ablation also tempered any adverse remodelling which may have ensued following PMCA1 deletion, and contradicts the view that cardiac hypertrophy during pressure overload is a necessary adaptation. Evidence from studies which have directly targeted hypertrophic signalling pathways would support the notion that the growth response at the initial onset of insult is essentially redundant in preserving function, whereas its blockade actually improves the long term outcome (Hill et al., 2000, Esposito et al., 2002). Therefore, one might consider from the data presented here that even early compensated hypertrophy is maladaptive, and rather that reactive calcium cycling is the essential response to the increased demands of pressure overload.

4.4.2.3 Possible mechanisms through which PMCA4 ablation attenuates pressure overload induced hypertrophy

Despite conflicting reports from elsewhere, the results presented in this chapter add to the growing body of evidence from within our group that silencing of PMCA4 in the heart may be able to reduce pathological hypertrophic growth, whilst not compromising and perhaps even improving cardiac function. The mechanism through which this is achieved remains unclear; however knowledge of PMCA4’s function in the resting heart and other cell types would suggest that it would likely be through a signalling or structural role.

The simplest hypotheses would involve the regulation of hypertrophic signalling via an already established interaction partner such as calcineurin or RASSF1A (Buch et al., 2005, Armesilla et al., 2004), and in the case of RASSF1A the relief of its inhibition theoretically could reduce hypertrophy through downstream regulation of ERK1/2 activation (Oceandy et al., 2009). A further possibility would be through PMCA4’s interaction with nNOS and subsequent regulation of NO signalling (Schuh et al., 2001), already well known to modulate contractility in the heart (Oceandy et al., 2007, Mohamed et al., 2009, Mohamed et al., 2011). nNOS demonstrates rapid upregulation in response to pressure overload.
(Loyer et al., 2007), and data from nNOS overexpressing and knockout mice has shown its ability to modulate the hypertrophic response (Loyer et al., 2008, Niu et al., 2012), yet neither model served to attenuate hypertrophy making this an unlikely mechanism in the current study.

In terms of the current study, mechanistically each of these pathways shares a common problem – their regulation by PMCA4 has been demonstrated when the molecule is overexpressed. In light of this, a recent report identifying PMCA4 silencing to prevent NF-κB nuclear translocation in a breast cancer cell line appeared to present a more attractive hypothesis (Curry et al., 2012). The inhibition of NF-κB has been demonstrated to ameliorate the response to pressure overload by a number of authors through reducing hypertrophic growth and fibrosis, whilst preserving systolic function and preventing lung congestion (Li et al., 2004, Liu et al., 2012, Tanaka et al., 2012). Therefore, not only would this mechanism have the benefit of being dependent on PMCA4 ablation, but the phenotype in PMCA1:4<sup>dcko</sup> hearts following TAC was also comparable to that after inhibition of NF-κB. In order to test this hypothesis preliminary experiments are underway to assess whether similar prevention of NF-κB nuclear translocation and activation can occur upon PMCA4 silencing in cardiomyocytes and show early promise.

4.4.3 The PMCA does not regulate hypertrophy induced by chronic exercise

The data discussed thus far in this chapter indicate that both isoforms 1 and 4 of the PMCA may play a significant role in determining the progression of adverse remodelling during the onset of pathological hypertrophy induced by pressure overload. However, the growth response to physiological stimuli such as endurance training is known to occur through very different pathways and thus the role of the PMCA during exercise induced hypertrophy was also examined.

PMCA1:4<sup>dcko</sup> and floxed control mice underwent a course of swimming for a period of 4 weeks to induce hypertrophy. Assessment of heart rate and left ventricular mass at 2 weeks revealed significant bradycardia in both groups compared to rested controls but little
increase in mass, however by the end of the swim period left ventricular hypertrophy was evident. The extent of hypertrophy did not differ between the two swim groups as evidenced by a comparable rise in normalised heart weight and wall thickness. A small increase in chamber size was also witnessed in swimmers and relative wall thickness was unchanged demonstrating an eccentric pattern of growth typical of volume overload induced by exercise (Bernardo et al., 2010). Moreover, consistent with previous reports in trained rats inotropic and lusitropic function was not altered in either swim group (Stones et al., 2008), whilst resting heart rate was similarly reduced compared to sedentary controls independent of genotype.

These results indicate that the concomitant deletion of both isoforms of the PMCA does not affect physiological hypertrophic growth or function. Given that a previous study has shown the extent of hypertrophy after swimming to be unaffected by either PMCA4 overexpression or gene targeting (Wu et al., 2009) this would suggest neither PMCA1 nor PMCA4 regulates exercise induced hypertrophy.

4.4.4 Study limitations

This study aimed to investigate the roles of PMCA1 and 4 during the development of pressure overload and exercise induced hypertrophy. The TAC model of pressure overload was selected as it induces a reliable and reproducible pattern of pathological hypertrophic growth amongst subjects providing the workload they are subjected to is comparable. As such it is important to assess the pressure gradient in all TAC mice to ensure that this is the case. Typically the pressure gradient is measured across the constriction via Doppler ultrasound, however in this study the maximal ventricular pressure during systole was used as an approximation of the aortic pressure for simplicity’s sake.

Also, a number of assumptions were made when interpreting the data in this study. In the PMCA1<sup>cko</sup> TAC experiments, a time point of 1 week post-TAC was selected to examine intracellular calcium handling, firstly because hypertrophy should already be established and secondly because mice had not yet begun to display external signs of failure. It would
have been useful to assess *in vivo* function prior to sacrifice to confirm that the progression into decompensation had not yet begun on a mouse by mouse basis so that a proper comparison could be drawn with the 2 week data. Similarly, it would be useful to have calcium data following 2 weeks TAC to avoid having to make assumptions as to the correlation between calcium and contractility between the two time points in control mice. Furthermore, calcium handling should be assessed in αMHC-Cre⁸ controls to confirm that the transgene had no effect. Another assumption which had to be made was that PMCA1 served the same function in single and double knockout mice when interpreting the role of PMCA4 in PMCA1:4⁴dko mice. In order to confirm the role of isoform 4 a third dataset from PMCA4 knockout mice would be required.

4.4.5 Conclusions

Despite having little impact on cardiac function under normal physiological conditions, the results presented here suggest that PMCA1 and 4 can act independently to profoundly affect the cardiac response to pressure overload. PMCA1 may be critical in coordinating necessary adaptations to improve calcium cycling during systole, and may play a protective role during the development of pathological hypertrophy. In contrast, PMCA4 is able to regulate the extent of cardiac hypertrophy, and its inhibition may provide a novel target for a future anti-hypertrophic therapy through averting the development of maladaptive left ventricular hypertrophy. In contrast, neither PMCA1 nor 4 plays a role in regulating exercise-induced hypertrophy.
Chapter 5
5. THE PROGRESSIVE DEVELOPMENT OF CARDIOMYOPATHY WITH AGE IN MICE CONTAINING THE αMHC-CRE TRANSGENE

5.1 Introduction

In the subsequent decades since the pioneering work of Nobel Laureates Mario Capecchi, Martin Evans and Oliver Smithies enabled the advent of gene targeting in mice, our knowledge of gene function has undoubtedly blossomed. The ability to knockout specific genes has provided a most powerful tool with which to study their roles in mammalian development and physiology in both health and disease, and permitted great advancement in the field of biomedical research. As the quest to expand our knowledge of gene function has grown, the available technologies have evolved allowing for greater control of transgene expression. However, with the added complexities that this brings to a knockout model, stringent controls must be employed in order to avoid misinterpretation of results.

