PMCA as a regulator of calcium/calmodulin-dependent signal transduction pathways

PMCA AS A REGULATOR OF CALCIUM/CALMODULIN-DEPENDENT SIGNAL TRANSDUCTION PATHWAYS

MARYLOUISA HOLTON (BSc)

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Signature: Marylouisa Holton

Date:

ABSTRACT

Plasma membrane calcium/calmodulin-dependent calcium ATPases (PMCAs) are high affinity calcium pumps regulating many calcium-dependent processes and advances in its characterisation have discovered that it may play a novel role in signal transduction pathways. It was the aim of this work to further characterise and confirm the role PMCA plays in regulating calcium/calmodulin-dependent signal transduction pathways.

PMCA4 has already been shown to inhibit the NFAT family of transcription factors by its interaction with calcineurin A in mammalian cells when ectopically expressed. This prompted the investigation into other isoforms of PMCA that may interact with the calcium/calmodulin-dependent calcineurin, to determine if this interaction was isoform-specific in a variety of cell lines. Endogenous proteins were isolated by immunoprecipitation with calcineurin A antibody and the presence of PMCA isoforms was determined by western blot using isoform-specific antibodies. This work has demonstrated that the PMCA and calcineurin interaction occurs *in vitro* at endogenous levels in MCF-7 human breast adenocarcinoma cells and endothelial cells and is isoform specific, predominantly for PMCA2. The characterisation of the PMCA2b-calcineurin A interactive domain was performed and it was demonstrated that PMCA2b significantly inhibits the NFAT/calcineurin pathway. These results indicate that PMCA2 is important in regulating the calcineurin/NFAT pathway in tissues where it is highly expressed. This work also demonstrates that the Flag-tagged, characterised interaction domain of PMCA2 with calcineurin, F-PMCA(462-684) when overexpressed, can disrupt the inhibitory PMCA2/calcineurin interaction in endothelial cells and significantly increase calcineurin activity.

The expression of PMCA in endothelial cells prompted the investigation of calcium/calmodulin-dependent proteins in endothelial cells as evidence for the important role of PMCA in regulating signal transduction pathways.

Nitric oxide synthases have been shown to be regulated by PMCA4 in cardiac cells. To further characterise the regulation of NOS by PMCA, this work shows that there is a novel molecular interaction between endogenous eNOS and the plasma membrane calcium ATPase (PMCA) in HUVEC primary endothelial cells. PMCA2 has been identified as the major isoform interacting with eNOS in endothelial cells.

The interaction between the two proteins has been mapped to the region 735-934 of eNOS and 462-684 of human PMCA2b. NO production was found to be inhibited by ectopic expression of PMCA2b in HUVEC cells. Moreover, disruption of the interaction between endogenous PMCA and eNOS by overexpression of theFlag-tagged, PMCA2b interaction domain, F-PMCA2(462-684), significantly increased NO levels in activated HUVEC endothelial cells. In summary, these results offer strong evidence for a novel functional interaction between endogenous PMCA and eNOS in endothelial cells, suggesting a role for endothelial PMCA2 as a negative modulator of eNOS activity, and, therefore, NO-dependent signal transduction pathways.
Overall this is a novel discovery which clearly demonstrates that PMCA is an important regulator of calcium/calmodulin-dependent signal transduction pathways in various cell types.

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ABBREVIATIONS

ADMA – Assymetric Dimethylarginine

AGE – Agarose Gel Electrophoresis

AI - Autoinhibitory

AKAP-79 – A Kinase Anchoring Protein -79

AMPK – AMP-Activated Protein Kinase

AP-1 – Activator Protein 1

APS – Ammonium Persulphate

ATCC – American Type Culture Collection

ATPase – Adenosine Triphosphatase

BAD – Bcl-2-Associated Death Promoter

BCA – Bicinchoninic Acid

Bcl-2 – B-Cell Lymphoma-2

BD – Binding Domain

bFGF – Basic Fibroblast Growth Factor

BH4 - Tetrahydrobiopterin

BSA – Bovine Serum Albumin

Ca^{2+} - Calcium

Cabin – Calcineurin Binding Protein

Cain – Calcineurin Inhibitor Protein

CaM – Calmodulin

CBP1 – Calcineurin Binding Protein 1

cDNA – Complimentary Deoxyribonucleic Acid

cGMP – Cyclic Guanosine Monophosphate

CHP – Calcineurin Homologous Protein

CK1 – Casein Kinase 1

CK2 – Casein Kinase 2

CNB – Calcineurin B

Cox-2 – Cyclooxygenase – 2

CsA – Cyclosporine A

CVD – Cardiovascular Disease

DMEM – Dulbeccos Modified Eagle Medium

DMSO – Dimethyl Sulphoxide

DNA – Deoxyribonucleic Acid

dNTP – Deoxynucleoside Triphosphates

DR – Diabetic Retinopathy

E.Coli – Escherichia Coli

EDTA – Ethylene Diamine Tetra Acetic Acid

Eef1A1 – Translation Elongation Factor 1 Alpha-1

EGF – Epidermal Growth Factor

eNOS – Endothelial Nitric Oxide Synthase

ERK – Extra-Cellular Signal Regulated Kinases

EtBr – Ethidium Bromide

FAD – Flavin Adenine Donor

FAK – Focal Adhesion Kinase

FasL – Fas Ligand

FBS – Fetal Bovine Serum

FGF – Fibroblast Growth Factor

FGF2 – Fibroblast Growth Factor 2

FKBP12 – FK506 Binding Protein 12

FKBP38 – FK506 Binding Protein 38

FMN – Flavin Mononucleotide

GA-1000 – Gentimycin

GM-CSF – Granulocyte Macrophage Colony-Stimulating Factor

GSK-3 – Glycogen Synthase Kinase-3

GTP – Guanosine Triphosphate

HEK293 – Human Epithelial Kidney 293

HEGF – Human Epithelial Growth Factor

HEPES – 4-(2-hydroxyethyl)-1-Piperazineethanesulfonic Acid

hFGF – Human Fibroblast Growth Factor

HSP90 – Heat Shock Protein 90

HUVEC – Human Umbilical Vein Endothelial cells

IBMX – 3-Isobutyl-1-Methylxanthine

IL – Interleukin

INF – Intrinsic Factor

iNOS – Inducible Nitric Oxide Synthase

IP - Immunoprecipitation

IP_3R – Inositol 1,4,5-Triphosphate Receptor

JNK2 – Jun N-Terminal Kinase

Kb – Kilobase

kD - kilodalton

KO – Knockout

LB – Luria Broth

MAGUK – Membrane Associated Guanylate Kinases

MAPK – Mitogen-Activated Protein Kinase

MCIP – Myocyte-Enriched Calcineurin Interacting Protein

MCS – Multiple Cloning Site

MEKK1 – MAP Kinase Kinase Kinase

$MgCl_2$ – Magnesium Chloride

mRNA – Messenger Ribose Nucleic Acid

MS – Multiple Sclerosis

NaAc – Sodium Acetate

NADPH -Nicotinamide Adenine Dinucleotide Phosphate

NEM – Non-Essential Amino Acids

NES – Nuclear Export Sequence

NFAT – Nuclear Factor Of Activated T-Cells

NFkB – Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells

NHERF – Na^+/H^+ Exchanger Regulatory Factor 2 (NHERF2)

NIHL – Noise Induced Hearing Loss

NLS – Nuclear Localisation Sequence

nNOS – Neuronal Nitric Oxide Synthase

NO – Nitric Oxide

NOS – Nitric Oxide Synthase

NOSIP – Nitric Oxide Synthase Interacting Protein

NOSTRIN – Nitric Oxide Synthase Trafficker

OD – Optical Density

PAGE – Polyacrylamide Gel Electrophoresis

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PDGF – Platelet-Derived Growth Factor

PI3/AKT1 – Phosphatidylinositol-3 Kinase/V-Akt Murine Thyoma Viral Oncogene Homologue 1

PIP2 – Phosphatidylinositol Bisphosphate

PISP – PMCA-Interacting Single-PDZ Domain Protein

PKA – Protein Kinase A

PKC – Protein Kinase C

PMA – Phorbol Myristate Acetate

PMCA – Plasma Membrane Calcium/calmodulin-Dependent ATPase Pump

PMSF - PhenylMethaneSulphonylFluoride

PP1 – Protein Phosphatase 1

PP2A – Protein Phosphatase 2A

PP2C – Protein Phosphatase 2C

R3-IGF-1 – R3-Insulin-Like Growth Factor

RIPA – Radio-Immune Precipitation Assay

RLU – Relative Luciferase Units

RNA – Ribose Nucleic Acid

ROS – Reactive Oxygen Species

SAPs – Synapse Activated Proteins

SDS – Sodium Dodecyl Sulphate

SERCA – Sarcolemmal Endoplasmic Reticulum Calcium ATPase

SHR – Spontaneous Hypertensive Rats

SOD – Superoxide Dismutase

SP -1, -2 and -3 – Serine-Proline Rich Regions -1, -2 and -3

SRR-1 and -2 – Serine-Rich Regions

TAE – Tris-Acetate-EDTA

TBS – Tri Buffered Saline

TE – Tris-Chloride/EDTA

TEMDED – N,N,N,N-Tetramethyl-Ethylenediamine

TGF – Tumour Growth Factor

TNF – Tumour Necrosis Factor

UTR – Untranslated Regions

UV – Ultraviolet

VCAM-1 – Vascular Cell Adhesion Molecule-1

VEGF – Vascular Endothelial Growth Factor

VSMC – Vascular Smooth Muscle Cells

WB – Western Blot

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1. CHAPTER ONE:

INTRODUCTION

1.1 PLASMA MEMBRANE CALCIUM/CALMODULIN-DEPENDENT ATPASE

The Plasma Membrane calcium/calmodulin-dependent ATPase (PMCA) is a P-type calcium ATPase pump (Pederson et al., 1978 and Pederson et al., 1987). Its main function is to export calcium, against a concentration gradient from the intracellular matrix to the extracellular milieu (Rosado et al*.,* 2004). This is important because calcium is a regulator of gene transcription and therefore, many cellular processes such as, angiogenesis and cell proliferation, (Rosado et al*.,* 2004). There are other proteins besides PMCA that control intracellular calcium concentrations such as Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) or Na^{+}/Ca^{2+} pumps. PMCA has not been characterised to the same degree as other calcium pumps and research into this ATPase will provide a more detailed explanation for the role of calcium and the effects it can have on various processes, as well as providing another, possibly more specific, avenue for the manipulation of intracellular calcium levels and the cellular processes controlled by them.

1.1.1 PMCA genomic structure

There are four PMCA isoforms (PMCA1, 2, 3 and 4) which are the products of four independent genes located on chromosomes 12, 3, X and 1 respectively (reviewed in Strehler and Zacharias, 2001). The structure of PMCA genes have only been partially characterised due to their large size, which is between 70kb to over 100kb depending on the isoform, and information on regulatory domains is scarce (Burk and Schull, 1992 and Hilfiker et al., 1993). The four isoforms of PMCA share a high degree of sequence homology. All show the features described in fig. 1.1.1 and are predicted to have a very similar structure (Strehler and Zacharias, 2001).

Research into the characterisation of the PMCA isoforms has shown that they are distributed differently in certain tissues. PMCA1 and 4 are ubiquitously expressed in most tissues, PMCA2 is expressed mainly in cerebellar Purkinje cells with some heightened expression in the uterus, liver, kidney and lactating mammary glands, thought to provide the high calcium levels found in breast milk, and PMCA3 is the least expressed found only in the brain and, at certain time points, in the skeletal muscle, (Strehler and Zacharias, 2001).

1.1.2 PMCA protein structure

The structure of PMCA consists of ten transmembrane domains with the bulk of the protein residing on the cytosolic side of the membrane. Domains of interest include the transduction domain, the ATPase domain and the C-terminal domain which contains a calcium/calmodulin binding domain and a PDZ-binding domain. PDZ binding domains bind to the carboxyl end of proteins or form dimers with other PDZ domain containing proteins, they are named after PSD-95, discs large, zona occludens 1 proteins (Kim et al., 2004). The transduction domain lies between transmembrane domains 2 and 3 (Fig. 1.1.1), it is important in the conformational change of the protein during activation and also contains an important domain for phospholipid activation (Niggli et al., 1981).The transduction domain also contains a site for alternative isoform splicing, known as splice site A. The ATPase domain lies between transmembrane domains 4 and 5 (Fig. 1.1.1), this is also known as the catalytic domain of the protein and includes an ATP binding site and aspartate residues which during ATP hydrolysis are important for the formation of an acyl phosphate intermediate. The C-terminal domain contains a binding site for proteins containing PDZ domains and also a site for alternative splicing, splice site C that overlaps the site for calcium/calmodulin binding and the autoinhibition domain. This domain is considered the major regulatory domain of PMCA (Penniston et al., 1998). See fig. 1.1.1 for a structural representation of PMCA.

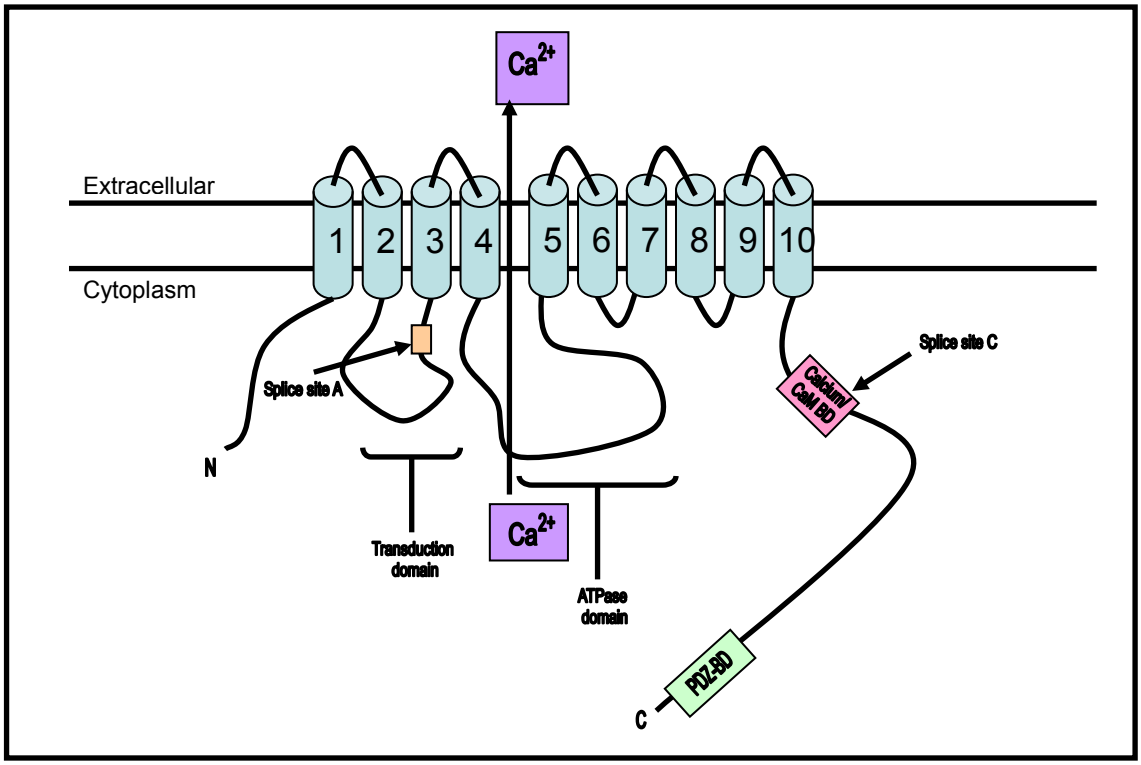

Fig. 1.1.1 The structure of PMCA. (adapted from Strehler and Zacharias, 2001). All isoforms of PMCA contain the same important domains which are important for its function and regulation of its activity.

1.1.3 PMCA splice variants

Over twenty splice variants of the PMCA pumps have been described (Fig. 1.1.2). These splicing events occur in splicing sites A and C located in the transduction domain and the c-terminal domains respectively (Di Leva et al., 2008).

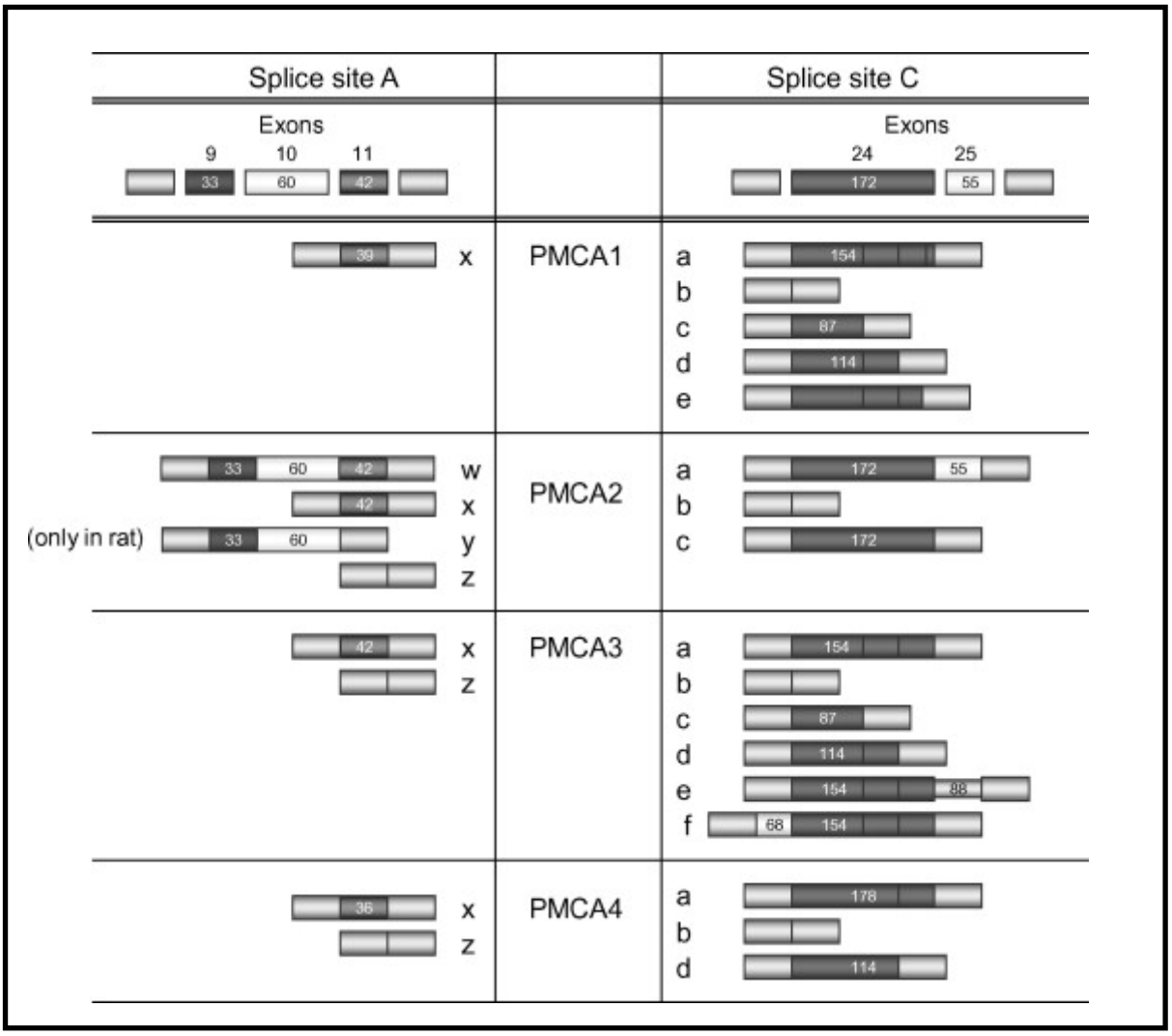

Fig. 1.1.2 The alternative splice variants for each PMCA isoform (Di Leva et al., 2008). All the variation of PMCA splice variants occurs at either the A-splice site within the first intracellular loop or the C-splice site in the C- terminal domains of the protein.

Each splice variant is thought to involve the localisation and specialisation of PMCA to a particular cell type or function, for example splice variant PMCA2*w/a* is mostly in the stereocilia of hair cells (Hill et al., 2006).

1.1.4 Regulation of PMCA activity

PMCA is an important regulator of intracellular calcium levels therefore, the control of its activity is of paramount importance and is exerted through various mechanisms at several levels.

1.1.4a Calcium calmodulin

PMCA is regulated by the intracellular concentrations of calcium/calmodulin. In an inactive state PMCA activity is suppressed by binding of an autoinhibitory domain (located on the C-terminus) to two regions located in the transduction and catalytic domains (Fig. 1.1.3). When calcium/calmodulin binds to PMCA it displaces the autoinhibitory domain from the active site of PMCA allowing it to pump calcium from the cell via the ATPase pump. It has been discovered that the binding of calcium/calmodulin to PMCA can occur with two different effects.
Calcium/calmodulin can bind to PMCA without dissociating the autoinhibitory domain, therefore rendering the pump inactive, or can cause the displacement of the autoinhibitory domain and activate the pump (Osborn et al., 2004). This indicates that the pump exists in three different states; inactive, partially inactive and active depending on the local calcium concentration, it is hypothesised that the partially inactive state would allow rapid response to changes in intracellular calcium concentrations (Osborn et al., 2004).

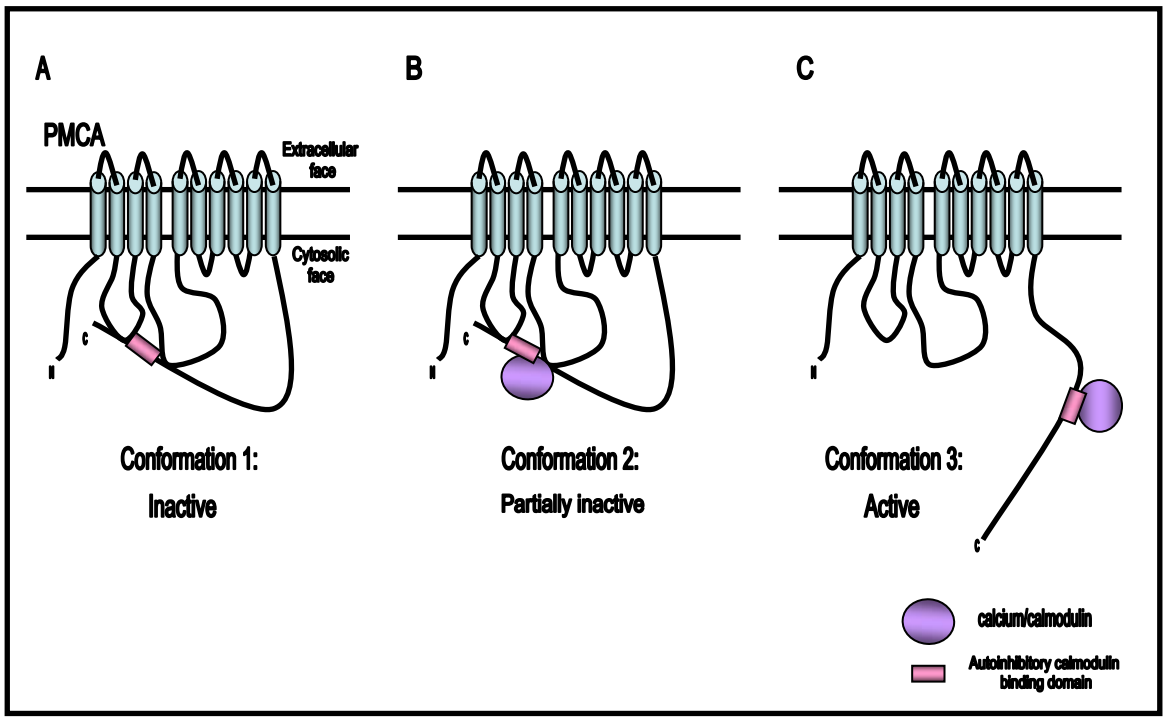

Fig. 1.1.3 Regulation of PMCA by calcium/calmodulin adapted from Osborn et al., 2004. PMCA exists in 3 regulatory conformations. A) Conformation 1 represents the inactive form of PMCA where the autoinhibitory domain blocks PMCA activity. B) Conformation 2 represents the partially active form where calcium/calmodulin is bound to PMCA but the autoinhibitory domain is also still bound. C) Conformation 3 represents the active form where calcium/calmodulin is bound and displaces the autoinhibitory domain resulting in PMCA activation.

1.1.4b Transcriptional control

The transcriptional control of PMCA has not been studied extensively; however, a few groups have reported that the expression of PMCA isoforms is regulated by different mechanisms. Ximenes et al. (2003) have demonstrated that in pancreatic islet cells the transcription and expression of all PMCA isoforms, in particular PMCA2, is decreased by high glucose levels. The reason is thought to be the cells switching from a low efficiency calcium pump to a higher efficiency pump, as calcium plays an important role in insulin release.

In neurons it is thought that PMCA4 expression is controlled directly by calcineurin or indirectly through NFAT-mediated pathways as they found that fully active calcineurin reduced the expression of PMCA4 during the neuron maturation process, no effect was observed on the expression of the other PMCA isoforms (Guerini et al., 2000).

Caride et al. (1999) have shown that by decreasing the level of protein in diet and decreasing calcium reabsorption the transcription of PMCA2 and 3 was markedly decreased in kidney parenchyma cells, whereas, the transcription of PMCA1 and 4 remained the same. The effect is thought to be mediated by calcium levels resulting in reduced calcium reabsorption and excretion in the kidney tubules.

Similarly Guerini et al. (2000) have shown that in cerebellar granule neurons the transcription of PMCA genes isoforms was dependent on sustained intracellular calcium increases, however the stability of the mRNA and its translation efficiency may also be upregulated by calcium.

Pannabecker et al. (1995) also describe another mechanism for PMCA1 and 4 transcriptional regulation in the intestine by vitamin D_3 (1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]). Vitamin D_3 is thought to exert its effects by the presence of a vitamin D_3 regulatory response element in the PMCA promoter, however, this has not been fully characterised (Lowe et al., 1992).

PMCA expression is controlled in a tissue-specific manner in muscle during myogenic differentiation, Hammes et al. (1994) have shown that a myogenic determination factor can result in the expression of different PMCA isoforms and splice variants and it is thought to exert its effects on the PMCA promoter at it's specific DNA motif. Overall it seems the transcriptional control of PMCA is essential for the tissue, isoform-specific expression of PMCA.

1.1.4c Post-translational modifications

1.1.4c(i) Phosphorylation

PMCA is phosphorylated by protein kinases, such as PKC, PKA, Src kinase and focal adhesion kinase (FAK) (reviewed in Di Leva et al., 2008). The sites for these phosphorylations are either serine/threonine or tyrosine residues (Di Leva et al., 2008). Phosphorylation of tyrosine residues of PMCA results in a significant reduction of PMCA activity, it is hypothesised that this inhibition is important in platelet activation (Dean et al., 1997).

1.1.4c(ii) Acidic Phospholipids

Phospholipids are the main component of cell membranes, many different types of phospholipid exist and the ratio of acidic phospholipids within the membrane has been found to regulate PMCA (Tang et al., 2006) by increasing the affinity of PMCA for calcium (Niggli et al., 1981). One of the most interesting phospholipids with respect to PMCA activation is phosphatidylinositol bisphosphate (PIP2), because its composition in the plasma membrane is greatly affected by external stimuli (Di Leva et al., 2008).

1.1.4c(iii) Lipid rafts

Lipid rafts are specialised domains of the plasma membrane high in cholesterol and glycosphingolipids, which are important in signal transduction (Sepulveda et al., 2005). PMCA4 has been found in high concentrations at lipid rafts suggesting it has an important role in cell signalling, especially in cerebellum synapses, (Sepulveda et al., 2005). Tang et al. (2006) have shown that PMCA4 activity depends on the lipid composition and structure of the membrane, this changes with age and disease and may affect PMCA activity leading to calcium imbalance within cells.

1.1.4c(iv) Proteolytic cleavage

PMCA has been shown to be digested by calpain and caspases. Calpain cleaves PMCA at a site upstream from the calmodulin binding domain resulting in an irreversible constitutively active form of the pump (James et al., 1989). The activity of caspases on PMCA activity has been investigated in more detail than calpains. Caspase-3 and to a lesser extent Caspase-7 has been shown to cleave PMCA in apoptotic cells resulting in irreversible activation of the pump and alteration of intracellular calcium concentrations, known to be important in apoptosis (Paszty et al., 2002). Paszty et al. (2005) have also shown that PMCA4 is targeted to the plasma membrane and is fully active without the need for calmodulin during apoptosis. However, Schwab et al. (2002) have evidence that cleavage by caspases can result in complete inactivation of PMCA possibly due to the further degradation of the pump during the apoptotic process leading to secondary necrosis.

1.1.5 Knockout models

Knockout models are often used to determine any biological process in which the expression of a protein is essential, they usually involve the production of a mutant mouse that is lacking the gene for a certain protein or a mouse that has been genetically engineered not to express a certain protein. PMCA knockout mice for PMCA isoforms are described here.

1.1.5a PMCA1

Attempts to produce knockout systems for PMCA1 have failed as it results in embryonic lethality (Prasad et al., 2004), indicating its importance in embryogenesis. One study by Okunade et al. (2004) has demonstrated that PMCA4 knockout mice that are also heterozygous for PMCA1 have increased VSMC apoptosis. This heterozygous mouse model also indicates that PMCA1 is important in the removal of calcium from bladder smooth muscle (Liu et al., 2006).

1.1.5b PMCA2

PMCA2 knockout studies have shown that lack of PMCA2 results in problems with hearing and balance, producing a mouse model known as deafwaddler. The balance phenotypic characteristics of the knockout mouse were thought to be a result of high calcium levels in cerebellar purkinje cells, however, although changes are seen in the number of purkinje cells, they were deemed not significant enough to produce the severe balance defects (Kozel et al., 1998). The current theory is that PMCA2 is essential for the formation of otoconia, calcium carbonate crystals found in the inner ear involved in sensing acceleration and gravity, important in balance (Kozel et al., 1998).

Mice homozygous for a null-mutation of the *PMCA2* gene produced milk with 60% less calcium than wild type animals suggesting an important role for PMCA2 in macrocalcium secretion (Reinhardt et al., 2004). Kurnellas et al. (2005) have demonstrated that PMCA2 may be important in the clearance of calcium from spinal cord motor neurons and PMCA2 null mice display neuronal cell damage.

1.1.5c PMCA3

A PMCA3 knockout mouse has not been reported (Prasad et al., 2007).

1.1.5d PMCA4

PMCA4 knockout studies show that lack of PMCA4 causes low sperm motility due to its necessary presence in the flagellar apparatus of the sperm tail, (Schuh, et al., 2004). Liu et al. (2005) have also shown that PMCA4 is involved in the contractility of bladder smooth muscle, where PMCA4 accounts for 25-30% of the muscles relaxation. PMCA4 has also been implicated in the negative regulation of B lymphocytes by interacting with CD22, a transmembrane glycoprotein found only in B cells, and increasing calcium expulsion after B cell receptor activation (Chen et al., 2004).

1.1.6 PMCA in physiological processes

The importance of PMCA in controlling calcium levels within cells means that it plays a significant role in many physiological processes that use calcium as a signalling mediator. *In vivo* and *In vitro* studies have shown the relevance of PMCA function in several physiological processes described here.

1.1.6a Apoptosis

Apoptosis or programmed cell death occurs either by the activation of death receptors or by limited growth/survival factors due to stress (Peluso et al., 2003). Apoptosis due to stress usually involves an increase in intracellular calcium concentration (Yu et al., 2001). Schwab et al. (2002) have shown that PMCA2 and 4 are cleaved by caspases at a site close to the C-terminal end of PMCA during neuronal cells apoptosis resulting in a lack of PMCA function, degradation and a subsequent increase in intracellular calcium levels. This hypothesis is further confirmed in work by Chami et al. (2003) that demonstrated that PMCA is inactivated by caspase-3 cleavage.

1.1.6b Tumour Necrosis Factor (TNFα)-induced cell death

TNFα is a proinflammatory signalling molecule and has been shown to induce cell death in certain cell types by apoptosis or necrosis (Beyaert et al., 1994). Ono et al. (2001) have demonstrated that when PMCA4 was mutated in murine fibrosarcoma cells they became resistant to TNFα-induced cell death. This was discovered to be due to an elevation in intracellular calcium and the subsequent effect on lysosome function and their inability to increase in volume.

1.1.6c Neuronal development

Neuronal cell differentiation is known to be controlled by calcium concentrations. Usachev et al. (2001) have shown that calcium efflux by PMCA increased significantly during neuronal cell differentiation and that the PMCA2, 3 and 4 isoforms were upregulated. Conversely Brandt et al. (1996) demonstrated, using a derivative of the same cell line, that PMCA1 was important in neurite extension. Schwab et al. (2002) have shown that PMCA2 is important in the apoptosis and secondary necrosis of neuronal cells after ischemia. PMCA2 has also been demonstrated to be highly important in purkinje neurons where it is involved in signalling for motor coordination, synaptic plasticity, synapse elimination and learning (Kurnellas et al., 2007). These investigations suggest that different isoforms of PMCA may be important at different stages of neuronal cell development.

1.1.6d Platelet aggregation

Platelets are activated by increases in cytosolic calcium (Sargeant et al., 1994). It is hypothesised that PMCA is an important regulator of platelet activation. It has been observed that PMCA is often overexpressed in platelets of diseased individuals such as diabetics and hypertensives as described below. Inhibition of PMCA activity by tyrosine phosphorylation has been shown to be important during thrombin-mediated activation of platelets suggesting an essential role for the inhibition of PMCA in rapid platelet aggregation (Bozulic et al., 2007).

1.1.6e Calcium influx into milk

The mammary gland requires high levels of calcium from the blood for inclusion in the production of milk (Reinhardt et al., 1998). This influx of calcium into mammary secretory cells is tightly controlled. The expression of PMCA2 has been shown to be upregulated during lactation and its expression is clearly important in the production of milk calcium and milk proteins in mammary gland milk production (Reinhardt et al., 2004).

1.1.6f Kidney reabsorption of calcium

In the kidney, the renal tubules are important in the reabsorption of calcium. Blankenship et al. (2001) demonstrate that the regulation of this reabsorption depends on the inhibition of PMCA. Magyar et al. (2002) have shown that the predominant isoforms of PMCA expressed in the renal tubules are 1 and 4. The regulation of PMCA expression and membrane localisation in the distal kidney is affected by hormones, in particular vitamin D_3 (Kip et al., 2004) and oestrogen (Oz et al., 2007).

1.1.6g Sperm motility

Sperm motility is essential for the successful fertilisation of the female egg. Schuh et al. (2004) have demonstrated that PMCA4 is highly expressed in the testis and localised to the flagellar of the sperm tail, implying its importance in sperm motility. By knocking down the expression of PMCA4 sperm motility was inhibited, however the sperm still had the capacity for fertilisation (Okunade et al., 2004).

1.1.7 PMCA in diseases

As well as having a significant role in certain physiological processes PMCA has also been shown to be important in many pathological conditions either through aberrant expression or activity.

1.1.7a Cancer

Cancer is the unregulated growth of cells resulting in the formation of detrimental tumours. An imbalance in calcium levels has been shown to contribute to cellular proliferation and tumorigenesis (Hanahan and Wein, 2000). Lee at al. (2002) suggest that calcium transport proteins may play an important role in regulating calcium levels. The expression of PMCA in breast cancer cells has been demonstrated to be upregulated compared to non-tumorigenic breast epithelial cells supporting this hypothesis (Lee et al., 2005). Delgado-Coello et al. (2003) have shown that this is also the case in murine hepatocarcinomas compared to normal or regenerating liver, higher levels of ATPase protein were found in cancerous liver cells. Aung et al. (2007) have demonstrated that during colon cancer cell differentiation the levels of PMCA 4 mRNA are upregulated however the functionality of this upregulation has yet to be determined. Ribiczey et al.

(2007) have also shown that PMCA expression is aberrant in colon and gastric cancer cells. In Human oral cancer cells Saito et al. (2006) hypothesise that the *PMCA1* gene may function as a tumour suppressor as a lack of PMCA1 expression is associated with oral cancer progression. *PMCA1* has been found to be underexpressed in oral cancer cells caused by inhibition of its transcription/translation by DNA methylation (Saito et al., 2006). Clearly the PMCA isoform and its expression are different depending on the cancer cell type.

1.1.7b Heart disease

Hypertension means an increase in blood pressure. Zwadlo et al. (2005) have observed that the expression of PMCA2 is significantly increased in hypertensive individuals and is possibly involved in regulating myocardial growth (Hammes et al., 1998). However Blankenship et al. (2000) have also demonstrated that PMCA is often tyrosine phosphorylated in platelets from hypertensive individuals reducing the activity of the pump and therefore increasing the levels of cytosolic calcium enhancing platelet activation and increasing the risk of thrombosis. PMCA has also been implicated in myocardial infarction, where Mackiewicz et al. (2008) have shown that PMCA activity decreases during the three months after an attack and this decrease may cause myocyte shortening.