5.1.1 Gene targeting using Cre-loxP technology

It is predicted that germline knockout of up to 30% of the approximately 23000 coding genes in the mouse genome would result in embryonic lethality (Ayadi et al., 2012), of which the gene encoding PMCA1 is one such example (Okunade et al., 2004). In order to circumvent this issue an alternative strategy must be employed, and this can be achieved through spatial and/or temporal confinement of the deletion. Cre-loxP technology allows the site-specific recombination of DNA at a chosen point in the genome, with the ability to restrict this to a desired tissue or cell type by driving the reaction through a tissue-specific promoter (Gu et al., 1994).

The 38kDa P1 bacteriophage enzyme Cre (named due to its action of causing recombination) acts to recombine specific base pair sequences in the P1 genome named as loxP sites (for locus of crossing over (x), P1) (Sternberg and Hamilton, 1981). In the late 1980s Brian Sauer developed this prokaryotic system for use in eukaryotic cells, demonstrating efficient recombination after incorporation first into yeast, and then mammalian cells (Sauer, 1987, Sauer and Henderson, 1988). LoxP sites are 34 base pairs
in length, and consist of 2 inverted 13bp repeats flanking an asymmetric 8bp core region determining \textit{loxP} orientation. Using the principle of homologous recombination in embryonic stem cells, \textit{loxP} sites can be incorporated into the mouse genome flanking critical exons in the gene of interest. Once these floxed mice have been generated, they can be crossed with a second transgenic line expressing Cre-recombinase in order to bring about excision of the flanked region, and by placing this reaction under the control of a tissue/cell specific promoter the deletion can be restricted to a particular cell lineage (Gu et al., 1994).

5.1.2 Gene targeting in cardiac research

When it comes to gene targeting in cardiac research there are a variety of strategies available, with a number of suitable promoters having been shown to produce reliable levels of excision (Davis et al., 2012). Each of these has their own intricacies in terms of expression pattern allowing control over the time and cell types in which recombination will occur. For example, the most widely used promoter for cardiac muscle-specific transgenesis – that of the $\alpha$ myosin heavy chain ($\alpha$MHC) – is highly specific to ventricular and atrial myocytes and expressed shortly after birth, persisting throughout adulthood (Agah et al., 1997). The myosin light chain 2v (MLC2v) promoter, on the other hand, can cause recombination to occur from as early as day E8.75 in the developing embryo, and is restricted mainly to ventricular myocytes (Chen et al., 1998).

However, the choice available to researchers is not simply restricted to the choice of promoter. Most promoters, as is the case for MLC2v, are expressed in the developing embryo and begin to facilitate recombination at this stage (eg. $\beta$ myosin heavy chain ($\beta$MHC), cardiac troponin T (cTnT) and Nkx2.5). This would prove problematic if the gene of interest impacted on cardiac development, and therefore inducible knockout models allowing temporal control have also been developed. These methods require the use of additional transgenes, which prevent induction of Cre expression until administration of a pharmacological activator. One system of inducible gene targeting fuses Cre to a mutated ligand binding domain of the oestrogen receptor (Cre-ER), and activates it upon administration of the drug tamoxifen (Feil et al., 1996), a strategy used in
cardiac research most commonly in the αMHC-MerCreMer line (Sohal et al., 2001). More recently, an inducible knockout system has been generated using a reverse tetracycline transactivator (rtTA), where Cre expression can be turned on upon the administration or withdrawal of tetracycline or its analogue doxycycline, for use with either the cTnT or αMHC promoter (Wu et al., 2010, Xiong et al., 2010).

5.1.3 Important considerations when using conditional gene targeting; the potential for Cre-toxicity

Each of these technologies has its own advantages and disadvantages in terms of spatial and temporal control of gene expression, and one must elect which will be best suited to the needs of a particular study. Cre activity must be high enough to ensure efficient recombination, tightly controlled to avoid leak into alternative cell types which may produce non tissue-specific effects and sustained for the duration of the experiment in order to maintain repression of the gene of interest.

One factor that must be taken into consideration irrespective of the strategy employed is the potential for Cre toxicity. More than a decade ago this phenomenon began to be documented following recombination in mammalian cell culture, with reports of reduced proliferation and chromosomal aberrations (Loonstra et al., 2001, Silver and Livingston, 2001). Moreover, many commonly used strategies for creating conditional knockouts have been shown to produce unwanted Cre-related effects in target tissues, such as glucose intolerance in pancreatic β-cells (Lee et al., 2006), impaired alveologenesis in the lung (Morimoto and Kopan, 2009) and dilated cardiomyopathy (DCM) in the heart (Buerger et al., 2006, Koitabashi et al., 2009).

With the wealth of strategies available for cardiac gene ablation, it is clear that careful consideration must be taken as to the most suitable approach for the purpose of a particular study. In this project, where we wished to investigate the phenotype following PMCA ablation over a sustained period of time, a standard conditional knockout driven by the widely used αMHC promoter was chosen (Agah et al., 1997), as it would provide robust
excision restricted to cardiomyocytes from early adulthood. Given the widespread documentation of Cre-induced side-effects, experiments in each knockout line were conducted alongside suitable controls in which the αMHC-Cre transgene was expressed on the same background strain (αMHC-Cre<sup>tg</sup> mice).

### 5.1.4 Aims

To assess whether Cre activity causes any cardiac phenotype on its own in αMHC-Cre transgenic control mice (αMHC-Cre<sup>tg</sup>), and if so from what age does this begin to occur. This will address the important issue of maximal experimental duration when using this transgenic line.
5.2 Methods

Recovery echo was performed under isofluorane anaesthesia. The MLC2v-Cre\textsuperscript{tg} mouse line (Minamisawa et al., 1999) has been previously used to generate knockout mice in our group and was hence readily available (Liu et al., 2011).
5.3 Results

One of the original aims of this project was to study the role of the PMCA in the heart during the ageing process as cardiovascular disease, and heart failure in particular, primarily affects elderly populations. As such, cardiac structure and function were examined by echocardiography periodically in a cohort of PMCA1:4\textsuperscript{dcko} and PMCA1:4\textsuperscript{flox} mice as they aged to identify any signs of spontaneous hypertrophy or dysfunction. As was the case in all experiments, this study was performed concomitantly in a cohort of αMHC-Cre\textsuperscript{tg} and αMHC-Cre\textsuperscript{neg} controls to account for any deleterious effects caused by the presence of the αMHC-Cre transgene, given previous reports of the development of cardiomyopathy upon high level Cre-recombinase expression (Buerger et al., 2006).

5.3.1 \textit{In vivo} cardiac phenotype in ageing αMHC-Cre\textsuperscript{tg} mice

Recovery echo was performed on αMHC-Cre\textsuperscript{tg} and αMHC-Cre\textsuperscript{neg} controls on a monthly basis from the age of 3 months. Up to the age of 8 months cardiac structure and function was similar amongst the two groups. Over the course of the ensuing weeks however, a number of αMHC-Cre\textsuperscript{tg} mice rapidly began to display external signs of ill health, which were accompanied by the development of severe contractile dysfunction and left ventricular dilation (figures 5.1A & B) whereupon they were euthanized in accordance with humane endpoints as stated in the project licence, along with age matched controls. As indicated by the Kaplan-Meier survival plot in figure 5.1C, this occurred between the ages of 9 and 10.5 months in all but one αMHC-Cre\textsuperscript{tg} subject, where cardiac function was maintained until the development of similar signs at 13.5 months. Age did not compromise cardiac performance in αMHC-Cre\textsuperscript{neg} control mice any age.
**Figure 5.1 The development of cardiomyopathy with age in αMHC-Cre\textsuperscript{tg} mice**

Echocardiography performed at monthly intervals in three αMHC-Cre\textsuperscript{tg} and control animals revealed A) a dramatic reduction in fractional shortening between the ages of 9 and 10.5 months in mice carrying the αMHC-Cre transgene with B) concurrent enlargement of the left ventricle. C) Kaplan-Meier survival curves for αMHC-Cre\textsuperscript{tg} and control mice. αMHC-Cre\textsuperscript{neg} mice were euthanized when healthy at age matched time points for tissue harvesting and considered as censored for the purpose of this plot, \(n = 5-6\). FS, fractional shortening; dLVD, diastolic internal left ventricular diameter.