1.1.7c Hearing loss

Calcium regulates mechanoelectrical transduction in the inner ear, therefore the expression of PMCA is important in modulating calcium levels (Brini et al., 2007). Furuta et al. (1998) demonstrated that the predominant isoform of PMCA in the inner and outer hair cells of the auditory systems is PMCA2. Kozel et al. (1998) characterised the PMCA2 knockout mouse to suffer from hearing loss due to the normally high expression of PMCA2 in the hair bundle, and Konrad-Martin et al. (2001) suggest that PMCA2 is essential for high frequency hearing. Also noted by Penheiter et al. (2001) was the fact that a lack of PMCA2 was not compensated by overexpression of any of the other PMCA isoforms.

1.1.7d Diabetes

Diabetes is a disease caused by a defect in the synthesis, secretion or function of insulin. β-cells express all 4 isoforms of PMCA and the effect of increased glucose on PMCA in β–cells is to switch from PMCA to a high capacity calcium pump to counteract the inflow of calcium (Herchuelz et al., 2007). The platelets of diabetic patients often have deregulated PMCA expression. Rosado et al. (2004) have demonstrated that PMCA tyrosine phosphorylation is higher is platelets from diabetic patients than from normal patients resulting in decreased calcium extrusion which may increase platelet aggregation and thrombosis formation. Chaabane et al. (2007) demonstrate that the isoform expressed most in diabetic patient platelets is PMCA4 and that the high expression of this PMCA during platelet development may lead to megakaryocytopoiesis or enlarged platelets leading to abnormal platelet function in diabetic patients leading to the cardiovascular disease often associated with diabetes.

1.1.7e Multiple sclerosis (MS)

MS is a chronic inflammatory disease resulting in demyelination, axonal and neuronal cell death (Hafler 2004). Kurnellas et al. (2007) summarise that PMCA2 is important in the balance of calcium in axonal and neuronal cells and that inhibition of PMCA2 results in cytoskeletal changes, neurite swelling and neuronal death. In fact they report that PMCA2 levels decline in MS models (Kurnellas et al., 2005).

1.1.7f Cataracts

Cataracts are thought to be caused mostly by an increase in intracellular calcium in the human lens resulting in increased opacity or lack of transparency, as calcium levels in cataractous lenses are often elevated by 3-3000 fold that of non-cataractous lenses (Marian et al., 2007). In human lens epithelial cells it has been shown that PMCA1 expression is upregulated in the presence of high intracellular calcium levels, suggesting that in some patients with cataracts a lack of functional PMCA1 may allow for the build up of intracellular calcium and increased opacity (Marian et al., 2007).

1.1.8 PMCA as a regulator of signal transduction pathways: Protein partners

PMCA participates in the regulation of physiological and pathological processes via its interaction with partner proteins that play essential roles in signal transduction pathways implicated in the control of these processes. PMCA interacts with many proteins at various regions, some of these regions are specific and can be predicted, such as PDZ-domains present in the interaction partner proteins, other interactions are more difficult to predict. Below is a review of the current known partner proteins of PMCA.

1.1.8a C-terminal interactions

The C-terminal domain of PMCA contains a PDZ-binding domain which is important for interactions with proteins containing this domain, these are described below.

1.1.8a(i) Membrane Associated Guanylate Kinases (MAGUK)

MAGUK are a family of multimodular proteins involved in the formation of cell-cell junctions and cellular signalling. A subfamily of MAGUK is the synapse-activated proteins (SAPs) which are thought to be scaffolding proteins (DeMarco and Strehler, 2001). Both PMCA isoforms 2 and 4 have been found to interact with MAGUK family members via their PDZ domain. Their interaction with PMCA2 and 4 is thought to be involved in localising multiprotein complexes to the plasma membrane, any functionality of the interaction has yet to be determined (Kim et al., 1998).

1.1.8a(ii) Ania 3/Homer protein

Ania 3/homer proteins are involved in the localisation of metaboltropic glutamatergic receptors with other channels and receptors in the brain and are thought to link extracellular signals with Ca^{2+} release and mediate PDZ protein interactions (Sgambato-Faure 2006). The Ania 3/Homer protein interacts with PMCA4 via its PDZ domain and inhibits the activity of PMCA4 (Rimessi et al., 2005)

1.1.8a(iii) Na^+/H^+ Exchanger Regulatory Factor 2 (NHERF2)

NHERF2 are responsible for the removal of Ca^{2+} from cells (DeMarco 2002). They have been reported to interact with many receptors and membrane transporters and are thought to be scaffolding proteins for the localisation of signalling molecules and transmembrane proteins (Bretscher 1999). The interaction has yet to be functionally characterised but only PMCA2 has been shown to be involved in the interaction with NHERF2 via its PDZ domain (DeMarco 2002).

1.1.8a(iv) Neuronal nitric oxide synthase (nNOS)

nNOS is responsible for producing NO in neuronal and cardiac cells. It contains a PDZ domain and is part of a large multi-protein complex. PMCA4 has been shown to interact with nNOS via its PDZ domain and inhibit its activity, this is thought to occur by the tethering of the protein to a low calcium microenvironment (Schuh et al., 2001). Oceandy et al. (2007) have shown that the interaction between PMCA4 and nNOS is functional *in vivo* where it regulates cardiac contractility.

1.1.8a(v) PMCA-interacting single-PDZ domain protein (PISP)

PISP is ubiquitously expressed in all tissues and interacts with all b-splice variant isoforms of PMCA. It is small in size and contains only one PDZ-domain making it unlikely to be involved in localising proteins. It is thought to function solely as a chaperone protein for b splice variants of PMCA in processes such as signalling, endocytosis and trafficking. (Goellner et al., 2003).

1.1.8a(vi) CLP36

CLP36 is a PDZ domain containing enzyme that also contains a LIM domain important in protein associations with the cytoskeleton (Sadler et al., 1992) CLP36 interacts with PMCA4 and also α-actinin-1 and actin tethering the pump to the cytoskeleton in platelets, possibly regulating the pumps activity in these cells (Bozulic et al., 2007).

1.1.8a(vii) CD22

CD22 is a transmembrane glycoprotein found only in B cells. Upon B cell receptor stimulation intracellular calcium concentrations increase resulting in the activation of B cell proliferation, differentiation and CD22 phosphorylation. PMCA4 seems to interact with phosphorylated CD22 where it is activated and increases calcium efflux (Chen et al., 2004). The site or mechanism of interaction has not been defined.

1.1.8b Large intracellular loop interactions

The large intracellular loop of PMCA lies between transmembrane domains 4 and 5, this is the site of the pump's ATPase activity and the point where hypothetically calcium-dependent proteins are inhibited by the low calcium microdomain created by PMCA's calcium extrusion activity.

1.1.8b(i) Rassf1

Armesilla et al., (2004) have shown that PMCA4 interacts with the tumour suppressor protein RASSF1. The interaction between PMCA4 and RASSF1 significantly inhibited EGF-dependent activation of the ERK pathway in HEK293 mammalian cells.

1.1.8b(ii) α–1 Syntrophin

Syntrophins interact with proteins involved in organising signal transduction pathways and are part of a dystrophin complex involved in important physiological processes (Suzuki et al., 1995). Williams et al. (2006) have demonstrated that PMCA1 and 4 interact with α-1 syntrophin in cardiac cells at a domain different to its PDZ domain, suggesting that PMCA, nNOS and α-1 syntrophin exist as a macromolecular protein complex.

1.1.8b(iii) Calcineurin (PP2B)

Calcineurin is a calcium-dependent serine/threonine protein phosphatase (Namgaladze et al., 2005). Buch et al. (2005) have demonstrated that PMCA4 interacts with calcineurin at a site located within the large intracellular loop between transmembrane domains 4 and 5 and inhibits calcineurin activity. It is hypothesised that PMCA4 tethers calcineurin to a low calcium micoenvironment created by the pump calcium extrusion capabilities and therefore inhibits its activity (Buch et al., 2005).

1.1.8c N-terminal interactions

The N-terminal domain of PMCA has not been identified to interact with many proteins, however, one has recently been discovered and is described below.

1.1.8c(i) 14-3-3ε

The 14-3-3 protein family are small acidic proteins thought to be involved in altering protein conformation to enhance phosphorylation events (Mackintosh 2004). Rimessi et al. (2005) have shown that 14-3-3 proteins interact with PMCA4 at a site within PMCA4 N-terminus resulting in inhibition of PMCA4 activity. This interaction is unusual as 14-3-3 proteins routinely interact at phosphoserine or phosphothreonine sites within proteins, of which the N-terminal domain of PMCA4 does not contain (Rimessi et al., 2005).

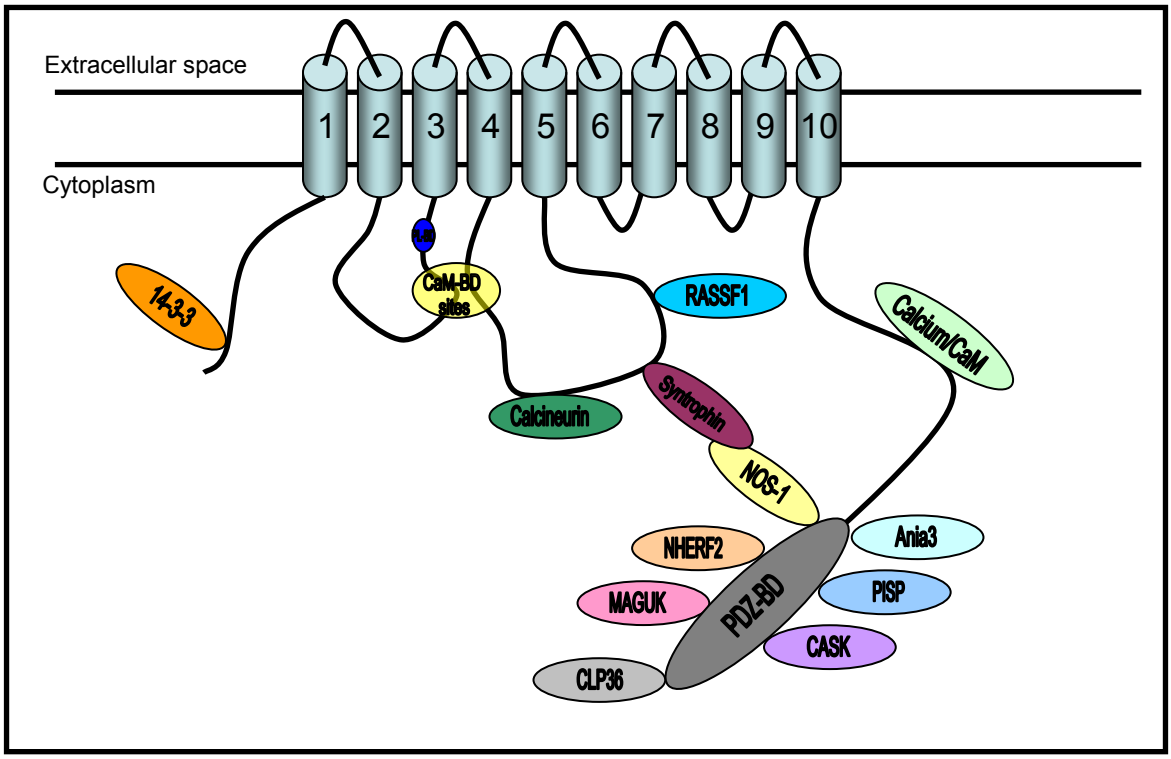

Fig. 1.1.4 PMCA interaction partners. (adapted from Di Leva et al., 2008). PMCA partner proteins all bind to the intracellular portion of the PMCA molecule. Their varying effects on PMCA allow for its regulation and in turn activity of the partner protein is often governed by PMCA.

1.1.9 Summary

Emerging evidence suggests a novel role for PMCAs as regulators of signal transduction pathways. This new function involves the interaction of PMCA with calcium-dependent proteins and tethering them to low calcium microdomains created by the pump's calcium extrusion function. The aim of this project was to demonstrate the importance of PMCA in regulating calcium-dependent signal transduction and to evaluate the role of different PMCA isoforms in the interaction/regulation of calcineurin. Moreover, the previous demonstration of PMCA as a regulator of NO production via interaction with nNOS in neuronal cells prompted us to investigate the possibility of a molecular interaction between PMCA and eNOS; the major NO synthase expressed in endothelial cells. For this reason the calcineurin and nitric oxide synthase signalling pathways will be described in more depth later in this introduction.

1.2 THE CALCINEURIN/NFAT SIGNAL TRANSDUCTION PATHWAY

Calcineurin, also known as, protein phosphatase 2B, is a member of the serine/threonine phosphatases protein family (Namgaladze et al., 2005). This family of phosphatases also includes protein phosphatases 1 (PP1), 2A (PP2A) and 2C (PP2C) all of which have been found to be important for signal transduction pathways (Shenolikar and Nairn, 1991). Calcineurin is ubiquitously expressed in eukaryotic cells and is involved in the regulation of apoptosis (Wang et al., 1999), angiogenesis (Hernandez et al., 2001), immune response (Winslow et al., 2003) and other biological processes (reviewed in Crabtree and Olson. 2002).

1.2.1 Calcineurin genomic structure

The protein itself is made up of two subunits; one catalytic (subunit A) and one regulatory (subunit B) encoded by separate genes. Calcineurin A exists as three isoforms in humans; α, β and γ that are produced by 3 separate genes located on chromosomes 4, 10 and 8 respectively (Giri et al., 1991). Calcineurin B exists in two variants generated from one gene found on chromosome 2, one variant is ubiquitously expressed and the other seems to be expressed in the testes only (Chang et al., 1994).

1.2.2 Calcineurin protein structure

Calcineurin A includes four functional domains (Fig. 1.2.1); an autoinhibitory domain, a catalytic domain, a calmodulin binding domain and a binding site for the regulatory subunit calcineurin B (Qin et al., 2003). These functional domains are conserved among species, however, the COOH and NH_2 termini vary greatly between species as do the calcineurin A genes (Kincaid 1993). Calcineurin is self-regulated by the presence of an autoinhibitory domain which allows binding of the regulatory subunit B. Calcineurin B is displaced upon activation by calcium/calmodulin leading to functional calcineurin (Klee et al., 1998). The affinity of calcineurin B for calcineurin A is so high that only strong denaturation can disassociate the two proteins (Merat et al., 1987).

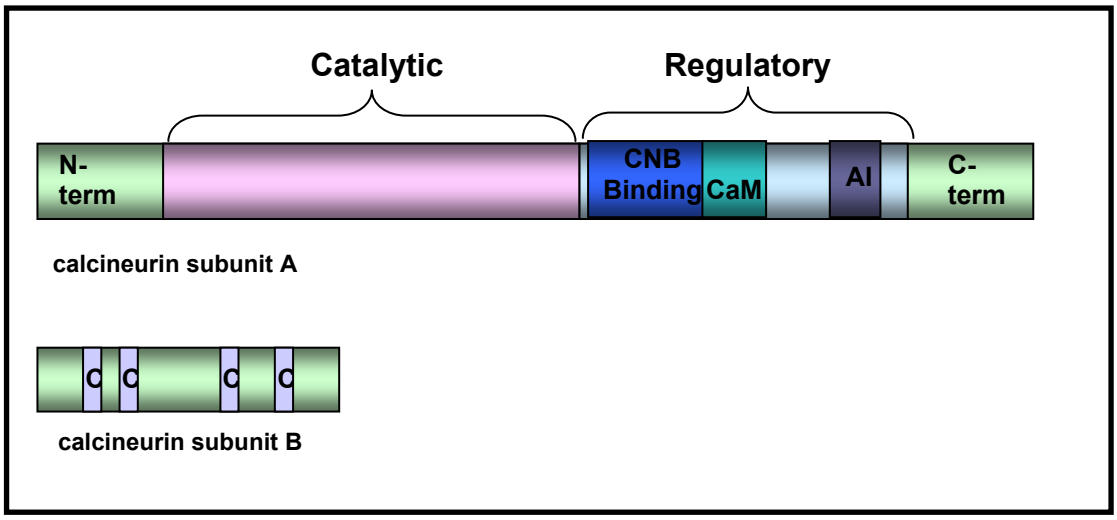

Fig. 1.2.1 Calcineurin protein structure, (adapted from Schulz and Yutzey, 2004). Calcineurin is divided into two definable protein subunits; calcineurin A and calcineurin B. A contains catalytic and regulatory domains which interact with calcineurin B (CNB) calmodulin (CaM) and allow for autoinhibition (AI). B contains sites for calcium binding, (**C**) which ultimately regulates the activity of calcieurin.

The active site of calcineurin, responsible for protein dephosphorylation, contains iron and zinc ions at a binuclear centre. This iron molecule can be subjected to oxidisation by superoxides resulting in a marked reduction in calcineurin activity (Namgaladze et al., 2002).

1.2.3 Calcineurin-dependent activation of Nuclear Factor of Activated T-cells, (NFATs)

NFATs are one of the main substrates for calcineurin. NFAT are a family of transcription factors including five members; NFAT1, 2, 3, 4 and 5 (Rao et al, 1997). The NFAT proteins consist of an N-terminal transactivation domain, followed by a regulatory domain, a conserved DNA binding domain and a C-terminal domain (Fig. 1.2.2)

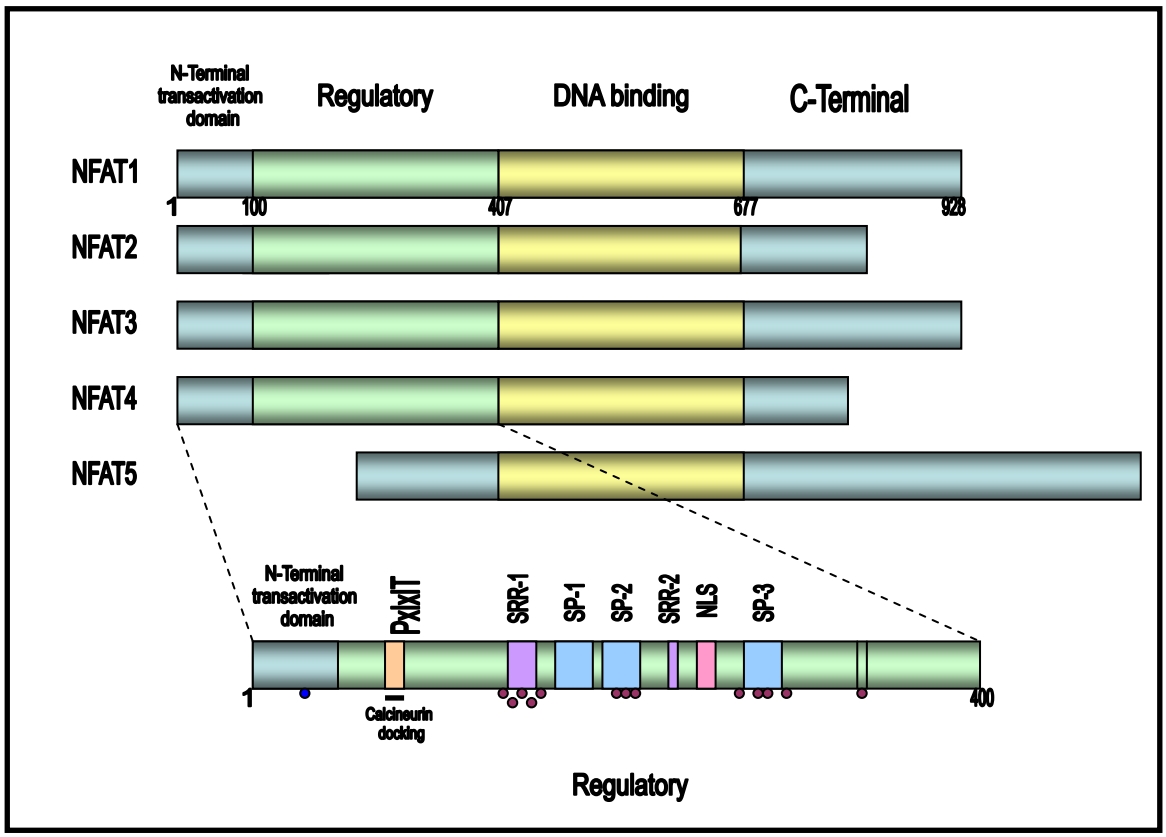

Fig. 1.2.2 NFAT isoforms and domain structure. (Adapted from Macian et al., 2001 and Martinez-Martinez et al., 2006). The regulatory domain has been enlarged to demonstrate important motifs involved in activating NFAT and regulating its activity. The regulatory domain contains two serine-rich regions (SRR-1 and SRR-2), three serine-proline rich regions (SP-1, -2 and -3), a calcineurin docking domain (PxIxIT) and a nuclear localisation domain (NLS). Conserved phosphoserines that become dephosphorylated upon activation are shown as purple circles. The inducible phosphorylation site is shown as a blue circle.

NFAT5 is not activated by calcineurin but shares a high degree of homology with the DNA binding domain of the other NFAT family members (Pan et al., 2000). During the remainder of this thesis when NFAT is mentioned it will refer to NFAT1-4 unless specifically stated. NFAT are activated by a sustained increase in intracellular calcium leading to calcineurin activation. The binding of calcineurin to NFAT involves a conserved sequence motif, PxIxIT, located in the regulatory domain of NFAT (Amburu et al., 1998). Calcineurin dephosphorylates NFAT at multiple serine residues found in the NFAT regulatory domain causing the exposure of the nuclear localisation sequences (NLS) (Im et al., 2004). Upon complete dephosphorylation by calcineurin, a nuclear export sequence (NES) is hidden resulting in the nuclear translocation of NFAT. Once in the nucleus NFAT

binds to regulatory motifs present on the regulatory regions of NFAT target genes (Klemm et al., 1997). Inducers of the calcineurin/NFAT pathway involve stimuli that cause the release of intracellular calcium, such as VEGF (Armesilla et al., 1999). Once NFAT has activated the required genes it is rephosphorylated and translocated back to the cytoplasm. The rephosphorylation of NFAT is performed by kinases, however, the precise kinase involved is still unknown but possibilities include Glycogen synthase kinase-3 (GSK-3), Casein Kinase, MAP kinase kinase kinase (MEKK1), Jun N-terminal kinase (JNK2), protein kinase A or p38 (reviewed in Kiani et al., 2000).

1.2.4 Calcineurin/NFAT physiological effects

1.2.4a Knockout models

Knockout models are often used to identify pathways where proteins are essential. Calcineurin Aα knockout mouse models had decreased T cell activity, reduced calcineurin activity in the brain, heart, kidney and spleen and homozygotes were sterile, (Halloran et al., 1998). NFAT knockout mouse models had varying phenotypic results. NFAT1 knockouts had enhanced B and T cell response. NFAT2 knockouts were embryonic lethal due to a defect in the cardiac valve, also there was reduced B and T cell proliferation. NFAT3 knockout mice presented with defects in the axon outgrowth. NFAT4 knockout caused mildly decreased hyperactivation of T cells.

1.2.4b Immune response

The calcineurin/NFAT pathway is involved in the positive selection of T cells and the expression of genes essential for immune response including; IL-2-5, IL-8, IL-13, GM-CSF and IFN-γ. The promoters of these genes contain binding sites for NFAT. Of great importance to the control of immune response by calcineurin/NFAT is the transcription partner AP-1. It is a balance between the activation of NFAT and AP-1 that regulates the transcription of some cytokine genes and determines if the response results in normal T cell activation or T cell anergy (Macian et al., 2002).

1.2.4c Angiogenesis

Angiogenesis is the formation of blood vessels from pre-existing vessels. Upon VEGF stimulation it is thought that NFAT is involved in the activation of tissue specific genes which are important in regulating angiogenesis. The activation of NFAT by VEGF involves dephosphorylation of NFAT by calcineurin (Armesilla et al., 1999). When activated by VEGF, NFATs are involved in inducing the expression of genes such as cyclooxygenase (Cox-2) (Tsuji et al., 2001) and tissue factor which are important regulators of angiogenesis.

1.2.4d Apoptosis

Apoptosis is also known as programmed cell death, where a series of events and signals generate an apoptotic response. Calcineurin has been shown to dephosphorylate the BAD protein, a proapoptotic Bcl-2 protein, resulting in the dimerisation of other antiapoptotic Bcl-2 proteins and promoting mitochondrial death signals, such as cytochrome C release, caspase activation and subsequently apoptosis (Wang et al., 1999). Calcineurin is also important in calcium-dependent apoptosis involving the activation of calpain and the subsequent cleavage and deactivation of cain/cabin1 resulting in a release of calcineurin from inhibition and NFAT activation of pro-apoptotic genes (Kim et al., 2002).

1.2.4e Bone development

Calcineurin has been implicated in many aspects of bone maintenance including remodelling, formation and resorption (Sun et al., 2005 and Sun et al., 2006). In calcineurin Aα knockout models the mice had lower body weights and importantly lower tibia and femur weight indicating the importance of calcineurin in bone development (Sun et al., 2007). NFAT has been discovered to be critical to cell differentiation in both osteoblasts and osteoclasts (Asagiri et al., 2005). The use of calcineurin inhibitors such as CsA often results in severe bone loss in the patient, although the exact mechanism for this may involve calcineurin-independent pathways due to the unspecificity of CsA (Cunningham 2005).

1.2.4f Skeletal muscle development

NFAT has been found to be expressed in myoblasts and myotubes, with specific isoforms playing different roles at different stages of muscle development (Abbott et al., 1998). Knockout models showed reduced muscle mass due to a decrease in myofiber number and muscle growth (Kegley et al., 2001). Calcineurin is involved in the final stage of skeletal muscle differentiation and is also thought to be critical in overload induced hypertrophy and muscle fibre type specialisation. (reviewed in Schulz and Yutzey, 2004)

1.2.4g Neuronal development

Calcineurin is important in neuronal cells where it has a role in axonal guidance, memory and learning (Mansuy et al., 1998). Calcineurin signalling is involved in a positive feedback pathway which may reinforce synaptic connections (Genazzani et al., 1999). Calcineurin is also important in the regulation of apoptosis in neuronal cells as high expression increases their susceptibility to apoptosis under certain conditions, possibly by cytochrome c/caspase 3 mechanisms (Asai at al., 1999)

1.2.4h Heart development

Calcineurin/NFAT has been shown to be involved in the later stages of heart development, specifically NFAT1 is highly expressed in developing heart valves (de la Pompa et al., 1998). NFAT3 and 4 have also been found to be essential for mitochondrial maintenance and energy metabolism with knockout mice displaying defective cardiomyocyte maturation (Bushdid et al., 2003).

1.2.4i Cell cycle

The cell cycle defines the mechanism behind cell growth and division. There are four primary stages including G_1, gap phase, S, DNA synthesis, G_2, gap phase and M mitosis. Calcineurin has been shown to be important in the G_1 and G_2 phases for the control of cyclin-dependent kinases essential for transcription and translation (reviewed in Kahl and Means 2003).

1.2.5 Calcineurin/NFAT in disease

The calcineurin/NFAT pathway has also been described as an essential mediator of many pathological conditions as described below.

1.2.5a Cancer

Cancer is defined as uncontrolled cell proliferation resulting in malignant tumours and metastases. It has been discovered that certain cancer types have aberrant calcineurin expression thought to aid in cancer progression. A study by Padma et al. (2005) demonstrates the importance of calcineurin in cervical cancer, where calcineurin activity was found to be downregulated, possibly reducing the degree of calcineurin-dependent apoptosis. By suppressing the calcineurin-mediated dephosphorylation of BAD with CsA it is possible that cancer progression may increase (Hojo et al., 1999). Sanli et al. (2003) have also reported that in certain breast cancer cells the activity of calcineurin was markedly reduced due to an upregulation of the calcineurin B subunit. The inhibition of calcineurin can lead to increases in skin cancer rates indicating that calcineurin may be involved in DNA repair (Caforio et al., 2000). However some investigations have revealed that rectal cancer and breast cancer rates were decreased *in vivo* and protection against glioblastoma, leukaemia and bladder cancers was found when calcineurin activity was reduced (Reviewed in Weischer et al., 2007). There is some controversy about the effect of calcineurin in cancer and different results seem to be obtained from studies performed *In vitro* or *In vivo.*

1.2.5b Noise-induced hearing loss (NIHL)

NIHL is defined as the irreversible and cumulative loss of the sensory hair cells of the inner ear and damage to their stereocilia caused by overexposure to intense sound (Hawkins et al., 1976). This acoustic overstimulation also increases calcium concentration in the hair cells which has been shown to activate calcineurin in the auditory hair cells (Minami et al., 2004). Uemaetomari et al. (2005) suggest that increased calcineurin activity may result in increased calcineurin-dependent apoptosis resulting in hair cell death. They demonstrated that treatment with calcineurin inhibitors significantly protected mice from acoustic injury. In fact Vincente-Torres and Schacht. (2006) have provided convincing evidence that in response to noise stimulation, calcineurin dephosphorylates the proapoptotic regulator Bcl-2-associated death promoter (BAD) resulting in activation of mitochondrion-dependent death cascades and apoptosis.

1.2.5c Diabetes

Diabetes is a disease caused by a defect in the synthesis, secretion or function of insulin. Diabetes was found to be regulated in some way by calcineurin as the use of calcineurin inhibitor drugs during transplantation resulted in an increased incidence of the patient developing diabetes (Weir 2001). In fact reduced calcineurin has been found to lower the intracellular concentration of insulin and the transcription of the insulin gene (reviewed in Heit 2007). Complete calcineurin inactivation results in diabetes by β-cell failure (Heit et al., 2006).

1.2.5d Pathological Cardiac hypertrophy

Pathological cardiac hypertrophy is characterised as changes in wall thickness and chamber size, gene expression and collagen content resulting from the autocrine and paracrine actions of various growth factors induced by stress (Lorell et al, 2000). Calcineurin has an important role in transducing the cardiac hypertrophic growth response (Molkentin et al., 1998). It was discovered by Molkentin et al. (1998) that overexpression of constitutively active calcineurin in the heart of transgenic mice induced severe cardiac hypertrophy, treatment of the mice with CsA had the reverse effect further confirming the importance of the calcineurin/NFAT pathway in this process.

1.2.6 Regulation of calcineurin activity

The main function of calcineurin is to dephosphorylate proteins important in cellular regulation, therefore the regulation of calcineurin itself is also essential. Partner proteins and modifications that are involved in its regulation are described below.

1.2.6a Calcium/calmodulin

Calcineurin activity it induced by the binding of calcium ions to the regulatory domain and this increase in local calcium concentration stimulates the binding of calmodulin and subsequent calcineurin activation (Qin et al., 2003).

1.2.6b Calreticulin

Calreticulin is involved in binding and buffering calcium in the endoplasmic reticulum lumen (Michalak et al., 2002). Since calcineurin activation is dependent on intracellular calcium release calreticulin is seen as an important upstream regulator of calcineurin function. Calreticulin possibly activates calcineurin activity by increasing utilizable calcium release from the endoplasmic reticulum (Groenendyk et al., 2004).

1.2.6c Phosphorylation

Calcineurin is phosphorylated by protein kinase C, casein kinase I and II however the activity of the enzyme remains at a similar level to that of its unphosphorylated state indicating that phosphorylation of calcineurin has little regulatory value (Hashimoto et al., 1989).

1.2.6d Myristoylation

Calcineurin B has been found to be consistently myristoylated after translation indicating that this plays an important role in calcineurin regulation, however, the activity of the enzyme is unaltered and the only significance that the modification imparts is increased protein stability at low temperatures (Kennedy et al., 1996).

1.2.6e Superoxides

Calcineurin is inhibited by superoxide oxidation of the iron ion located in the active site of the enzyme. This inhibition has been described as reversible indicating that it is an important physiological regulatory mechanism for calcineurin and calcineurin-dependent processes (Namgaladze et al., 2005)

1.2.7 Exogenous commercial calcineurin/NFAT pathway inhibitors

Since calcineurin/NFAT is important in many pathological pathways it has prompted research and development of drugs to manipulate calcineurin activity and its subsequent pathways. There are three calcineurin inhibitors available for use as immunosuppressants during transplant therapy. All exert their effects by interfering with calcineurin activity and preventing NFAT dephosphorylation (Nghiem et al., 2002).

1.2.7a Cyclosporine A (CsA)

CsA is the most well known of the calcineurin inhibitors. It functions by first binding to cyclophilins, isomerase enzymes and this complex then binds to calcineurin inhibiting its phosphatase activity (Schreiber et al., 1992). This inhibition is caused by the cyclophilin complex blocking the active site of calcineurin resulting in a physical inability of calcineurin to dephosphorylate substrates (Schreiber et al., 1992).

1.2.7b Tacrolimus (FK506) and Pimecrolimus.

Tacrolimus and pimecrolimus are closely related and differ only slightly in their chemical structures and as so inhibit calcineurin in the same way, (Nghiem et al, 2002). Both bind to FKBPs, isomerases, and this complex then binds to calcineurin and inhibits its phosphatase activity by blocking the calcineurin active site.

1.2.8 Endogenous inhibitors of the calcineurin/NFAT pathway

The calcineurin/NFAT pathway is involved in many pathological pathways making it an attractive target for therapeutic drugs. Unfortunately the use of the exogenous inhibitory agents often results in severe side effects such as nephrotoxicity (Myres 1989), hypertension (Starling et al., 1990), reduced immune response and possibly enhancement of patient susceptibility to acquiring cancer (Reviewed in Dantel et al., 2007). Lesser side effects include hypertrichosis, tremors and fatigue (de Rie et al., 1990). These side effects highlight the need for more selective inhibitors and have prompted investigations into more specific endogenous inhibitors of the calcineurin/NFAT pathway. A number of cellular proteins have been reported to bind and inhibit the calcineurin/NFAT pathway, these proteins are described below.

1.2.8a Plasma membrane calcium/calmodulin-dependent ATPase (PMCA)

PMCA is a calcium pump responsible for maintaining intracellular calcium concentration and also plays a role in the regulation of signal transduction pathways. PMCA isoform 4 interacts with calcineurin and inhibits its activity resulting in a reduced NFAT activation, possibly due to the tethering of calcineurin to a low calcium microenvironment (Buch et al., 2005).

1.2.8b A kinase associated proteins (AKAP-79)

This family of proteins is responsible for targeting proteins to specific microenvironments. AKAP-79 is found specifically in neurons and T cells and inhibits the activity of calcineurin and subsequent NFAT activation, thought to occur by the tethering of calcineurin to the plasma membrane away from NFAT (Kashishian et al., 1998).

1.2.8c FK506-Binding protein (FKBP12)

FKBP belongs to a class of immunophilins, it interacts with calcineurin and anchors it in place for localised dephosphorylation of substrates in particular Inositol 1,4,5-triphosphate receptor IP_3R, however calcineurin remains active (Cameron et al., 1995). This complex is also important for the inhibition of calcineurin by FK506.

1.2.8d FKBP38

FKBP38 is involved in mediating the inhibition of calcineurin by FK506 and CsA, it has been shown to block calcineurin phosphatase activity and in this way reduce calcineurin-dependent apoptosis by aiding binding to Bcl-2 and reducing BAD dephosphorylation (Shirane et al., 2003).

1.2.8e Calsarcins

Calsarcins are a class of calcineurin interacting proteins specifically expressed in striated muscle. They interact and co-localise with α–Actinin and are thought to tether calcineurin to areas containing a local calcium pool in the sarcomere of cardiac and skeletal muscle resulting in regulation of calcineurin activity, it is as yet unknown as to the outcome of this regulation (Frey et al., 2000).

1.2.8f Cabin1/cain

Cabin1/cain has been described as a calcineurin binding/calcineurin inhibitor protein (Liu 2003). High mRNA levels of this protein have been found in the brain which is the site for maximal calcineurin expression, indicating a functional link between the two proteins (Lai et al., 1998). Cabin1/cain inhibits the phosphatase activity of calcineurin by binding to a C-terminal domain of the protein (Lai et al., 1998) during T cell activation (Sun et al., 1998) and during synaptic vesicle endocytosis (Lai et al., 2000).

1.2.8g Calcineurin homologous protein (CHP)

CHP is a novel inhibitor of calcineurin that contains sequence homology with calcineurin B and calmodulin (Lin et al., 1999). The mode of inhibition of calcineurin by CHP is under debate, theories suggest that it could compete with calcineurin B for binding or could serve as a calmodulin antagonist (Liu 2003).

1.2.8h Myocyte-enriched calcineurin interacting protein (MCIP/CALP1/calcipressin1)/endogenous calcineurin regulating proteins (RCAN)/calcineurin binding protein 1 (CBP1)

This family of proteins are the only endogenous regulators of calcineurin activity conserved from lower to higher eukaryotes indicating their importance as

regulators of this pathway (Kingsbury et al., 2000). At high concentrations this family of proteins inhibit calcineurin activity and at low concentrations they activate it (Vega et al., 2003). The mechanism for this regulation of calcineurin is under debate but theories are that MCIP1 is needed for calcineurin protein folding or recognition of substrate (MacKintosh et al., 1996).

The cumulative effect of all these interactions is demonstrated in Fig. 1.2.3.