Further analysis of the cardiac phenotype at the time of termination revealed significantly altered cardiac structure and signs of left ventricular failure in αMHC-Cre\textsuperscript{tg} mice compared to their age matched controls (table 5.1). Heart weight normalised to tibia length was increased by 31% (12.09 ± 0.56 vs 9.20 ± 0.52 mg/mm, \(p<0.01\)) indicative of cardiac hypertrophy, which was eccentric in nature as evidenced by a reduction in relative wall thickness (\(p<0.05\)). This was characterised by left ventricular dilation (dLVD 6.52 ± 0.17 vs 5.28 ± 0.18, \(p<0.001\)) and little overall increase in wall thickness, with a 19% thickening of the interventricular septum countered by thinning of the posterior wall by 13% (both \(p<0.05\)).
In addition to displaying evidence of dilation and hypertrophy, αMHC-Cre\textsuperscript{tg} hearts also experienced severely compromised inotropic function (fractional shortening 8.00 ± 1.18 vs 30.35 ± 2.15 %, p<0.001) and consequently exhibited significant signs of lung congestion with a 50% increase in normalised lung weight (11.82 ± 0.69 vs 7.87 ± 0.77 mg/mm, p<0.01). These results suggest that the presence of the αMHC-Cre transgene predisposed mice to the onset of dilated cardiomyopathy as they aged.

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Table 5.1 Cardiac structure and function in aged αMHC-Cre\textsuperscript{tg} mice BW, body weight; HW/TL, heart weight/tibia length; LW/TL, lung weight/tibia length; d, diastolic; s, systolic; LVD, left ventricular internal diameter; LVPW, left ventricular posterior wall thickness; IVS, interventricular septal thickness; RWT, relative wall thickness
5.3.2 The molecular phenotype in ageing αMHC-Cre<sup>tg</sup> mice

The in vivo data presented in section 5.3.1 suggested that the presence of Cre-recombinase under the control of the αMHC promoter predisposed mice to the fairly acute onset of dilated cardiomyopathy as they aged beyond 9 months. In order to gain insight into the mechanisms behind this seemingly rapid progression into heart failure, the molecular phenotype in αMHC-Cre<sup>tg</sup> mice was examined during the months preceding this.

5.3.2.1 Expression of the heart failure biomarker BNP in ageing αMHC-Cre<sup>tg</sup> mice

Brain natriuretic peptide (BNP) is a factor secreted by the heart during cardiac stress, and is commonly used as a diagnostic and prognostic biomarker in the clinical setting (Mukoyama et al., 1990). To examine whether the onset of heart failure in αMHC-Cre<sup>tg</sup> mice truly was an acute event occurring after the age of 9 months, mRNA transcripts of the NPPB gene encoding BNP were examined in heart extracts taken from mice aged 3, 6 and 8 months.

Figure 5.2 highlights a progressive induction of NPPB expression from 3 to 8 months in αMHC-Cre<sup>tg</sup> mice. At the age of 3 months expression was similar to αMHC-Cre<sup>neg</sup> controls; however, whilst levels remained fairly constant over the following months in the absence of Cre-recombinase, the presence of the αMHC-Cre transgene led to an approximately 2- and 5-fold increase in NPPB mRNA abundance at 6 and 8 months, respectively, compared to age matched controls (both p<0.01). In addition, expression was significantly higher in αMHC-Cre<sup>tg</sup> samples at 6 months compared to 3 months, and at 8 months compared to 6 months (both p<0.05). This suggests that transgenic hearts were undergoing changes at the molecular level long before the onset of failure.
Figure 5.2 Progressive induction of BNP expression as αMHC-Cre<sup>tg</sup> mice aged from 3 to 8 months mRNA transcript levels of the heart failure biomarker BNP as measured by qPCR revealed significantly higher expression in αMHC-Cre<sup>tg</sup> samples at 6 months (2.25 ± 0.36) compared to both age matched controls (0.80 ± 0.06, ** p<0.01) and 3 month transgenic mice (1.24 ± 0.07, † p<0.05), which increased further to 5.13 ± 0.91 in 8 month old αMHC-Cre<sup>tg</sup> hearts ( ** p<0.01 vs 8 month αMHC-Cre<sup>neg</sup>, † p<0.05 vs 6 months αMHC-Cre<sup>tg</sup>)
5.3.2.2 The development of myocardial fibrosis in ageing αMHC-Cre<sup>tg</sup> mice

The deposition of collagen fibres leading to interstitial fibrosis often underlies the development of heart failure, and therefore analysis of mRNA transcript levels of the precursors to types I and III collagen were examined in αMHC-Cre<sup>tg</sup> mice in the months leading up to the onset of failure.

Figures 5.3A & B show that, similar to the pattern of BNP expression, relative COL1A1 and COL3A1 mRNA abundance rose progressively in αMHC-Cre<sup>tg</sup> hearts from 3 to 8 months, whilst expression in controls remained fairly constant. Type I procollagen expression increased ~1.8 and ~2.9-fold from 3 month levels at 6 and 8 months, respectively, and reached significance at 8 months compared to αMHC-Cre<sup>neg</sup> controls (2.92 ± 0.42 vs 0.79 ± 0.12, p<0.001). COL3A1 abundance mirrored this pattern, rising to ~1.7 (at 6 months) and ~2.8 (at 8 months) times the levels seen at 3 months. These were significantly higher than those in age matched αMHC-Cre<sup>neg</sup> samples (both p<0.01), and significantly increased at 8 months compared to 6 (2.80 ± 0.35 vs 1.69 ± 0.22, p<0.05).