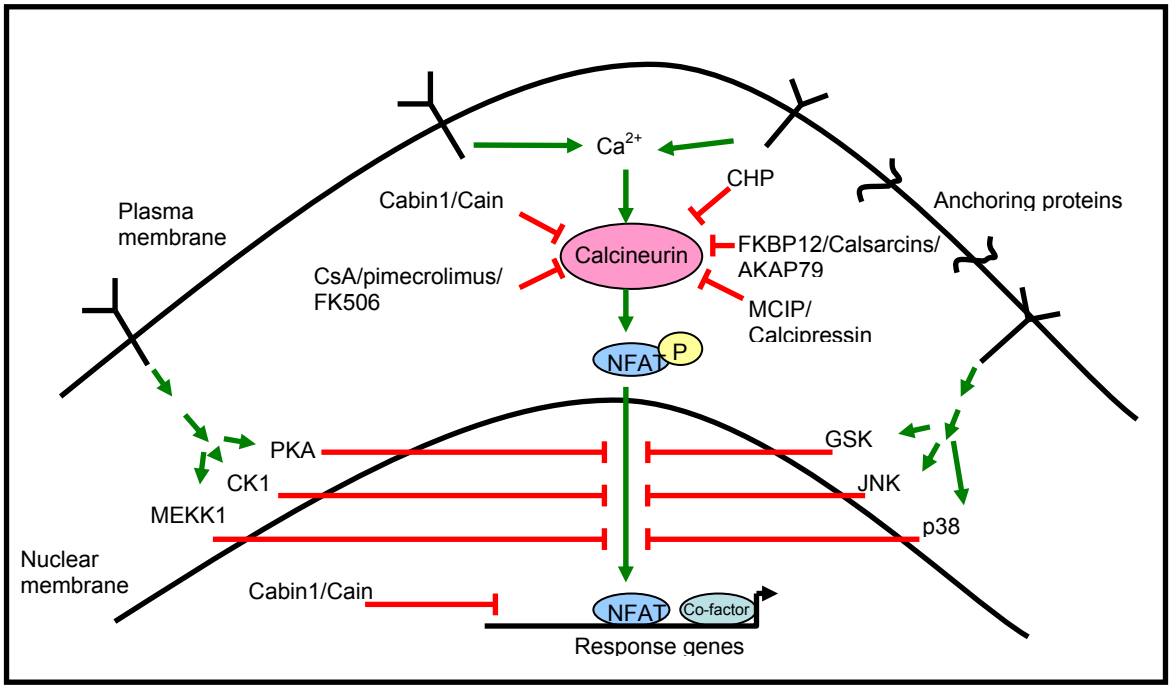

Fig. 1.2.3 Calcineurin/NFAT signalling pathway. (Adapted from Wilkins and Molkentin, 2004) A summary of the calcineurin signalling pathway including inhibitory factors of calcineurin and NFAT kinases. Green lines indicate activation. Red lines indicate inhibition.

1.2.9 Summary

The calcineurin/NFAT pathway is involved in many physiological and pathological pathways and it is important to characterise its role in these processes and identify ways to regulate its activity for therapeutic use. During this project the interaction between PMCA and calcineurin will been investigated in different cells types to help in fully characterising PMCA as a regulator of signal transduction pathways.

1.3 NITRIC OXIDE SYNTHASE

Nitric Oxide synthases (NOS) are heme containing enzymes, (Babaei and Stewart, 2002), which convert the amino acid L-arginine to Nitric Oxide (NO) in response to mechanical stress and a wide range of agonists. NO is a free radical gas used in cells as a signalling molecule, (Pan et el*.,* 2005). It is also involved in regulating key physiological pathways as well as being a cytotoxic molecule with low levels at or below 100nM producing anti-apoptosis and protection against oxidative stress-induced cell death and high levels at 400nM or more giving rise to p53 activation (Thomas et al., 2004). A study by Qiu et al. (2003) demonstrated that in systems with increased microvasculature such as the liver and lungs, eNOS can act to prevent metastasis by releasing high levels of NO and triggering apoptosis under conditions of cell arrest.

NO is responsible for maintaining the vasculature, protecting from platelet aggregates and leukocyte adhesion and most importantly preventing the proliferation of vascular smooth muscle cells. Studies have shown that exogenous NO directly affects angiogenesis (Ziche et al, 1994) and its role as a vasodilator is inevitably crucial in this function. Many groups are currently trying to target the eNOS pathway as a means of regulating blood vessel growth.

1.3.1 NOS isoforms

There are three isoforms of nitric oxide synthase; iNOS (inducible NOS) regulated by cytokines causing it to produce vast quantities of NO comparable to the other isoforms, (Napoli et al., 2006) and calcium/calmodulin regulated nNOS (neuronal NOS) and eNOS (endothelial NOS). All three isoforms use NADPH, (Nicotinamide adenine dinucleotide phosphate) as an electron donor for the synthesis of NO, tetrahydrobiopterin (BH4), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Babaei and Stewart, 2002).

1.3.1a iNOS

iNOS (also known as NOSII) is not found in resting cells or tissues and its expression is induced by septic shock caused by bacterial lipopolysaccharides and cytokines (Schwartz et al., 1999) leading to an increase in systemic NO production. Primarily iNOS is expressed in vascular smooth muscle cells (VSMC) and only induced under stress or inflammation in the endothelium (Nasser et al., 2005)

1.3.1b nNOS

nNOS (also known as NOSI) is constitutively expressed, mainly in neuronal tissue and excitable cells. Its function is regulated by calcium/calmodulin and research has shown that nNOS forms a macromolecular complex together with Syntrophin and PMCA4b that results in negative regulation of its enzymatic activity (Schuh at al., 2001, Schuh et al., 2003 and Williams et al., 2006). nNOS control by PMCA4 has also been implicated in the regulation of cardiac contractility in the heart. (Oceandy et al., 2007). nNOS may function as a fail save mechanism when the activity of eNOS is affected in certain pathophysiological conditions (Schwartz et al., 1999).

1.3.1c eNOS

eNOS also known as NOSIII is the predominant isoform producing NO within the endothelium. This is the isoform on which my project has focussed and as such a more detailed review is provided.

1.3.2 eNOS genomic structure

The gene encoding eNOS is located on chromosome 7, as a single copy in the human genome (Marsden et al., 1993). The eNOS gene contains 26 exons and introns, including intron 13 which contains the alternative splicing exons for production of eNOS splice variants A, B and C during RNA processing, (Lorenz et al., 2007) (Fig. 1.3.1). The functional significance of these splice variants seems to be one of activity inhibition by the formation of heterodimers of truncated and full length eNOS (Lorenz et al., 2007).

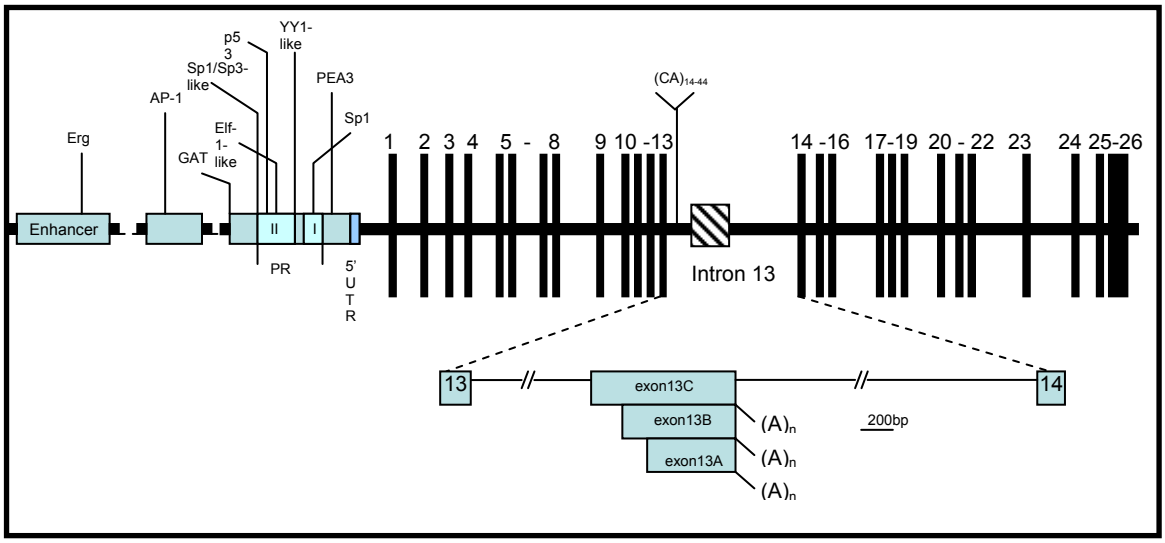

Fig. 1.3.1 The genomic structure of eNOS (adapted from Lorenzo et al., 2007 and Li et al., 2002). A summary of the promoter region and exons and introns of the eNOS gene. Intron 13 is of interest as it contains 3 exons responsible for the 3 known splice variants of eNOS.

The promoter sequence for eNOS contains many transcription factor binding sites including p53 and AP-1 confirming the role of eNOS as a possible regulator of angiogenesis, particularly under cancerous conditions. (Mortensen et al.,1999 and Navarro-Antolin et al., 2000). The regulatory domains of the human eNOS promoter also share a 75% homology with bovine eNOS indicating these sites are important (Venema et al., 1994). It has been demonstrated that the stability of eNOS mRNA can be altered by various growth factors including VEGF and TNFα (Bouloumie et al., 1999 and Yoshizumi et al., 1993).

1.3.3 eNOS protein structure

The molecular mass of the predicted protein is 133kDa. eNOS exists as a homodimer consisting of an oxygenase domain and a reductase domain. The oxygenase domain contains binding sites for arginine, haem iron and BH_4. The reductase domain contains binding sites for NADPH, FAD and FMN with the binding site for calmodulin lying between the two domains (Fig. 1.3.2).

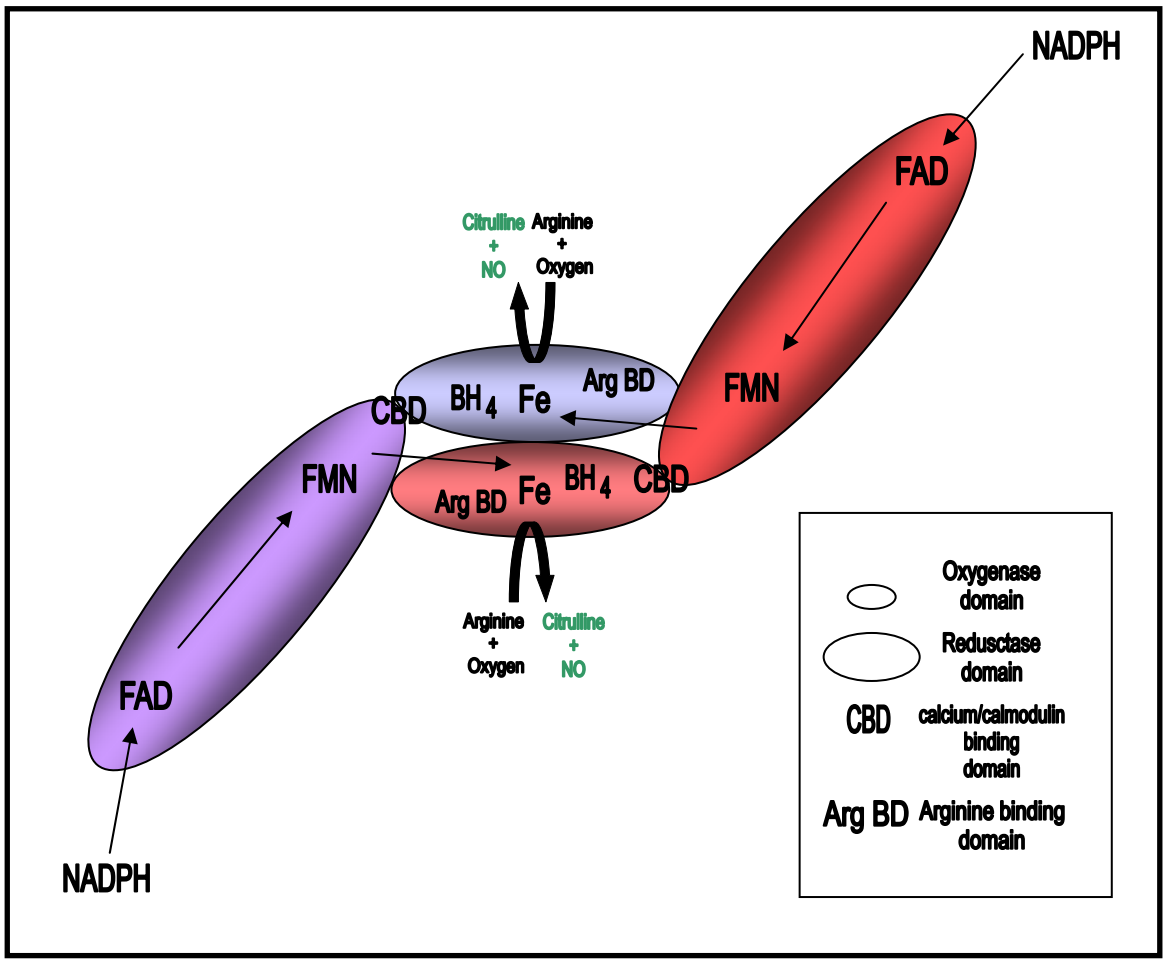

Fig. 1.3.2 eNOS protein structure (Adapted from Alderton et al, 2001 and Fleming et al., 2003). The eNOS protein exists as a homodimer. Each dimer has the same function but uses the oxygenase domain of its partner to catalyse the conversion of Oxygen and Arginine to NO and Citrulline. eNOS uncoupling can result in lack of NO production.

1.3.4 Regulation of eNOS activity

eNOS protein activity is regulated on many levels including transcriptional and post-transcriptional control, growth factors, hormones, phosphorylation, calcium/calmodulin, protein interactions and physiological conditions.

1.3.4a Transcriptional control

Transcriptional control of eNOS occurs mainly by the Sp1 and GATA binding sites in the eNOS promoter. These sites are essential for eNOS promoter activity. Upon mutation of these sites the activity of the promoter is reduced dramatically (Zhang et al., 1995). There are multiple binding sites for transcription factors on the eNOS promoter demonstrating the complexity of eNOS transcriptional regulation (See Fig. 1.3.1). Methylation of the eNOS promoter has been shown to

be important in the expression of the eNOS gene in specific cell types particularly in non-endothelial cells where the eNOS promoter has been found to be highly methylated compared to endothelial cells resulting in an impaired eNOS promoter activity (Chan et al., 2004). A negative feedback mechanism for regulation of eNOS transcription has been described by Grumbach et al. (2005) which hypothesises that NO inhibits NFkB activity, a transcription factor for eNOS itself. Also NO can inhibit eNOS activity and modify the expression of the enzyme by affecting cGMP levels (Abu Soud et al., 2000 and Vaziri et al., 1999).

1.3.4b Post-transcriptional control

The stability of eNOS mRNA is an important factor in determining eNOS translation. The Untranslated Regions (UTR) of the mRNA construct mediate most of the post-transcriptional control. TNFα has been shown to destabilise eNOS mRNA by preventing the binding of a cytosolic protein to the 3'-UTR region of eNOS mRNA important for the stability of the transcript (Gonzalez-Fernandez et al., 2001). Cell proliferation enhancement of eNOS expression has been shown to be a completely post-transcriptional mechanism, where the half-life of eNOS mRNA was increased by over three times but the transcription of the eNOS gene was not altered (Searles et al., 1999). Lipopolysaccharides have been shown to have opposite effect on mRNA half-life, (Lu et al., 1996). FGF and PDGF have both been shown to increase the induction of eNOS mRNA (Cuevas et al., 1996 and Guillot et al., 1999). Under long incubation of eNOS with VEGF, eNOS expression has been shown to be upregulated (Hood et al., 1998).

1.3.5 Regulation of eNOS enzymatic activity

Regulation of eNOS activity is clearly essential due to its involvement in important physiological and pathological conditions. Many mechanisms exist for the regulation of eNOS activity, these are described below.

1.3.5a Acylation

eNOS is the only isoform of NOS acetylated by both palmitate and myristate and provides evidence for a preferential localisation to the cell membrane (Feron et al., 1997). The process of myristoylation is irreversible, however palmitoylation is reversible and thought to be one of the mechanisms for regulating the translocation of eNOS to and from caveolae (Milligan et al., 1995).

1.3.5b S-nitrosylation

The NO produced by eNOS itself is used in the S-nitrosylation of Cys 94 and Cys 99 resulting in eNOS activity inhibition. The reaction occurs in the membrane and it is thought to modify binding of substrate or co-factors to eNOS, however the exact mechanism has yet to be defined, (Erwin et al., 2005)

1.3.5c Phosphorylation

The eNOS protein contains multiple sites for eNOS phosphorylation. There are currently five known sites on eNOS, four serine residues and one threonine residue (Fig. 1.3.3). Three of the four serine residues have a positive effect on eNOS activity when phosphorylated. The serine residue Ser^{114} is the only phosphorylation site in the oxygenase domain of eNOS and its function on eNOS activity has yet to be defined as inhibitory or stimulatory, its effects are thought to change depending on the type of stimuli causing the phosphorylation (Mount et al., 2007). Of the other phosphorylation sites Ser^{1177} has been studied the most and is considered to be the most important phosphorylation site for eNOS activity regulation. It is thought that the carboxy-terminal end of eNOS lies between the two eNOS monomers and causes autoinhibition when the Ser^{1177} phosphorylation site is dephosphorylated (Lane et al., 2002). Ser^{633} and Ser^{615} are both located near the calmodulin binding site of eNOS in the FMN binding domain. Ser^{633} is phosphorylated mostly by PKA, (Boo et al., 2002) and Ser^{615} by Akt (Michell et al., 2002). Interestingly, the Thr^{495} phosphorylation site is involved in the inhibition of

eNOS activity. Thr^{495} is phosphorylated by Protein Kinase C (PKC) and AMP-activated Protein Kinase (AMPK) thought to result in interference with the binding of calcium/calmodulin (Fleming, 2001). Dephosphorylation of Thr^{495} occurs via Protein Phosphatase 1 (PP1), Protein Phosphatase 2A (PP2A) and calcineurin (PP2B), (Thomas et al., 2002).