This suggested that collagen deposition might be apparent in αMHC-Cre<sup>tg</sup> samples, which could possibly contribute to the failing phenotype witnessed at more advanced ages. Masson’s trichrome staining for fibrosis in transverse histological sections prepared from αMHC-Cre<sup>neg</sup> and αMHC-Cre<sup>tg</sup> mice euthanized at 10.5 months revealed extensive interstitial collagen deposition throughout both the endo- and epicardium of transgenic hearts, whilst control hearts remained clear of fibrosis (figure 5.3C).
Figure 5.3 Cardiac fibrosis in ageing αMHC-Cre<sup>tg</sup> mice

A) Expression of the collagen type I precursor COL1A1 as measured by qPCR increased progressively with age in αMHC-Cre<sup>tg</sup> mice (*** p<0.001 tg vs neg at 8 months of age). B) COL3A1 transcript levels also increased with age in αMHC-Cre<sup>tg</sup> mice (ǂ p<0.05 8 vs 6 months), and were significantly higher in transgenic hearts than age matched controls at both 6 (** p<0.01) and 8 months (*** p<0.001). C) Representative sections cut from 10.5 month old αMHC-Cre<sup>neg</sup> and αMHC-Cre<sup>tg</sup> mice stained with aniline blue to highlight collagen deposition. Extensive interstitial fibrosis can be seen in epicardial and endocardial regions of the aged αMHC-Cre<sup>tg</sup> heart.
5.3.2.3 The progressive loss of dystrophin in ageing αMHC-Cre\textsuperscript{tg} mice

During phenotypic analysis of PMCA1:4\textsuperscript{dcko} mice, the expressions of proteins known to interact with the PMCA complex were examined by western blot. Both isoforms 1 and 4 interact physically with α1-syntrophin, with PMCA4 in particular forming a functional macromolecular complex with nNOS, syntrophin and dystrophin (Williams et al., 2006). During this analysis the expression of dystrophin was found to be reduced in older PMCA1:4\textsuperscript{dcko} samples, and as the cardiomyopathy witnessed in aged mice carrying the αMHC-Cre transgene closely matched that of the aged mdx mouse (Quinlan et al., 2004) (a dystrophin deficient mouse model used in the study of muscular dystrophy), it was hypothesised that this could be behind the development of heart failure in αMHC-Cre\textsuperscript{tg} mice.

Protein expression of dystrophin was therefore examined as αMHC-Cre\textsuperscript{tg} mice aged from 3 to 8 months. In younger mice levels were similar to controls; however, by the age of 6 months dystrophin was significantly downregulated by 62% (p<0.05), and this deteriorated further to just 15% of the level in αMHC-Cre\textsuperscript{neg} controls in 8 month samples (p<0.05 – figures 5.4A & B).

One of the factors thought to contribute to the ensuing cardiomyopathy in mdx mice is through the loss of the interaction between dystrophin and the cardiac sodium channel Na\textsubscript{v}1.5 (Gavillet et al., 2006), resulting in reduced expression of the sodium channel and consequent abnormalities in cardiac electrical activity. Na\textsubscript{v}1.5 expression was therefore examined in ageing αMHC-Cre\textsuperscript{tg} mice to see if this was affected by the dystrophinopathy. Figures 5.4A and C demonstrate that, as was the case with dystrophin, protein levels of Na\textsubscript{v}1.5 in αMHC-Cre\textsuperscript{tg} mice were not affected at 3 months. As mice aged to 6 months, αMHC-Cre\textsuperscript{tg} hearts began to show signs of sodium channel downregulation, displaying a 17% reduction in Na\textsubscript{v}1.5 expression which approached significance, and this progressed further to a 40% decrease by the age of 8 months.
Figure 5.4 Disruption of the dystrophin-Na,1.5 complex in ageing αMHC-Cre<sup>tg</sup> mice

A) Representative western films depicting the progressive reduction in B) dystrophin expression at 6 (* p<0.05) and 8 (* p <0.05) months and C) Na,1.5 protein abundance with age.
5.3.3 In vivo cardiac phenotype in aged MLC2v-Cre\textsuperscript{tg} mice

The data presented in sections 5.3.1 & 5.3.2 provide evidence of the development of Cre-mediated cardiomyopathy with age in mice expressing Cre-recombinase under the control of the αMHC promoter, with progressive alterations of gene and protein expression at the molecular level. Gene targeting using the αMHC promoter is the most commonly used system for generating conditional knockouts in cardiac research; however, other techniques for achieving tissue-specific restriction are available.

Placing Cre-recombinase under the control of the MLC2v promoter provides a reliable method for the targeted recombination of loxP sites specifically within ventricular myocytes (Chen et al., 1998). To determine whether a similar situation ensued with ageing as was witnessed in αMHC-Cre transgenic mice, echocardiography was periodically performed on a cohort of MLC2v-Cre\textsuperscript{tg} mice as they aged beyond 8 months.

MLC2v-Cre\textsuperscript{tg} mice experienced no loss of cardiac function up to the age of 16 months, and appeared outwardly identical to littermate wild type controls. At this point mice were sacrificed for tissue harvesting. Terminal echo results are presented in table 5.2. When compared to the phenotype of the enlarged dilated heart displaying significant signs of heart failure following Cre-recombinase expression under the control of the αMHC promoter, no such effects were apparent in MLC2v-Cre\textsuperscript{tg} mice. Fractional shortening and normalised lung weights were comparable to controls, and rather than displaying the pattern of eccentric hypertrophy seen in αMHC-Cre\textsuperscript{tg} mice, heart size if anything was a little smaller than in controls with a slight reduction in left ventricular diameter (3.90 ± 0.15 vs 4.35 ± 0.11, p<0.05). This data suggests that conditional knockouts generated using the MLC2v-Cre transgene are unlikely to have the same unwanted side-effects with ageing as were witnessed in αMHC-Cre\textsuperscript{tg} mice.
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**Table 5.2 Cardiac structure and function in aged MLC2v-Cre\textsuperscript{tg} mice** BW, body weight; HW/BW, heart weight/body weight; LW/BW, lung weight/body weight; d, diastolic; s, systolic; LVD, left ventricular internal diameter; LVPW, left ventricular posterior wall thickness; IVS, interventricular septal thickness; RWT, relative wall thickness
5.4 Discussion

Cre toxicity is an issue of the utmost importance, yet often overlooked when it comes to studies involving conditional gene targeting (Schmidt-Supprian and Rajewsky, 2007). The experiments described in this chapter were conducted alongside those in PMCA1:4\textsuperscript{dcko} mice to control for any possible side-effects related to Cre toxicity caused by the presence of the αMHC transgene. In\textit{ vivo}, αMHC-Cre\textsuperscript{tg} mice developed severe systolic dysfunction and DCM after passing nine months of age, whilst littermate controls remained healthy well into their second year. Molecular analysis revealed that this decline in function was preceded by fibrotic changes some months in advance, and degradation of the structural protein dystrophin. Interestingly, Cre expression under the control of the MLC2v promoter did not appear to affect cardiac function at any point studied. This discussion will address the nature of the observed Cre toxicity, and the problems that this phenomenon poses the researcher when designing a conditional knockout mouse.

5.4.1 The development of spontaneous dilated cardiomyopathy in aged αMHC-Cre\textsuperscript{tg} mice

By performing echocardiography at monthly intervals in batches of αMHC-Cre\textsuperscript{tg} and wild type control mice, we were able to observe the onset of a phenotype of spontaneous DCM in transgenic mice as they aged beyond 9 months. This was characterised by dramatically reduced left ventricular contractility, chamber enlargement and thinning of the free wall, and associated with extensive endo- and epicardial fibrosis and signs of pulmonary congestion at the time of sacrifice due to ill health. Heart weight was markedly increased, with an overall reduction in relative wall thickness despite thickening of the interventricular septum, a possible sign of hypertrophic changes emanating from the right ventricle.

These are by no means the first reports of this phenotype in mice carrying the αMHC-Cre transgene. The Molkentin lab state in a recent review that mice from the same Cre line used here (that developed by Michael Schneider (Agah et al., 1997)) encounter spontaneous cardiotoxicity between the ages of 8 and 12 months (Davis et al., 2012), prompting them to generate several further Cre lines of which two developed spontaneous
dilation with reduced fractional shortening by 6 months of age. Surprisingly, this occurred in the highest and lowest expressing Cre lines, whilst intermediate levels of expression were associated with preserved function. Another study found high levels of αMHC-Cre expression to induce the same phenotype at the age of 10 to 12 weeks, whilst lower expressing lines remained healthy (Buerger et al., 2006). Interestingly, over the course of this study we found all αMHC-Cretg subjects to develop cardiomyopathy within a 6 week period with one exception, where systolic function was preserved until 13.5 months, presumably a consequence of lower Cre expression.