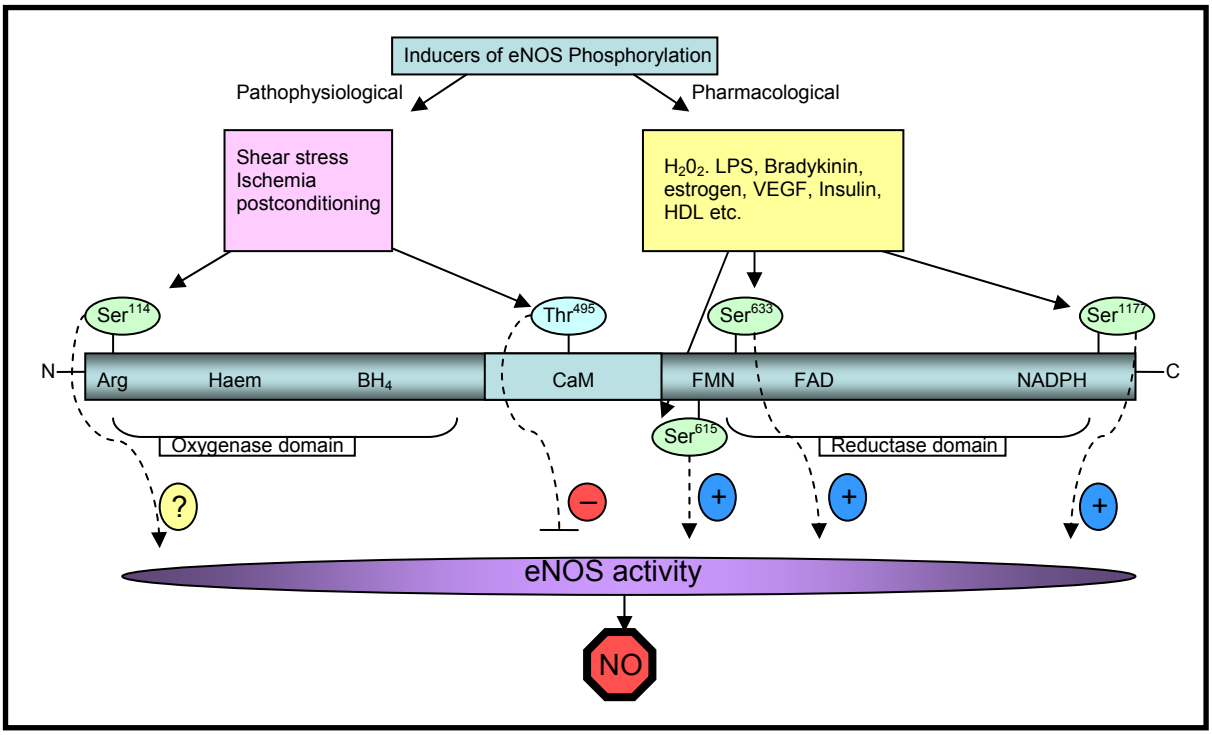

Fig. 1.3.3 Phosphorylation of eNOS (adapted from Kukreja et al., 2007 and Mount et al., 2007). eNOS activity is induced by physiological, pathological and pharmacological stimulators. These stimulators have an effect on eNOS phosphorylation. There are five sites of possible phosphorylation of eNOS three of which are known to activate eNOS activity and NO production, one of which has a negative effect on eNOS activity and a phosphorylation of unknown effect, possibly biphasic.

Differing effects are produced from the phosphorylation of eNOS and all are physiologically relevant. The phosphorylation of eNOS is thought to modulate other modifications of eNOS and allow for interaction of NO with other signal transduction pathways (Michel et al., 1997).

1.3.6 Physiological regulation

As well as regulation by protein modification eNOS activity is also modulated by mechanical and hormonal pathways such as shear stress and growth factors.

1.3.6a Shear stress

Shear stress is the force produced as blood flows through arteries. eNOS is regulated by shear stress (Grumbach et al., 2005). Cai et al. (2004) have shown that NO production from activation of eNOS by shear stress may be important in adaptive arteriogenesis (the development of pre-existing arterioles in to larger arterioles or arteries) a process essential to the bypassing of an occluded main artery or providing a blood supply to ischemic tissues (Yu et al., 2005). It is hypothesised that shear stress induces tyrosine phosphorylation of caveolae proteins resulting in a 3-dimensional reorganisation of the caveolae and release of eNOS from caveolin-1 inhibition, (Rizzo et al., 1998). Shear stress has also been shown to enhance eNOS gene transcription and stabilise eNOS mRNA (Davis et al., 2001).

1.3.6b Growth factors and cytokines

VEGF has two separate effects on eNOS, over the short-term eNOS becomes activated, whereas long-term results in upregulation of eNOS expression. VEGF is also important in the regulation of angiogenesis and atherosclerosis indicating that eNOS is also important to these processes. Angiogenesis, the formation of new blood vessels from pre-existing vessels, is mediated by eNOS via VEGF. The production of NO from eNOS results in apoptosis inhibition (Rössig et al., 1999) and increased endothelial cell proliferation (Ziche et al., 1997) and migration (Ziche et al., 1994). Fibroblast growth factor 2 (FGF2) has also been implicated in controlling the expression of eNOS via extracellular signal-regulated kinase 2/1 (ERK2/1), Jun N-terminal kinase (JNK1/2) and phosphatidylinositol-3 kinase/v-akt murine thyoma viral oncogene homologue 1 (PI3K/AKT1) activation, important members of signal transduction pathways (Meta-Greenwood et al., 2008). Transforming growth factor-β1 (TGF-β1) has been shown to increase mRNA levels in endothelial cells this increased transcription was a result of TGF-β1 recruitment of various important transcription factors (Saura et al., 2002). TNFα has been

shown to significantly reduce the half life of eNOS mRNA by destabilising it through enhancing an interaction with translation elongation factor 1 alpha-1 (eEF1A1) (Yan et al., 2008).

eNOS physiological effects

eNOS and the production of NO in endothelial cell is important in regulating physiological processes such as angiogenesis, vascular tone and apoptosis.

1.3.7a Knockout models

Studies into the effects of eNOS physiologically have been performed in knockout (KO) mice models. The KO mice were found to be hypertensive and lacking endothelium-dependent, NO-mediated vasodilation (Huang et al., 1995 and Gödecke et al., 2001). KO mice also suffered from hind limb ischemia due to impaired arteriogenesis and angiogenesis all indicative of defective VEGF and PDGF pathways (Yu et al., 2005).

1.3.7b Angiogenesis

It has been discovered that angiogenesis is decreased when eNOS activity is lowered, this occurs in diseases such as hypercholesterolemia, (Cooke, 2003). Also Babei et al. (1998) have shown that when endothelial cells are stimulated to differentiate by bFGF the expression of eNOS mRNA and NO production is also increased and important in tube formation. The overexpression of eNOS in smooth muscle cells in a co-culture model was also shown to promote endothelial cell migration and tube formation demonstrating the paracrine effects of NO on angiogenesis (Babaei et al., 2002). It is still thought that the mechanism for eNOS mediated angiogenic response involves activation of endothelial cell matrix proteins such as integrins and adhesion molecules (Dejana et al., 1996 and Koch et al., 1995).

1.3.7c Vasodilation

Vasodilation is the relaxation of the vascular smooth muscle resulting in an increase in the vessel diameter. NO is involved in this process by activating guanylyl cyclases which in turn activate cGMP-dependent kinases thought to be important in vasodilation, their exact mechanism of action is still unknown (Surks 2007).

1.3.7d Apoptosis

Apoptosis also known as programmed cell death is a defined pathway used by cells to control the cell proliferation. Mortensen et al. (1999) have demonstrated that high exogenous levels of eNOS produced NO result in increased apoptosis and that lower endogenous levels protect against apoptosis, it is hypothesised that high exogenous levels may induce apoptosis in invading cells. The mechanism for apoptosis induction is implied to be activation of MAPK and subsequent caspase 3 activation (Kwak et al., 2006). NO species such as peroxynitrite have also been implicated in triggering apoptosis in many cell types (reviewed in Pacher et al., 2007).

1.3.8 eNOS in disease

Since eNOS is involved in the regulation of many important physiological processes abnormal eNOS functioning leads to the progression of many pathological conditions.

1.3.8a Heart disease dysfunctional endothelium and the metabolic syndrome

NO plays a protective role in vascular smooth muscle cells by suppressing abnormal proliferation making NO a homeostatic mediator of vascular health. One important hallmark for cardiovascular disease (CVD) is the reduced bioavailability of NO. The bioavailability of NO can be reduced in many ways, L-arginine availability is one such possibility, however, circulating levels of this amino acid are thought to be high enough to eliminate it from being a rate-limiting step. Several strategies for treatment of CVD have focussed on increasing levels of L-arginine in the endothelium and some beneficial results have been produced (Drexler et al., 1991). Also endogenous inhibitors of eNOS (L-arginine analogues ADMA and L-NMMA) compete with L-arginine and it is hypothesised that an increase in their

circulating concentrations may occur in the diseased state (Vallance and Leiper 2004). A reduction in the availability of the essential eNOS co-factor BH4 is clearly an avenue to explore, however studies have been conducted in vitro only and demonstrated short term benefits when BH4 levels were increased (Maier et al., 2000). Finally, NO can be destroyed by reactive oxygen species (ROS). These ROS react with NO and result in the production of nitrogen/oxygen species which induce protein nitration and lead to coronary heart disease.

1.3.8b Atherosclerosis

Atherosclerosis is the formation of a plaque made up from the aggregation of fatty deposits on the endothelium wall in blood vessels (Fig. 1.3.4). Often caused by underlying conditions such as; hypercholesterolaemia, diabetes and hypertension (Naseem, K.M. 2005). The formation of atherosclerotic plaques is highly complex, however, it is promoted by a lack of NO bioavailability which causes the already abnormal endothelial surface to become more susceptible to platelet aggregation and adhesion. Under normal, physiological conditions NO is vasoprotective and prevents the build up of platelets and inhibits the release of growth factors involved in proliferation, such as VEGF and PDGF. Proliferation is a key event in atherosclerosis formation.

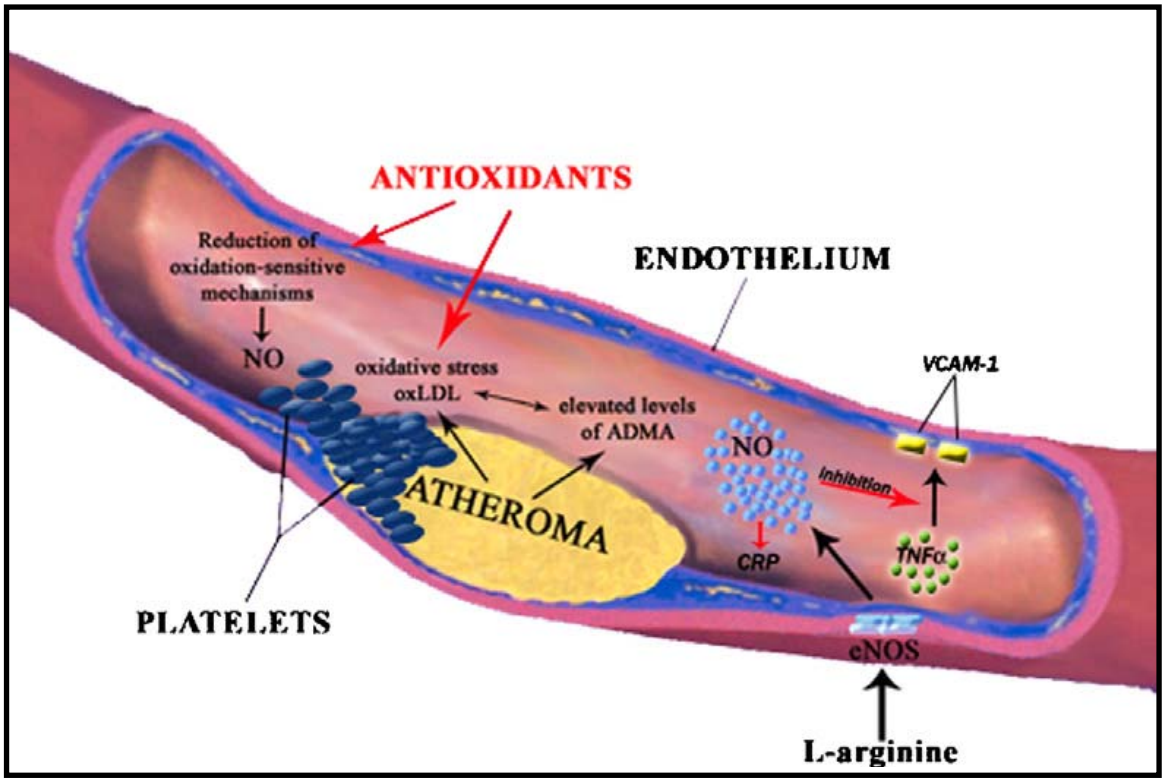

Fig. 1.3.4 The involvement of NO in atherosclerosis formation. (Napoli 2006). eNOS NO production inhibits inflammatory signalling such as the C reactive protein (CRP), Tumour Necrosis Factor α (TNFα) and subsequent stimulation of vascular cell adhesion molecule 1 (VCAM-1). Atheroma are produced when levels of NO are reduced causing platelet aggregation and cause the release of asymmetric dimethylarginine (ADMA) resulting in further inhibition of eNOS activity.

1.3.8c Diabetic retinopathy

Diabetic retinopathy (DR) is a disease manifestation of diabetic microvascular disease causing blindness or reduced vision in both eyes of diabetic patients. The metabolic pathway involved is still to be elucidated, however, endothelial cell dysfunction has been implicated as one of many causes (Khan and chakrabarti, 2007). DR involves increased angiogenesis, tissue ischemia and vascular permeability (Suganthalakshmi et al., 2006). In patients with DR the levels of VEGF expression are elevated and this may affect eNOS activity increasing the levels of NO which again stimulate VEGF expression in a positive feedback loop.

1.3.8d Cancer

Abnormal eNOS expression has been described in certain cancers and it is thought to be regulated by cytokines, growth factors and hormones and also by tumour suppressor inactivation and oncogene activation, (Geller et al., 1998). Tumour-derived eNOS has been shown to promote metastasis and tumour growth by releasing NO and stimulating tumour cell migration and angiogenesis (Jadeski et al, 1999). Studies have shown that the over-expression of eNOS is crucial for angiogenesis and that tumour cells are also capable of producing it. When produced from the eNOS of endothelial cells NO affects surrounding cells to promote proliferation and migration, when produced by tumour cells NO promotes the formation of vessel walls (Pan et al., 2005). VEGF and eNOS exist in cells in a positive feedback loop and it has been discovered that in some cells the distribution of eNOS and VEGF is co-localised helping to produce a cooperative effect (Pan et al., 2005). In malignant melanoma it has been suggested that the overexpression of VEGF and eNOS may be novel markers for the disease as their expression is normal in benign melanocytes (Tu et al., 2006). In gastric cancer eNOS is overexpressed and relates to poor prognosis as it confers cancer progression and angiogenesis, therefore, screening for eNOS may be a useful preoperative marker (Wang et al., 2005). It is hypothesised that combined inhibition of eNOS and the VEGF receptor would be a more effective chemotherapy and that their combined over-expression may be used as markers for some cancers (Tu et al*.,* 2006). However, Chen et al. (2004) stress that the type of tumour must be taken into account before modulating eNOS expression. NO plays a very important role in angiogenesis as inhibition by L-NAME results in reduced angiogenesis (Gallo et al., 1998). It has also been hypothesised that VEGF and eNOS may work in conjunction to increase the number of blood vessels reaching tumours (Shang and Li, 2005) research in this field has shown that eNOS is present in vascularised regions of tumours in the endothelial cells of capillaries (Kucera at al., 2004). NO has also been implicated in reacting with ROS, activating telomerase activity, and delaying endothelial cell senescence (Vasa et al., 2000), providing evidence of a role for eNOS in the promotion of cancer (Jadeski et al., 1999).

1.3.9 Protein interactions

Protein interactions with eNOS modulate its production of NO. The characterisation of the molecular interaction partners that control eNOS activity would undoubtedly constitute an essential step in the development of specific therapeutic regulators of NO production in endothelial cells.

1.3.9a Calcium/Calmodulin

eNOS is a calcium/calmodulin-dependent enzyme. Increases in intracellular calcium caused by external stimuli result in the formation of the calcium/calmodulin complex. This complex displaces an autoinhibitory loop of eNOS resulting in eNOS activity upregulation (Fleming et al., 2003). Calcium/calmodulin activation of eNOS can be disrupted by CK2 kinase which causes the dissociation of calcium/calmodulin from eNOS in a specific manner (Greif et al., 2004).

1.3.9b Caveolin-1

Caveolin-1 coats the caveolae of blood vessel cells and is an important modulator of signalling pathways by compartmentalisation of the cell membrane (Bernatchez et al., 2005). Caveolin-1 has been shown to interact with and inhibit eNOS activity by blocking the calcium/calmodulin binding site of eNOS (Garcia-Cardena et al., 1997). This inhibition can be released by displacing caveolin-1 with a calcium/calmodulin complex (Feron et al., 1998). The caveolin-1 interaction with eNOS is not necessary for eNOS localisation to caveolae (Drab et al., 2001).

1.3.9c Guanine nucleotide binding protein coupled receptors (GPCR) - Bradykinin B2 receptor, angiotensin II and endothelin-1.

Bradykinin B2, angiotensin II and endothelin-1 receptors are G-protein coupled cell surface receptors which span the plasma membrane (Hess et al., 1992). The ligands of these receptors activate eNOS activity, however, the receptors have been found to interact directly with eNOS and inhibit its activity (Marrero et al., 1999). The mechanism of this inhibition is unknown but does not seem to involve disruption of calcium/calmodulin binding as in caveolin-1 inhibition (Ju et al., 1998).

1.3.9d Heat shock protein 90 (Hsp90)

Hsp90 is a chaperone protein essential in the folding of proteins and important in many signal transduction pathways. Hsp90 binds eNOS when it is tyrosine phosphorylated (Harris et al., 2000). Hsp90 enhances the binding of calmodulin by increasing Akt-dependent phosphorylation (Fontana et al., 2002). Endoglin is expressed on endothelial cell membranes and is responsible for stabilising the association of eNOS with Hsp90 (Toporsian et al., 2005).

1.3.9e eNOS interacting protein (NOSIP) and eNOS trafficking inducer protein (NOSTRIN)

NOSIP binds to eNOS and aids the translocation of the enzyme from the plasma membrane to the intra-cellular membranes reducing eNOS activity, (Dedio et al., 2001). NOSTRIN, discovered at the same time as NOSIP, is important in the formation of protein associations with eNOS and *in vivo* aids in the binding of caveolin-1 with eNOS (Schilling et al., 2006).

1.3.10 Summary

eNOS and its production of NO in endothelial cells are clearly an essential part of many physiological and pathological processes. It is important to characterise how eNOS activity is modulated and whether it can be manipulated as part of a therapeutic treatment. This project will look at characterising the involvement of PMCA in the regulation of eNOS in endothelial cells.

1.4 BASIS FOR THIS PROJECT

Previous work has demonstrated that PMCA regulates the signal transduction pathway of calcineurin/NFAT (Buch et al., 2005). This work provided evidence for the important role played by PMCA in inhibiting the activity of calcineurin in mammalian cells and its subsequent signal transduction pathway. The hypothesis that PMCA inhibits calcineurin activity by tethering the calcium/calmodulin-dependent protein to a low calcium microdomain created by the pumps calcium extrusion function, was raised.