5.4.2 Cardiomyopathy in aged αMHC-Cretg mice is preceded by progressive changes at the molecular level

It appeared that the decline in systolic function and left ventricular dilation in αMHC-Cretg mice was fairly sudden in onset, generally occurring in a matter of weeks. This is consistent with the observations of Buerger et al, who noted the phenotype to develop over 4 weeks (Buerger et al., 2006). However, examination of the expression of the clinical biomarker for heart failure BNP and markers of fibrosis found an increase at 6 months, 3 months prior to the onset of failure, which continued to rise during the following weeks. In addition, dystrophin protein abundance was dramatically reduced by the age of 6 months and virtually absent in 8 month old transgenic mice, while expression of the cardiac sodium channel Na\textsubscript{v}1.5 which exists in a complex with dystrophin also appeared reduced at advanced ages. This provides a possible molecular pathway through which the DCM develops, as the phenotype bears similarities to both human and murine dystrophic cardiomyopathies where a cardiac phenotype will typically present as mild and progress to cardiac dilation and severe systolic dysfunction in later life (Fayssoil et al., 2010, Quinlan et al., 2004).

It is thought that altered mitochondrial metabolic function with subsequent increases in ROS production and susceptibility to cell death may contribute to adverse remodelling in the dystrophic heart (Burelle et al., 2010), and this may explain the progressive increase in procollagen levels witnessed in αMHC-Cretg mice. Additionally, it is hypothesised that observed reductions in Na\textsubscript{v}1.5 channel expression and activity may contribute to disease
progression in the dystrophin-deficient mdx mouse, with sodium current density declining with age in the months preceding the onset of cardiomyopathy (Gavillet et al., 2006, Koenig et al., 2011). Furthermore, several studies have noted inherited mutations in the SCN5A gene encoding the cardiac sodium channel to be linked to familial DCM (McNair et al., 2004, Olson et al., 2005, Ge et al., 2008), and the degree of repression of the gene in mice has been shown to correlate strongly with disease severity and age of onset (Hesse et al., 2007). Thus it is quite possible that the decline in Na\textsubscript{v}1.5 expression which we begin to observe in αMHC-Cre\textsuperscript{tg} mice as they age beyond 6 months is the driving force behind the subsequent progression into DCM.

What these experiments also underline is that even in the absence of an obvious presenting phenotype, the presence of Cre-recombinase may cause substantial changes at the molecular level which could confound any positive results which may be accredited to the ablated gene of interest. Therefore, this highlights the necessity for full phenotyping of a suitable transgenic control in any study utilising Cre-mediated conditional gene targeting technologies.

5.4.3 The potential for illegitimate Cre-mediated excision at loxP-like sites

This study was not able to evaluate the mechanism behind the degradation of dystrophin in αMHC-Cre\textsuperscript{tg} mice; however one might hypothesise that it could arise through Cre-mediated disruption of regions of the dystrophin gene at sites resembling the sequence for loxP. It has been demonstrated that Cre is able to efficiently recombine not only authentic loxP sites, but also ones which differ significantly in nucleotide sequence as long as they adhere to certain characteristics of the loxP structure ie. two palindromic 13bp repeats sharing a certain degree of homogeneity with loxP separated by an 8bp spacer region (Sauer, 1996). So-called cryptic or pseudo lox sites have been identified in both the mouse and human genomes which demonstrate close to 100% recombination efficiency in bacterial excision assays, despite exhibiting several nucleotide mismatches in residues thought previously to have been critical for successful recombination (Thyagarajan et al., 2000). In this study, Calos and colleagues also assayed the potential for pseudo lox sites to catalyse excision events in human cells and found this to occur with over 50% of the
efficiency of wild type loxP. Coincidentally, the most efficient pseudo lox site identified in the human genome in this study was located on the X chromosome (hXp22), not too distant from the dystrophin gene (hXp21.2).

Recently, in vivo recombination of pseudo lox sites at a specific locus was demonstrated for the first time following tamoxifen administration in combination with ubiquitous expression of Cre-ER (Higashi et al., 2009). In addition, a further study expressing Cre in mouse sperm without the insertion of any loxP sites noted abortive pregnancies with 100% penetrance, with evidence of chromosomal rearrangements resulting from Cre enzymatic activity (Schmidt et al., 2000). The authors drew the conclusion that this illegitimate activity was likely to be the recombination of pseudo lox sites.

It may be reasonable to assume that due to the size of the dystrophin gene (at around 2.3 megabases it is the largest in the genome (Mandel, 1989)), it would be as likely as any to contain pseudo lox sites capable of recombination upon Cre expression. However, given that this comprises less than 0.1% of the total size of the mouse genome (Waterston et al., 2002), it would be highly unlikely to be unique to dystrophin.

5.4.4 Alternative strategies to using the αMHC-Cre transgenic line in the heart

The data presented in this study suggests that the standard approach to gene targeting using the αMHC-Cre transgene is not a suitable model for use in experiments lasting 6 months or more. Without data covering the intervening months in the lead up to this, it is not possible to say at exactly what age the dystrophic phenotype manifests itself, however with no apparent molecular or in vivo phenotype at the age of 3 months it should still be a viable option for studying gene function in young adult mice as long as suitable transgenic controls are included.

So what is the optimum strategy to use if one wishes to examine gene function in older mice? The knock-in strategy adopted by Kenneth Chien in generating the MLC2v-Cre
transgenic line appears a promising option, providing approximately 80% excision restricted to ventricular myocytes (Chen et al., 1998). In addition, no gene dosing effects were witnessed after performing TAC in this transgenic line (Minamisawa et al., 1999) and here, in following a cohort of MLC2v-Cre<sup>ie</sup> mice we were unable to detect any overt signs of cardiac dysfunction well into their second year. Heart size did appear a little smaller compared to controls, and further examination would be warranted to completely rule out the absence of any Cre-related side effects, however the line does appear a viable candidate for studying gene function with age.

One issue with the MLC-2v promoter however, is that it is active during embryonic development which therefore may rule out its use if one wished to study a gene of importance to early cardiac formation. In this case it may be prudent to select an inducible knockout strategy, although these too present the potential issue of Cre toxicity. Tamoxifen administration in doses sufficient to allow efficient recombination in the αMHC-MerCreMer line is known to induce acute and severe DCM in the days following delivery (Koitabashi et al., 2009, Hall et al., 2011). Depending on the tamoxifen dosage, this can be transient in nature without mortality, with systolic function and chamber dimensions returning to normal in the days following tamoxifen withdrawal. Each of these studies noted substantial but reversible alterations at the molecular level, for example the downregulation of SERCA and reduced mitochondrial ATP. In addition, Hall et al. found cardiac hypertrophy to persist even after drug withdrawal. Furthermore, tamoxifen administration alone has been shown to alter the cardiac action potential, resting membrane potential and intracellular calcium handling (Pahlavan et al., 2012), whilst others have witnessed evidence of apoptosis, fibrosis and DNA damage in the activated αMHC-MerCreMer line accompanied by significant mortality (Bersell et al., 2013).