Other work by Schuh et al. (2001) demonstrated that PMCA was also responsible for regulating the activity of nNOS in cardiac cells and the subsequent NO signalling pathway, highlighting the novel role of PMCA as a regulator of signal transduction pathways.

1.5 AIMS OF THIS PROJECT

Previous research suggests that PMCA plays an important role as a regulator of signal transduction pathways. The aim of this project was to further define the role played by PMCA in signal transduction pathways in different cells types.

Further characterisation of the interaction between PMCA and calcineurin in various cells types to determine any isoform-, cell type-specific regulation will be investigated. The expression profile of PMCA in breast cancer cells has recently been established, therefore, the effect on the calcineurin/NFAT signal transduction pathway will be assessed.

The hypothesis that PMCA may be important in regulating calcium/calmodulin-dependent proteins and has already been shown to interact with NOS, prompted the investigation for its role in regulating calcium/calmodulin-dependent eNOS in endothelial cells. To further investigate the role of PMCA as a regulator of signal transduction pathways in endothelial cells any interaction with calcineurin will also be determined.

1.6 HYPOTHESIS

The overall hypothesis for this project is that PMCA is an inhibitor of the activity of the calcium/calmodulin-dependent proteins calcineurin and eNOS and their subsequent signal transduction pathways in mammalian cells. This work will provide further circumstantial evidence in support of the hypothesis that PMCA inhibits the activity of calcium/calmodulin-dependent proteins by tethering them to a low calcium microdomain created by the pump itself (fig 1.6.1).

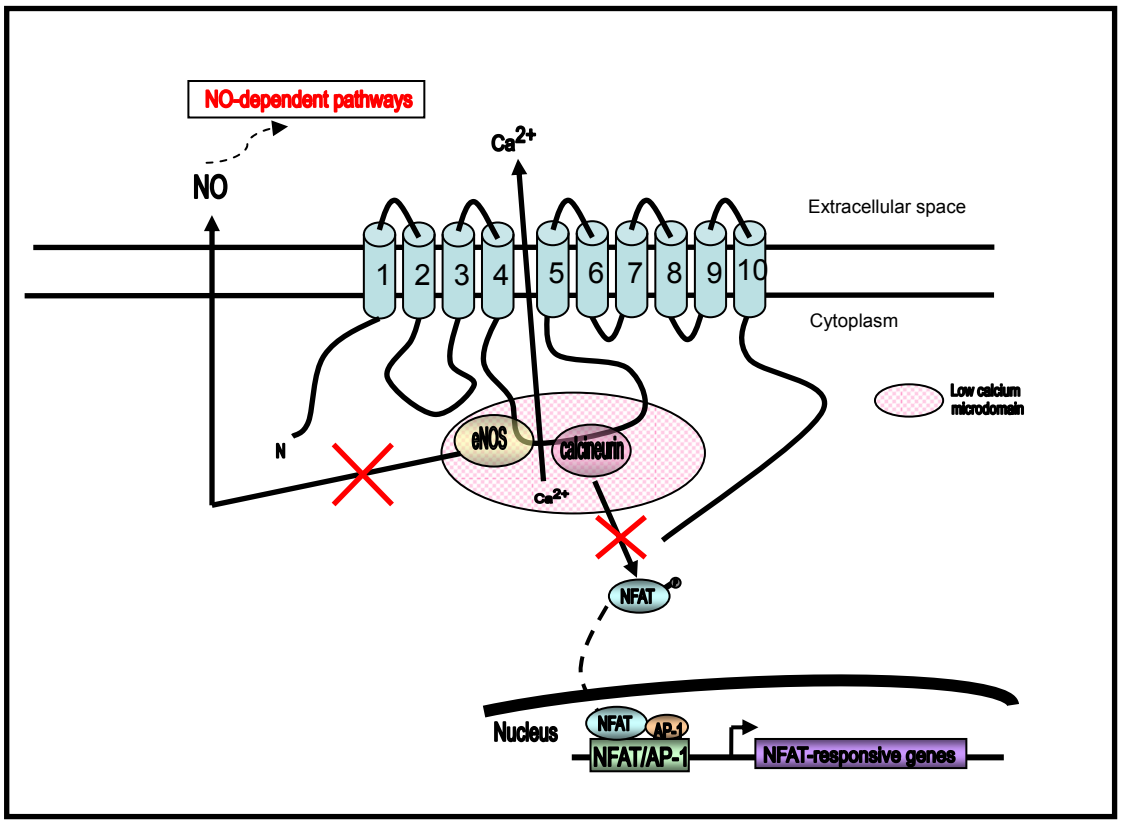

Fig. 1.6.1 Hypothetical involvement of PMCA in the regulation of calcium/calmodulin-dependent proteins and their subsequent signalling pathways.

2. CHAPTER TWO:

GENERAL METHODS

(All materials were produced by Sigma-Aldrich unless otherwise stated. See appendix section A2 for address details on each company mentioned and appendix A3 for details of solutions used in the methods section 2.1).

2.1 Cell culture methods

2.1.1 Primary cells and cell lines

Each primary cell culture and cell line was handled separately from each other to avoid cross contamination. The morphology of each cell line was monitored at each passage and the use of primary cells was stopped at the point of senescence.

2.1.2 Cell culture medium

Hek293 cells (Human epithelial Kidney cells) and MCF-7 (human breast adenocarcinoma) were obtained from the ATCC (American Type Culture Collection) and were grown in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 5% HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1% penicillin/streptomycin (100x), 1% L-Glutamine (200mM) and 1% non-essential amino acids (NEM).

EAhy926 cells (endothelial cell line derived from the fusion of Human Umbilical Vein Endothelial Cells, HUVEC, with A549 carcinoma cell line) were grown in RPMI supplemented with 10% FBS, 1% penicillin/streptomycin (100x) and 1% L-Glutamine (200mM).

HUVEC were cultured in Endothelial Growth Medium 2, (EGM-2) (Cambrex) supplemented with the EGM-2 growth factors kit (Cambrex) containing 10mL FBS, 1μg/mL Hydrocortisone, 1ng/mL human Fibroblast Growth Factor-B (hFGF-B), 0.5ng/mL Vascular Endothelial Growth Factor (VEGF), 20ng/mL R3-Insulin-like Growth Factor-1 (R3-IGF-1), 1μg/mL Ascorbic acid, 5ng/mL Human Epidermal Growth Factor (HEGF), 1μg/mL Gentamycin (GA-1000), 22.5μg/mL Heparin, 10000 U/mL penicillin and 10mgmL streptomycin.

The EAhy926 cell line was the generous gift of Dr Lora-Jean S. Edgell (University of North Carolina, Chapel Hill, North Carolina, USA) and HUVEC cells were purchased from Cambrex.

2.1.3 Freezing and thawing of cells

Primary cells, such as HUVEC, were frozen on arrival and the cell lines were frozen down at various time points so that a stock was maintained. Cells were prepared for freezing by trypsinisation and centrifugation (explained in section 2.1.4). The pellet was resuspended in freezing solution containing 90% FBS and 10% Dimethyl Sulphoxide (DMSO). DMSO is a cryoprotectant and stops the formation of crystals during freezing that may damage the integrity of the cells. Cells were stored at -80°C for one week to allow for slow freezing and then transferred to liquid nitrogen until needed. Cells were defrosted by rapid thawing and resuspended in pre-warmed medium. The cell suspension was centrifuged at 1400 rpm for four minutes and the supernatant containing DMSO, which is toxic to cells, was aspirated away. The cells were then treated as normal.

2.1.4 Passaging cells

Each cell line and primary cells were passaged when they reach 80-100% confluence. The cell medium was aspirated away and the cells were washed with 1x Phosphate Buffered Saline (PBS) to remove any remaining FBS that may inactivate the trypsin. This PBS was aspirated away and trypsin-Ethylene Diamine Tetra acetic Acid (Trypsin-EDTA) (2.5g/L Trypsin and 0.2g/L EDTA) was added and left for approximately five minutes until all cells had detached. Depending in the cell type, complete pre-warmed medium was then added and the cell suspension was collected and centrifuged at 1400rpm for five minutes to pellet the cells. The supernatant was aspirated away and the pellet was resuspended in pre-warmed medium and transferred to a tissue culture flask. The amount of medium used depended on the size of the flask, 175mL flask requires 30mL of medium, 75mL requires 20mL and a 25mL flask requires 5mL.

2.1.5 Cell quantification

Cell density was determined so that results obtained were consistent and cells were at confluence. The cells were passaged until they needed to be transferred to a new tissue culture flask, at this point 1mL of the cell suspension was removed and 20μL of this solution was used to determine cell density. A Neubauer haemocytometer was used and the cell number was determined by counting the number of cells in two 4x4 grids and taking an average. The 4x4 grid represents the number of cells x 10^4 in 1mL of cell suspension.

2.1.6 Transfection of mammalian cells

All Lipofectamine transfection efficiencies were confirmed using a ß-galactosidase expression plasmid and Amaxa transfections efficiencies were confirmed using a GFP expression plasmid as positive controls.

2.1.6a(i) Lipofectamine method

For immunoprecipitation experiments 4.5x10^6 HEK293 cells were plated in 100mm tissue culture plates the day before transfection. 15μg of each plasmid was incubated with 25μL of cationic lipid-based transfection reagent, Lipofectamine (invitrogen) per plate and made up to a final volume of 500μL with Opti-mem (invitrogen). This was incubated at room temperature for thirty minutes to allow for the formation of DNA-lipid complexes. The solution was then pipetted onto the cells and swirled to mix. The cells were left overnight. The following morning the medium was removed, the cells were washed with 1x PBS to remove any Lipofectamine, as this is toxic to cells and 10mL of fresh medium was added. The cells were incubated at 37°C overnight.

2.1.6a(ii) Luciferase gene reporter analysis

For Luciferase gene reporter analysis 1x10^6 HEK293 cells were plated per well in a six well plate the day before transfection. The required amount of plasmid (up to 5μg) was incubated with 5μL of Lipofectamine per well and made up to a final volume of 520μL with Opti-mem. This solution was incubated at room temperature for 30 minutes to allow for the formation of DNA-lipid complexes. The solution was then pipetted onto the cells and swirled to mix. The cells were left overnight. The

following morning the medium was removed along with any dead cells. Viable cells were washed with 1x PBS to remove any Lipofectamine and 5mL of fresh medium was added. The cells were incubated at 37°C overnight.

2.1.6b AMAXA method

Cells were quantified (as described in section 2.1.5) and pelleted. $0.5x10^6$ HUVEC cells were then resuspended in Nucleofector solution (Amaxa AG), 100μL required per transfection. Up to 5μg of plasmid was added to the cell solution and transferred to a nucleofector cuvette. Cells were electroporated according to the manufacturer instructions. Transfected cells were resuspended in 5mL of fresh medium and plated onto a six well plate. After overnight incubation the medium was aspirated away removing any dead cells and viable cells were incubated for another 24 hours in 5mL of fresh medium.

2.2 Methods for DNA preparation and cloning

2.2.1 Polymerase Chain Reaction (PCR)

All PCR products were sequenced prior to use.

The programme used for each reaction included an initial denaturation step of 94°C for two minutes, thirty cycles of denaturation at 94°C for one minute, primer annealing at a temperature relevant for the primers for one minute and elongation at 72°C for one minute thirty seconds. The programme ended with a final elongation at 72°C for ten minutes. The samples were kept at 4°C until needed. A high fidelity PCR master kit from Roche (Roche Diagnostics Limited) was used to setup PCR reactions which contained 4mM of each deoxynucleoside triphosphates (dNTPs), 3mM Magnesium Chloride ($MgCl_2$), Taq DNA and Tgo DNA polymerases and reaction buffer. A standard reaction included 25μL of the master mix described above, 10-500ng of template DNA, 200ng/μL of each specific forward and reverse primer and PCR grade dH_2O to a final volume of 25μL. For each reaction different primers and template DNA were used. The annealing temperature of the primers was determined using the following equation:

$$Tm = [(G + C)4 + (A + T)2] - 4°C$$

All primers were created manually using DNA sequence data from NCBI and ordered from VHBio. The Primer concentration was determined by spectrophotometry. The single stranded DNA was measured at 260nm. To calculate nucleotide concentration 1 optical density unit (OD) at 260nm corresponded to 20μg of single stranded DNA.

2.2.2 Agarose Gel Electrophoresis (AGE)

Agarose gel solution was prepared using electrophoresis grade agarose (Invitrogen) and 1x Tris-Acetate-EDTA (TAE) buffer (invitrogen). Ethidium Bromide (EtBr) (invitrogen), an intercalating agent which fluoresces when excited by light in the ultraviolet spectrum, was also added to allow visualisation of the DNA. The quantity of agarose and ethidium bromide added to TAE buffer was dependent on the size of the DNA and the size of the gel being prepared. A 2% w/v gel was prepared for DNA below 1000 base pairs (bp). 0.7% w/v gel was prepared for anything over 1000bp. For a 50mL gel 2μL of 10mg/mL EtBr was used and for a 100mL gel 3.5μL was used. The gels were electrophoresed at 80 Volts for a 0.7% w/v and 120 Volts for a 2% w/v. Samples were prepared by addition of 6x loading buffer containing bromophenol blue and glycerol. The samples were loaded at a volume of 12μL/well. In addition to the samples a 100mg/mL 1 kilobase (kb) DNA ladder (invitrogen) was used as a DNA length marker loaded at a volume of 12μL/well. The length of time the gels were electrophoresed was dependent on the size of the DNA but was approximately thirty minutes. The gels were viewed under ultraviolet light in a syngene genesnap gel documentation system (Geneflow).

2.2.3 DNA precipitation

DNA produced from PCR experiments was precipitated using 5M Sodium Acetate (NaAc) at a volume $1/10^{th}$ that of the volume of the solution containing the DNA and 100% ice cold Ethanol at a 2.5x volume of the volume of the solution containing the DNA. The precipitating DNA was incubated at -20°C overnight and centrifuged at 13000rpm for seven minutes to pellet the DNA. The supernatant was discarded and the pellet was resuspended in autoclaved dH_2O to the required volume.

2.2.4 Restriction enzyme digestion of PCR product and plasmid

DNA was digested using restriction enzymes from Promega in a reaction containing the relevant buffer, 1x 10mg/mL Bovine Serum Albumin (BSA), DNA sample and dH_2O. The digestion reaction was incubated at the corresponding temperature in a water bath overnight. The following morning an additional aliquot of fresh restriction enzyme was added to the reaction and samples were incubated for an additional three hours. All restriction enzymes and buffers were purchased from Promega.

	Buffer	**Temperature (°C)**
Restriction enzyme digest pair		
EcoRI - BamHI	E	37
HindIII-BamHI	E	37
HindIII-BglII	B	37
EcoRI - XbaI	H	37

Fig 2.2.1. Table of restriction enzymes, buffers and optimum digestion temperatures.

2.2.5 Preparative gel

A large agarose gel was prepared, the percentage depended on the size of the DNA, three wells were joined together to allow 60μL of sample to be loaded. 1Kb ladder was used to allow identification of the DNA size.

2.2.6 Purification of DNA from agarose gels

DNA was visualised by using a UV light box and excised from the gel using a scalpel blade. The excised DNA was transferred to a microfuge tube. A PCR gel clean up wizard kit (Promega) was used to elute the DNA. 300μL of membrane binding solution was added (10μL/10μg) and the agarose containing DNA was incubated at 50°C-60°C for ten minutes, vortexing occasionally to dissolve the agarose. The agarose/DNA solution was transferred to a silica resin minicolumn and incubated for one minute to allow the DNA to attach to the column resin. The column was centrifuged for one minute at 13000rpm, the eluate was discarded

from the collection tube. 700μL of wash buffer was added and the column was centrifuged at 13000rpm and the eluate was discarded again. The column was washed with 500μL of wash buffer and after centrifugation 50μL of nuclease free water was added and allowed to incubate for one minute. The column was centrifuged at 13000rpm for one minute and the eluate containing the DNA was transferred to a fresh microfuge tube and stored at 4°C until needed.

2.2.7 Plasmid dephosphorylation

Plasmids were dephosphorylated before ligation to reduce the amount of plasmid that may re-ligate. Precipitated plasmid DNA was resuspended in 80μL of dH_2O. 10μL of alkaline phosphatase buffer was added and 5μL of 1U/mL shrimp alkaline phosphatase (Promega). The reaction was incubated at 37°C in a water bath for thirty minutes. Then another 5μL of alkaline phosphatase were added to the reaction mixture and that was further incubated for thirty minutes or more. A preparative agarose electrophoresis gel was carried out to purify the plasmid.

2.2.8 Plasmid ligation

Insert DNA and plasmid vector DNA were incubated at a 3:1 Molar ratio, respectively, with bacteriophage T4 DNA ligase (3U/mL) and 1x ligase buffer (Promega) at 15°C overnight.

2.2.9 Plasmid transformation

200μL Competent Escherichia Coli (E.coli), JM109 (Promega) were incubated with plasmid DNA or the ligation reaction at 4°C for ten minutes. The mixture was then incubated at 42°C for two minutes. This heat shock caused the bacterial cell wall to perforate and allowed the DNA to enter the cell. 300μL of fresh Luria Broth (LB) were added to the solution that was incubated at 37°C for one hour to enable the bacteria to produce antibiotic resistance. Transformed bacteria were plated on LB/Agar plates containing ampicillin (100μg/mL) and incubated overnight at 37°C.

2.2.10 Minipreparation

Individual colonies were selected and aseptically transferred to 11mL of LB culture media containing ampicillin (100μg/mL). The culture was incubated on an orbital

shaker at 37°C overnight. 1mL of the culture was aseptically transferred to a microfuge tube as stock. The rest was then centrifuged at 3500rpm for ten minutes and the bacterial pellet was resuspended in 250μL of P1 (resuspension buffer) from a QIAGEN spin miniprep kit from (QIAGEN). The bacteria were lysed by the addition of 250μL of P2 (lysis buffer) and inverted for five minutes. Lysis was stopped with 350μL of P3 (Neutralisation buffer). The lysed bacteria were centrifuged at 13000rpm for ten minutes. The supernatant containing the plasmid DNA was transferred to a purification column and incubated for one minute to allow the plasmid DNA to attach to the column resin. The column was centrifuged at 13000rpm for one minute and the eluate was discarded. 700μL of wash buffer was added to the column and centrifuged at 13000rpm for one minute. The column was transferred to a fresh collection tube and 50μL of elution buffer was added and allowed to incubate for one minute. The column was centrifuged at 13000rpm for one minute and the eluted solution containing plasmid DNA was transferred to a fresh microfuge tube.