The toxic effects of tamoxifen administration in the αMHC-MerCreMer line have even been used as a disease model by some groups to test the protective or potentiating effects of certain genes. For example, mice carrying a heme oxygenase-1 transgene did not experience the cardiac toxicity, myocyte necrosis or neutrophil infiltration that was induced by tamoxifen-mediated Cre activation in controls (Hull et al., 2013), whilst deletion of the leptin receptor was found to exacerbate the transient nature of the toxic
effects of Cre in the MerCreMer line and induce irreversible and lethal heart failure (Hall et al., 2012). I would speculate that these studies were not originally designed this way, but rather that the phenotype presented itself during the generation of the transgenic mice, for given the seemingly unpredictable nature of Cre-toxicity and the lack of any clinical relevance it would appear a poor choice for a disease model.

Optimisation of the tamoxifen dosage and administration route does appear to limit Cre-mediated toxicity. For example, adding the drug into dry food as opposed to administration through injection appears favourable (Andersson et al., 2010), and if injections are to be used then reducing the concentration and number of doses may avoid any overt cardiomyopathy (Bersell et al., 2013, Hall et al., 2011, Hougen et al., 2010). In the last of these studies, Hougen et al. performed microarray analysis of over 35,000 genes in αMHC-MerCreMer mice and found tamoxifen administration to significantly regulate around 11% of the transcriptome. Interestingly, dystrophin was found to be downregulated by 80%. One further strategy might be to induce recombination with the oestrogen receptor modulator raloxifene rather than tamoxifen, which appears to circumvent any transient DCM (Koitabashi et al., 2009), although limited phenotyping was performed in this study.

Given the number of reports of toxicity in the αMHC-MerCreMer line it would appear that this strategy is far from ideal. Recently, inducible knockout technologies have been generated activating Cre upon doxycycline withdrawal or tetracycline administration using a reverse tetracycline transactivator (rtTA), and showed no obvious signs of Cre-toxicity (Xiong et al., 2010, Wu et al., 2010). However, Xiong et al. did not include a control expressing activated Cre in their study, whilst similar technologies utilising the rtTA transgene have noted toxicity in other tissues (eg. lung (Sisson et al., 2006, Morimoto and Kopan, 2009)). It would therefore be naïve to assume, given the prevalence of Cre toxicity in other gene targeting models, that these are 100% clean.
5.4.5 Conclusions

It is abundantly clear that Cre toxicity presents a substantial problem when interpreting data obtained using many commonplace gene targeting strategies. The data presented in this chapter indicates not only that the use of the αMHC transgenic mouse can cause cardiomyopathy in later life, but also that it is possible that illegitimate recombination at pseudo lox sites in the genome may cause alterations at the molecular level before any observable gross phenotype, and that all experiments using this line should be terminated before mice reach 6 months of age. Placing Cre under the control of the MLC2v promoter may be a more suitable model in which to study gene function in older mice; however it is essential that all studies must include sufficient and appropriate controls. Currently many studies omit to include a transgenic control expressing active Cre, and the potential issue of toxicity is frequently overlooked by researchers and reviewers alike (Nature-editorial, 2007). Conditional gene targeting has proved an invaluable tool in the advancement of our knowledge of gene function and, with a little prudence with regards to experimental design and scrutiny during the review process, will no doubt continue to benefit the field of biomedical research for years to come.
Chapter 6
6. GENERAL DISCUSSION

For many years it has been postulated that the presence of 4 isoforms and more than 25 splice variants of the PMCA in the genome suggests that the calcium pump has evolved to perform specialised functions adapted to particular cell types (Strehler and Zacharias, 2001). The ubiquitous distribution of PMCA1 and 4 led to the belief that these isoforms may serve general housekeeping duties in order to maintain a desirable concentration of intracellular calcium in many tissues; however the minor contribution of the PMCA to global calcium clearance during cardiac EC coupling suggested that in cardiomyocytes the pumps may have an alternative role to play in coordinating calcium signalling.

The key aim of this thesis was to assess the specific functions of PMCA1 and 4 in the adult myocardium under basal conditions and during the development of pathological and physiological left ventricular hypertrophy. Consistent with the hypothesis of isoform-specific roles, PMCA1 and 4 were found to be capable of independently influencing intracellular calcium dynamics although this had little impact on cardiac structure or function under normal physiological conditions. Moreover, this study identified an additional role for each isoform in regulating the development and progression of pathological left ventricular hypertrophy in response to pressure overload. Meanwhile, experiments in control mice carrying the αMHC-Cre transgene detected the development of age-related DCM preceded by a progressive deterioration in dystrophin abundance providing novel mechanistic insight into the occurrence of Cre-mediated toxicity.

6.1 The generation and basal characterisation of a novel mouse line with cardiomyocyte-specific deletion of PMCA1 and 4 (PMCA1:4^dcko mice)

The first objective of this project was to generate a novel transgenic mouse in which both isoforms 1 and 4 of the PMCA were deleted from the myocardium (PMCA1:4^dcko mice). Demonstration of the near absence of PMCA1 and 4 protein in cardiomyocytes and normal levels in other tissues confirmed that this had been successfully achieved. This provided the first in vivo model in which to study the effect of concomitant deletion of both isoforms...
from the myocardium. Cardiac structure and function in PMCA1:4^dcko mice was similar to controls up to the age of 8 months indicating that neither isoform played a significant role in regulating growth or maintaining normal cardiac function in the adult heart under basal conditions. Unfortunately, due to the toxic effects of Cre-recombinase, analysis beyond 8 months could not be performed as was originally planned and therefore it is not possible to comment on whether PMCA deletion affects the ageing heart. When conducting similar studies in the future it would be pertinent to select an alternative gene targeting strategy to that of Cre expressed under the control of the αMHC promoter.

6.2 Isoform-specific roles of PMCA1 and 4 in the regulation of basal intracellular calcium handling

Through the comparison of systolic and caffeine-evoked calcium transients in PMCA1:4^dcko and PMCA1^cko myocytes this study provides the first evidence of the specific roles of isoforms 1 and 4 in basal intracellular calcium handling. The overall PMCA contribution to diastolic calcium clearance was found to be minor, accounting for approximately 1% of global calcium in agreement with previous reports (Negretti et al., 1993, Bassani et al., 1994). In confirmation of the hypothesis, this was achieved almost exclusively via PMCA1, compatible with data from PMCA4 knockout mice which have found the contribution of this isoform to calcium extrusion to be negligible (Mohamed et al., 2011). In PMCA1:4^dcko mice, the deletion of PMCA4 instead appears to increase peak levels of intracellular calcium during systole, possibly through the phosphorylation of RyR_{Ser2808}. From reports in PMCA4 knockout mice this is likely achieved through activation of PKA following altered compartmentalisation of nNOS-mediated signalling and may serve to increase basal contractility (Mohamed et al., 2011). In hindsight it would have been useful to measure cell shortening by edge detection in PMCA1:4^dcko myocytes to confirm whether a similar correlation between systolic calcium and contractility was apparent. These results further confirm that PMCA4 functions primarily as a regulator of signalling pathways in the heart.
6.3 PMCA1 ablation leads to systolic dysfunction and a progression into left ventricular failure following pressure overload

To date no study has examined the role of PMCA1 during the development of pathological hypertrophy. In order to achieve this, pressure overload was induced by TAC in PMCA1cko mice for two weeks. PMCA1 ablation did not affect the degree of hypertrophic growth. Despite this, hearts displayed significant signs of systolic and diastolic dysfunction, pronounced left ventricular dilation and pulmonary congestion indicative of the progression into decompensation, whilst function remained preserved and LVD unaltered in TAC controls. Haemodynamic analysis revealed that contractility was sufficiently improved in control hearts to maintain efficient arterial-ventricular coupling in response to the TAC-induced increase in afterload, but this response was not reciprocated in PMCA1cko hearts. The precise chronological sequence of events leading to decompensation however is unclear from this data and it would have been useful to perform intermediate assessments of cardiac function by echocardiography.

6.4 PMCA1 ablated myocytes demonstrate a lack of adaptive calcium cycling in response to pressure overload

It was hypothesised that PMCA1 may take on a greater role in calcium clearance at times of haemodynamic stress, and therefore intracellular calcium handling was assessed following 1 week of TAC before the progression into heart failure was evident. Analysis of the relative contributions of SERCA, NCX, PMCA1 and PMCA4/mitochondrial uptake to global calcium clearance found that TAC did not affect the proportion of calcium removed by each system, thus disproving the hypothesis.

However, a significant increase was noted in the amplitude of the systolic transient in control myocytes, which from a review of the literature would be most likely to occur as a result of greater SR fractional release and content (Ohkusa et al., 1997, Carvalho et al., 2006, Toischer et al., 2010). Indeed the amplitude of the caffeine-evoked transient was also increased in control myocytes following TAC compatible with increased SR load. These adaptations in calcium cycling were accompanied by an upregulation of NCX expression, which it is believed may provide a trigger for increased calcium release from the SR during
hypertrophy by increasing calcium entry via its reverse mode (Sipido et al., 2002, Chorvatova et al., 2004). Interestingly, calcium transients were not altered in PMCA1\textsuperscript{cko} cells following TAC and NCX expression did not increase, suggesting a potentially critical role for PMCA1 in initiating adaptive changes in calcium cycling required to maintain function after the onset of pressure overload. These results provide some mechanistic insight into how cardiac contractility may have improved in PMCA1\textsuperscript{fl} controls but not PMCA1\textsuperscript{cko} mice after TAC. However, they do not explain the mechanism through which PMCA1 ablation prevents the initiation of these adaptations and further work is required on this matter.

In contrast to calcium handling during systole, the rate of calcium decay was not affected by surgery in PMCA1\textsuperscript{cko} or control mice, suggesting that the diastolic dysfunction witnessed \textit{in vivo} in PMCA1\textsuperscript{cko} mice after 2 weeks of pressure overload most likely developed secondary to systolic dysfunction. Again, it would have been useful to measure \textit{in vivo} cardiac performance prior to cell isolation in these mice in order to correlate calcium handling with cardiac (dys)function.

6.5 PMCA4 deletion attenuates the hypertrophic response to pressure overload and protects PMCA1 ablated hearts from decompensation

Several reports have suggested that PMCA4 is capable of regulating the hypertrophic response to pathological stimuli (Hammes et al., 1998, Piuhola et al., 2001, Oceandy et al., 2007), however there is some disagreement as to whether its silencing during pressure overload improves or hinders outcome (Abou-Leisa, 2013, Wu et al., 2009). Given that PMCA1 didn’t appear to influence the extent of hypertrophy in response to pressure overload, it was hypothesised that by repeating the same 2 week TAC experiment in PMCA1:4\textsuperscript{dcko} mice it would be possible to deduce whether PMCA4 ablation positively or negatively regulated pathological growth, whilst also assessing whether the additional deletion of PMCA4 would ameliorate or exacerbate the decompensated phenotype in PMCA1\textsuperscript{cko} mice. This would also provide novel insight into the effect that concomitant downregulation of both isoforms has under pathological conditions, a situation known to occur in human heart failure (Borlak and Thum, 2003).
Consistent with reports of pharmacological PMCA4 inhibition (Abou-Leisa, 2013), PMCA4 ablation attenuated the extent of hypertrophic growth in PMCA1:4\textsuperscript{dcko} mice following TAC, although further work is required in order to elucidate the mechanism behind this. Remarkably, systolic function was completely preserved, with less evidence of interstitial fibrosis and left ventricular dilation was not evident indicating that PMCA4 deletion impeded adverse remodelling and prevented the transition into decompensation witnessed in PMCA1\textsuperscript{cko} mice. The mechanisms through which this is achieved are again unclear, but I would postulate that enhanced calcium cycling witnessed under basal conditions in PMCA1:4\textsuperscript{dcko} mice as a result of PMCA4 ablation corrects the need to initiate an adaptive response following pressure overload. Furthermore, these results confirm previous reports stating that compensatory hypertrophic growth is not a required adaptation during pressure overload (Hill et al., 2000, Esposito et al., 2002), and that targeting its development may be beneficial to long term outcomes.

6.6 The PMCA does not regulate exercise-induced hypertrophy

An additional aim of this study was to assess whether PMCA 1 or 4 played a role in regulating physiological hypertrophy. A study has already shown PMCA4 to not be involved in this process (Wu et al., 2009), and therefore by swimming PMCA1:4\textsuperscript{dcko} mice one could likely attribute any observed phenotype to PMCA1. Swimming induced a similar extent of hypertrophy and bradycardia in PMCA1:4\textsuperscript{dcko} and control mice, and did not affect resting cardiac performance in PMCA ablated hearts indicating that neither isoform played a role in regulating the response to exercise.

6.7 PMCA1 – a major player in cardiovascular disease

The experiments described in this thesis potentially highlight a novel role for PMCA1 in coordinating the adaptive response in calcium cycling required to overcome the increased demands of pressure overload, and that the failure to initiate this response following PMCA1 silencing contributes to the progression into decompensation. This would suggest that defects in the \textit{ATP2B1} gene could potentially increase susceptibility to the development of heart failure under conditions of pressure overload. Considering that in recent years SNPs at multiple loci in or around \textit{ATP2B1} have been identified to have the
strongest known genetic association to hypertension (Levy et al., 2009, Cho et al., 2009, Kato et al., 2011), whilst also demonstrating associations with coronary artery calcification and MI (Ferguson et al., 2013), this would highlight PMCA1 as a major player in CVD. Given that reports suggest that ischaemia and hypertension account for the underlying aetiology in 70-80% of heart failure patients (McMurray and Stewart, 2000), there is the possibility that mutations in the ATP2B1 gene may also prove one day to be a heritable risk factor for the development of heart failure.

6.8 PMCA4 inhibition as a therapeutic target

This study adds to evidence from within our group suggesting that silencing PMCA4 can attenuate the development of pathological hypertrophy. Moreover, the data also indicates that PMCA4 deletion can prevent the occurrence of adverse remodelling and progression into decompensation witnessed in PMCA1cko mice. Given that hypertrophic growth does not appear to be an essential response to pressure overload and that LVH in itself is an independent risk factor for cardiovascular morbidity and mortality (Levy et al., 1990), anti-hypertrophic therapies would seem a promising strategy for the prevention of hypertensive heart failure, and as such specific inhibition of PMCA4 may be a suitable candidate. In support of this, global PMCA4 knockout mice are viable and show no adverse phenotype other than a defect in sperm motility (Schuh et al., 2004), which in itself could hold potential therapeutic benefits in the field of contraception, suggesting that PMCA4 inhibition may have limited systemic side-effects. Furthermore, a GWAS conducted in a West African population has recently identified ATP2B4 to contain a novel resistance locus to severe malaria providing a further potential therapeutic benefit to PMCA4 inhibition (Timmann et al., 2012).

6.9 The progressive development of dystrophic cardiomyopathy in mice carrying the αMHC-Cre transgene

Gene targeting using Cre-loxP technology provides a powerful tool with which to study gene function in the heart, affording the researcher spatiotemporal control over the pattern of gene expression in order to circumvent issues such as embryonic lethality and extra-cardiac side-effects which may occur in a constitutive knockout. Cre-mediated toxicity has
been observed to cause chromosomal defects in mammalian cells (Loonstra et al., 2001, Silver and Livingston, 2001), most likely due to the recombination of non-targeted pseudo lox sites (Thyagarajan et al., 2000, Higashi et al., 2009). In the heart reports suggest that Cre toxicity can induce a phenotype of DCM when driven by the αMHC promoter and that this may be dependent on age and level of transgene expression (Davis et al., 2012, Buerger et al., 2006). In light of this all in vivo experiments in PMCA1 cko and PMCA1:4 dcko mice during this study were conducted alongside controls expressing the αMHC-Cre transgene, with the aim to determining the maximal experimental duration in these mice, and rule out the contribution of Cre related artefacts to any observed phenotype that might otherwise be attributed to PMCA ablation.

αMHC-Cre⁸ controls were indeed found to develop spontaneous DCM as they aged beyond 9 months, characterised by increases in heart and lung weight, chamber size, epicardial and endocardial fibrosis and severely reduced fractional shortening. Biomarkers of heart failure and fibrosis were elevated from the age of 6 months and progressively increased thereafter. Dystrophin abundance was found to be dramatically reduced in 6 month old αMHC-Cre⁸ mice, and near absent at 8 months, whilst Na,1.5 expression also appeared reduced at more advanced ages demonstrating parallels between the phenotype in αMHC-Cre⁸ mice and dystrophic cardiomyopathy in the dystrophin-deficient mdx mouse (Koenig et al., 2011, Gavillet et al., 2006). The size of the dystrophin gene makes it a likely candidate for the presence of pseudo lox sites.

Importantly, no functional or molecular phenotype was witnessed in mice at 3 months of age when basal calcium handling and hypertrophic responses in the PMCA mouse lines were assessed indicating that Cre toxicity did not affect the results in these experiments. Interestingly, a mouse line expressing Cre under the control of the MLC2v promoter did not develop cardiomyopathy over a period of 16 months suggesting that this may be a more viable promoter to use when assessing gene function in the hearts of older mice. These results provide mechanistic insight into the development of age-related DCM in αMHC-Cre⁸ mice, and indicate that studies utilising αMHC driven Cre should be terminated before mice reach 6 months of age. They also highlight the importance of full phenotyping of appropriate controls when studying conditional knockout mice.
6.10 Future work

This study provided evidence of a novel role for both cardiac isoforms of the PMCA in the pressure overloaded heart, but the mechanisms through which these roles are achieved are not yet apparent. Therefore the bulk of the future work arising from this project should focus on elucidating these mechanisms. With regards to PMCA1 further work is required in order to verify that the progression into decompensation occurs because of the lack of an adaptive response in calcium cycling. Firstly this would involve assessing in vivo function at the time point at which calcium handling was analysed. It would also be useful to examine an earlier time point to verify that a transient increase in calcium cycling followed by a regression with disease progression 1 week post TAC did not occur. Further analysis of calcium handling protein expression is required following TAC to confirm that NCX expression is altered as the n numbers are currently small, and it would be useful to assess RyR phosphorylation.

Using the methods available in our lab, to test the hypothesis that calcium entry via reverse NCX causes the increase in systolic calcium in controls through triggering SR release whilst this did not occur in PMCA1<sup>cko</sup> myocytes, calcium handling could be assessed in the presence of the NCX reverse mode inhibitor KB-R7943 (although this does have specificity issues) to see whether control TAC transients return to the levels of sham mice, whilst not affecting PMCA1<sup>cko</sup> TAC transients. In addition calcium entry in the presence of an LTCC antagonist such as verapamil could be assessed. If these experiments agree with the hypothesis, in vivo administration of KB-R7943 during TAC would be expected to prevent the compensatory changes in calcium cycling in controls and cause a similar progression into decompensation as was witnessed in knockout mice.

None of these experiments would address the issue of how PMCA1 ablation prevents NCX upregulation however. α- and β-adrenergic stimulation is known to induce NCX upregulation (Menick et al., 2013), therefore to confirm that PMCA1 does regulate NCX expression an assessment of whether PMCA1 knockdown using siRNA in NRCM prevents this could be made. Secondly, an assessment of whether PMCA1 interacts with or influences the subcellular localisation of known NCX regulators such as CaMKII or
HDACs by immunoprecipitation, immunocytochemistry or luciferase reporter assay could be performed.

With regards to PMCA4’s role in attenuating hypertrophy and preventing the PMCA1-mediated progression into decompensation in PMCA1:4\textsuperscript{dcko} mice experiments are currently underway to investigate whether this occurs through the regulation of NFκB. In addition to experiments assessing NFκB nuclear translocation and activity following PMCA4 knockdown in NRCM, an analysis could be made of the expression of downstream targets of NFκB signalling such as TNFα or IL-6 following TAC (Gordon et al., 2011). It would also be useful to assess calcium handling in PMCA1:4\textsuperscript{dcko} after TAC in order to compare the phenotype with PMCA1\textsuperscript{cko} hearts. It would also be highly interesting to assess whether the ablation of PMCA4 protects against the progression into decompensation in response to long term pressure overload, typically witnessed in wild type mice following 5-8 weeks TAC (Esposito et al., 2002, Toischer et al., 2010, Toischer et al., 2013).

Whilst assessing intracellular calcium handling in each knockout line all experiments were performed at a stimulation frequency of 1Hz which is roughly compatible with the resting human heart rate. It would be useful to assess whether PMCA ablation had a more pronounced effect on calcium handling when stimulated at frequencies more representative of the physiological heart rate in mice before and after TAC. It would also be interesting to examine whether diastolic calcium levels are altered in PMCA ablated hearts as has previously been witnessed in the presence of carboxyeosin (Choi and Eisner, 1999b), and if so which isoform is responsible.

In light of Cre-mediated toxicity in ageing mice it was not possible to study the effect of PMCA1 ablation beyond the age of 9 months. Therefore a different model would need to be used in order to study PMCA function at more advanced ages. Our laboratory has developed a heterozygous constitutive PMCA1 mouse line which is viable (Shaheen, 2010). This would appear a highly advantageous model with which to study PMCA1 function in the cardiovascular system in general 1) given the link between PMCA1 and hypertension 2) because CVD is more prevalent in the elderly and 3) as it is more relevant.
to human disease because patients will most commonly carry only one copy of a mutation. This might allow the elucidation of a link between PMCA1 mutations, hypertension and heart disease.

6.11 Overall conclusions

This thesis provides novel evidence that PMCA1 and 4 perform independent roles in cardiomyocytes. Under basal conditions PMCA1 is responsible for the small amount of calcium extruded from the cell via the PMCA during diastole, constituting around 1% of global calcium, whilst PMCA4 serves to modulate systolic calcium levels and possibly contractility. Despite the small contribution of the PMCA to global calcium clearance, each isoform acts independently to profoundly influence the response to pressure overload. This study is the first to identify a potentially protective role for PMCA1 in adapting to the demands of pressure overload, and maintaining or restoring PMCA1 levels during hypertrophy could prove to be of future therapeutic value. In addition, the work presented here provides further evidence that PMCA4 ablation attenuates maladaptive hypertrophic remodelling whilst preserving cardiac function, and that this protein may provide a suitable candidate to target in the ongoing search for an anti-hypertrophic therapy.
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