2.2.11 Maxipreparation of plasmids

400mL of LB culture media containing ampicillin (100μg/mL) was innoculated with 200μL of bacterial culture that contained the specific plasmid and incubated at 37°C in an orbital shaker overnight. The culture was then centrifuged at 3500rpm for ten minutes and the pellet was resuspended in 10mL of resuspension buffer from a maxiprep kit (Qiagen). The bacteria were then lysed using 10mL of lysis buffer by inversion for five minutes. 10mL of neutralisation buffer was then added and inverted for five minutes. The mixture was then centrifuged at 11400rpm for twenty minutes to pellet the genomic DNA. The supernatant was transferred to a pre-prepared column, (10mL of equilibration buffer was added previously), and allowed to run completely through the column. 60mL of wash buffer was added to the column and allowed to run through. 15mL of elution buffer was added to the column and the eluate was mixed with isopropanol to precipitate the plasmid DNA. This was centrifuged at 11400rpm for thirty five minutes to pellet the plasmid DNA. The pellet was resuspended in 300μL of Tris-chloride/EDTA buffer (TE). The plasmid was then restriction enzyme digested to confirm the presence of the correct DNA insert.

2.3 Methods for protein analysis

2.3.1 Protein extraction from cultured cells

The medium was removed and discarded from the cells and the cells were washed with 1x PBS. The cells were then lysed in RIPA buffer containing; 2mL of 10x PBS, 2mL of 10% Igepal, 1mL of 10% Sodium Deoxycholate, 200μL of 10% Sodium Dodecyl Sulphate (SDS), 20μM PhenylMethaneSulphonylFluoride (PMSF), 500ng/mL pepstatin A, 500ng/mL leupeptin, 1μg/mL aprotinin made up to 20mL with dH_2O. The cells and RIPA buffer were incubated at 4°C on an orbital shaker for ten minutes. The lysed cells were then transferred to a microfuge tube and centrifuged at 13000rpm for one minute to remove any precipitated DNA. The supernatant was retained and stored at 4°C overnight or at -20°C until needed, protein quantification was performed at this step if required.

2.3.2 Protein quantification

Proteins were quantified using the BCA protein assay kit-(reducing agent compatible) from (Pierce) following the manufacturer instructions.

2.3.3 Immunoprecipitation

Agarose A beads (Roche) were prepared by centrifugation at 3000rpm for two minutes and washing with RIPA buffer twice. The protein cell lysate was incubated with 100μL of beads for every 1mL of cell lysate for one hour at 4°C with rotation. This step was called pre-cleaning and was necessary to remove any particles in the protein lysate that may have unspecifically bound to the agarose A beads, to improve the specificity of the immunoprecipitation. The agarose A beads and protein lysate solution was centrifuged at 3000rpm for two minutes. If necessary 12μL of supernatant was aliquoted into a fresh microfuge tube and mixed with 48μL of 4x protein loading buffer (invitrogen), for the analysis of plasmid expression levels. The remaining supernatant was incubated with 40μL of fresh agarose A beads and specific antibody overnight at 4°C with rotation. The immunoprecipitation mixture was then centrifuged at 3000rpm for two minutes and the supernatant was discarded. The remaining agarose A beads, protein and antibody complexes were washed with RIPA buffer twice to remove any unbound

proteins. The beads complex was then resuspended in 2x protein loading buffer and stored at -20°C until needed.

2.3.4 Purification of Flag-PMCA2(462-684) recombinant protein

HEK293 cells were transfected with p3xFlagPMCA2(462-684) using the lipofectamine method described in section 2.1.6a and cell lysates were incubated with anti-Flag®-M2 affinity gel agarose beads overnight. The immunoprecipitated protein was eluted with Flag peptide (125 ng/µl) in RIPA buffer according to the manufacturer's instructions.

2.3.5 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis requires two types of gel, a stacking gel and a resolving gel. The stacking gel was prepared using 1.4 mL of Bis-acrylamide, (Geneflow Ltd) 2.5mL of 4x 1.5M Tris buffer pH 6.8, 6.1mL of dH_2O, 100 µL of 10% Ammonium persulphate (APS) and 60 µL of N,N,N,N-tetramethyl-ethylenediamine (TEMED) added last. The resolving gel was prepared using 3.75mL of 4x 1.5M tris buffer pH 8.8, 100 µL 10% of APS, 60 µL of TEMED and depending on the size of the protein the concentration of Bis-acrylamide and dH_2O was altered. For a protein over 100kD a 6% gel was required containing 2.8mL of Bis-acrylamide and 7.35mL of water. For a protein under 100kD a 12% gel was required containing 5.6mL of Bis-acrylamide and 5.65mL of dH_2O. The resolving gel was prepared first and placed into the PAGE apparatus. 1ml of 100% Butanol was added to the top of the resolving gel to remove any bubbles that may have formed. The gel was then allowed to polymerise for ten minutes. The Butanol was washed away using dH_2O. The stacking gel was then prepared and placed on top of the resolving gel. Proteins were denatured by heating at 100°C for two minutes and centrifuged briefly to gather any evaporated sample. 20 µL of each sample were loaded per well and 12 µL of 1x protein marker Seeblue plus2 (Invitrogen) were added to the first and last wells. The gel was electrophoresed at 200V for one hour in running buffer (0.25M Tris base, 1.92M glycine and 1% w/v SDS) (Geneflow).

2.3.6 Western Blotting

Proteins were transferred from the gel to a nitrocellulose membrane by electrophoresis at 35V for ninety minutes in transfer buffer (0.2M Tris base/1.5M glycine/20% methanol). The membrane was removed and placed into a 5% w/v semi-skimmed milk solution prepared in 1x Tris Buffered Saline (TBS) solution. This was incubated overnight at 4°C on a shaker. The milk solution was discarded and the membrane was washed with TBS-0.1%TWEEN20. The membrane was incubated with the required amount of primary antibody for three hours. The membrane was washed again as before and, if necessary, was incubated with the required amount of secondary antibody for one hour. The membrane was washed again as before and transferred to the dark room. The antibody dilutions used are detailed in fig. 2.3.1.

	Company	**Useage**	**Dilution/concentration**	**Incubation period (Hrs)**
Antibody				
α-PMCA1 (rabbit)	Swant	WB/IP	WB (1:2000)/IP (1:100)	3
α-PMCA2 (rabbit)	Swant	WB/IP	WB (1:2000)/IP (1:100)	3
α-PMCA3 (rabbit)	Swant	WB/IP	WB (1:2000)/IP (1:100)	3
5F10 (mouse)	Abcam	IP	IP (1:1000)	overnight
α-Luc (mouse)	Promega	IP	IP (1:1000)	overnight
α-calcineurinA (mouse)	Sigma	IP	IP (1:1000)	overnight
α-Flag-hrp	Sigma	WB	WB (1:10000)	3
α-eNOS (rabbit)	Sigma	WB/IP	WB (1:1000)/IP (1:100)	3/overnight
α-eNOS (mouse)	Zymed	WB	WB (1:1000)	3
α-rabbit-hrp	Sigma	WB	WB (1:5000)	1
α-mouse-hrp	Sigma	WB	WB (1:5000)	1

WB: Western Blot

IP: Immunoprecipitation

Fig. 2.3.1. Details of Antibody dilutions, source, usage and incubation period.

2.3.7 Western blot development

Membranes were incubated for one minute with a mixture of 1mL solution A and 1mL solution B from an EZ chemiluminescence kit (geneflow). Antibodies were detected by exposing the membranes to autoradiographic film (Kodak).

2.4 Functionality assays

2.4.1 cGMP assay

Cells were transfected with 5μg of each plasmid required. Cells were saturated with L-arginine (30 min, 1 mM) and nascent NO was stabilized by addition of superoxide dismutase (SOD, 100 U/ml of culture medium) (Sigma-Aldrich) five min before lyses. In parallel, 3-isobutyl-1-methylxanthine (IBMX, 1 mM; Sigma-Aldrich) was added to the culture five min before lyses (to inactivate phosphodiesterase activity), and NO synthesis was induced by addition of 0.5 μM A23487 calcium ionophore three min before lyses. Cells were washed three times in cold 1x PBS and resuspended in buffer A from a cGMP enzymeimmunoassay biotrak (EIA) system (Amersham). Cells were resuspended in enough volume to allow protein quantification and duplicate cGMP assay, up to 1 x 10^6cells/mL. cGMP was measured according to the manufacturer's instructions. A thermo lab systems multiskan ascent machine was used to measure the optical density of the final samples.

2.4.2 Luciferase assay

Cells were transfected with the required plasmid and the ratio of the pNuclear Factor of Activated T-cells-Luciferase (pNFAT-TA-Luc) plasmid (Clontech) to the plasmid of interest was 1:3 respectively. Cells were stimulated with phorbol myristate acetate (PMA) (20 ng/ml) and the calcium ionophore A23187 (1 μM) for sixteen hours and luciferase activity determined using a kit from Promega. Cells were washed with cold PBS 1x and lysed with 100μL of 1x luciferase lysis reagent (Promega) for ten minutes. 100μL of luciferase substrate (Promega) was incubated with 20μL of sample and the relative luciferase units (RLU) were determined using a Berthold detection systems Sirius (Geneflow) for thirty seconds.

2.5 Plasmids used

2.5.1 Novel plasmids

Plasmids that were created during this project are described in sections 3.3.1 and 4.5.1.

2.5.2 Plasmids created previously or purchased

pNFAT-TA-Luc (Clontech)

P3xFlagcmv7.1 and pFlag5b (Sigma)

$pcDNA_3$ (Invitrogen)

pcDNA3-hPMCA2b contains the human PMCA2b cDNA and was a gift from Prof. Carafoli (University of Padova, Italy)

pFlag-PMCA4-(428-651) has been described previously (Buch et al., 2005)

$pcDNA_3$-PMCA1 has been described previously (Holton et al., 2007)

$pcDNA_3$-eNOShumanA contains the human eNOS cDNA and was a gift from Prof. S Lamas (Centro de Investigaciones Biologicas, Madrid, Spain)

2.6 Statistical methods

All data for the luciferase assays and cGMP assays were analysed using an unpaired student T-test, as the experiments were carried out independently of each other, with their own controls and samples were unpaired. All data analysis was performed using Graphpad prism software.

3. CHAPTER THREE RESULTS

PMCA AS AN INHIBITOR OF THE CALCINEURIN/NFAT SIGNAL TRANSDUCTION PATHWAY

3.1 Introduction

Dr Armesilla's group has recently reported an inhibitory interaction between ectopically expressed human PMCA4b and calcineurin A in HEK293 cells (Buch et al., 2005). In this thesis the interaction between endogenous PMCA and calcineurin A in human breast cancer cells and endothelial cells has been investigated further to extend the initial observations with PMCA4b to other PMCA isoforms and cell types.

Appendix, section A1 demonstrates the specificity of the primary antibodies used.

3.2 Analysis of the interaction PMCA-calcineurin in MCF-7 human breast adenocarcinoma cells

To analyse the interaction between endogenous PMCA1, 2 or 4 and calcineurin A, a monoclonal anti-calcineurin A antibody (Sigma) was used to immunoprecipitate protein extracts isolated from human MCF-7 breast adenocarcinoma cells. A western blot was then performed on the immunoprecipitated proteins using isoform-specific anti-PMCA antibodies (Swant).

A faint but reproducible band corresponding to PMCA4 was detected in samples probed with a rabbit anti-PMCA4 antibody (Fig. 3.2.1, upper panel). This result suggests that calcineurin A and PMCA4 interact weakly in MCF-7 cells. High levels of PMCA4 were found in MCF-7 proteins immunoprecipitated with the 5F10 anti-PMCA monoclonal antibody (Fig. 3.2.1, upper panel), thus confirming that low affinity of the anti-PMCA4 antibody or low expression of PMCA4 in MCF-7 cells were not responsible for the weak detection of PMCA4 co-precipitated with calcineurin.

Western blot performed using an anti-PMCA2 antibody revealed high levels of co-precipitated PMCA2 (Fig. 3.2.1, middle panel). This result suggests a strong interaction between endogenous calcineurin A and PMCA2 in MCF-7 cells.

PMCA1 failed to be co-precipitated with calcineurin A, as demonstrated by lack of PMCA1 detection during western blot analysis of the precipitated proteins using an antibody recognising specifically PMCA1 (Fig. 3.2.1, lower panel). This result suggests that PMCA1 and calcineurin do not interact in MCF-7 cells. The lack of interaction between PMCA1 and calcineurin A was not due to low expression of PMCA1 as control immunoprecipitation performed with MCF-7 protein extracts using the 5F10 anti-PMCA monoclonal antibody, and subsequent western blot with

the anti-PMCA1 specific antibody, precipitated high levels of PMCA1 (Fig. 3.2.1, lower panel).

The selectivity of the interactions was confirmed by performing control immunoprecipitations with an irrelevant antibody (anti-Luciferase) (Promega) in which no protein was precipitated (Fig. 3.2.1).

To summarise, these results demonstrate that the interaction between endogenous PMCAs and calcineurin A is isoform specific in breast cancer cells. They suggest that PMCA2 is the predominant isoform interacting with calcineurin A in MCF-7 cells, although calcineurin A also interacts weakly with PMCA4.

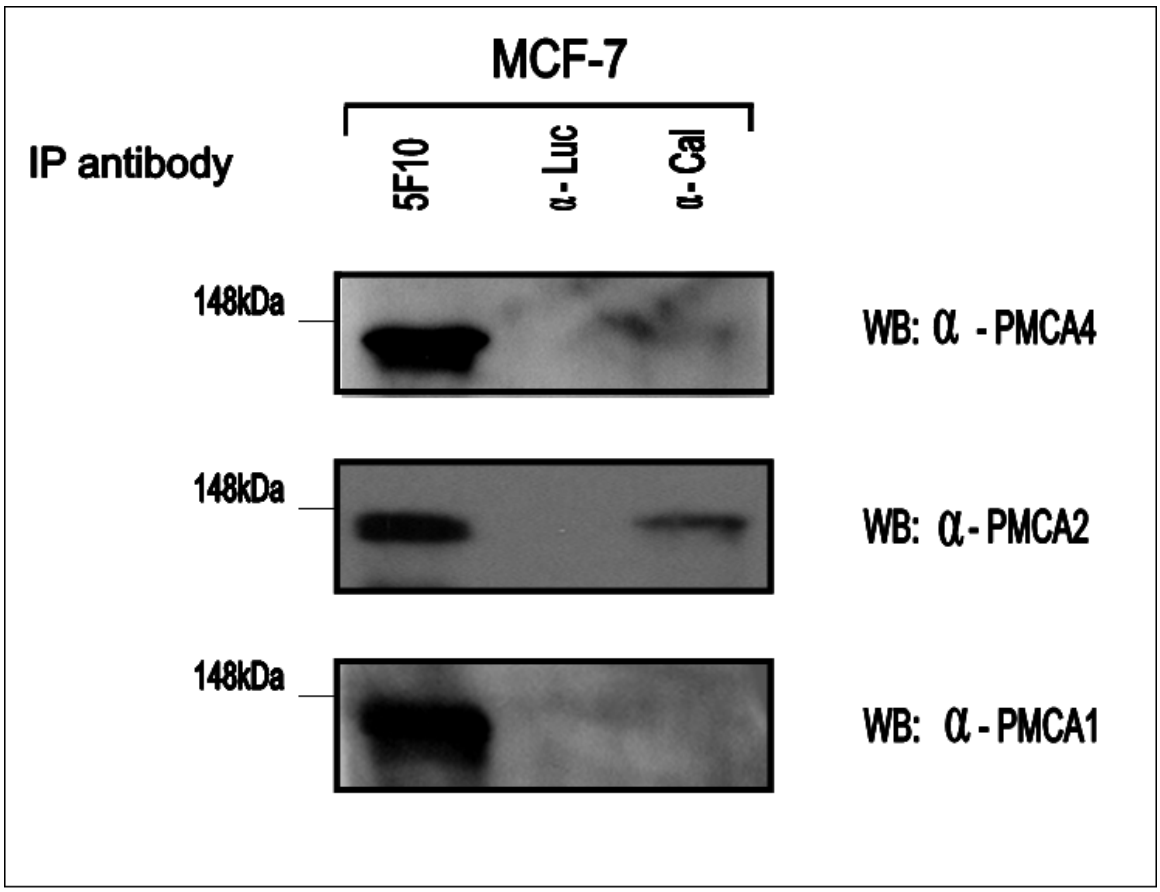

Fig. 3.2.1 The interaction between endogenous PMCA and calcineurin A in MCF-7 human breast adenocarcinoma cells is isoform specific. The interaction between PMCA and calcineurin in MCF-7 cells was demonstrated by co-precipitation of PMCA2 or –4 (but not PMCA1) and calcineurin from protein extracts immunoprecipitated with an antibody against calcineurin A. (α-CnA) anti-calcineurin A monoclonal antibody, (5F10) anti-PMCA monoclonal antibody, (α-luc) anti-luciferase antibody. Western blots of immunoprecipitated proteins were probed with antibodies specific for PMCA1, -2, or -4 (Swant) to detect PMCA1 (WB: α-PMCA1), PMCA2 (WB: α-PMCA2) and PMCA4 (WB: α-PMCA4) respectively. A representative result from three independent experiments is shown.

The strong interaction between PMCA2 and calcineurin A prompted the mapping of the domain of PMCA2 implicated in the interaction.

3.3 Determination of the domain of PMCA2 interacting with calcineurin A

Dr Armesilla's group has previously reported that the region 428-651 of human PMCA4b is implicated in the interaction with calcineurin A (Buch et al., 2005). Comparison of the equivalent region in PMCA2b revealed a high degree (76%) of amino acid homology suggesting that this region of PMCA2 might be implicated in the interaction with calcineurin A. To test this possibility, we have generated Flag-tagged fusion proteins including this and other regions of human PMCA2b.

3.3.1 Generation of Flag-tagged expression plasmids for identification of the PMCA2b domain interacting with calcineurin.

3.3.1a Cloning strategy

In order to generate expression vectors encoding Flag-tagged truncated proteins of human PMCA2, the corresponding fragments of human PMCA2b cDNA (gene bank accession number: NM_001683) were amplified by PCR and cloned into the expression vector p3xFlagcmv7.1 (Sigma).

Prior to primer generation, the sequence of the fragments of PMCA2b cDNA encoding the relevant regions of the PMCA2b protein were analysed for restriction site mapping. Primers for PCR reactions were designed containing appropriate restriction enzymes for cloning, not found within the fragment of PMCA2b cDNA to be amplified.

The same strategy was used to generate Flag-tagged PMCA1 truncated proteins using human PMCA1 cDNA (gene accession number: NM_001682).

The cloning of an expression plasmid encoding Flag-tagged PMCA4b(428-651) including the domain of PMCA4 responsible for the interaction with calcineurin A has been previously reported (Buch et al., 2005).

An overview of the Flag-tagged PMCA truncated proteins used in this study is shown in Fig. 3.3.1.

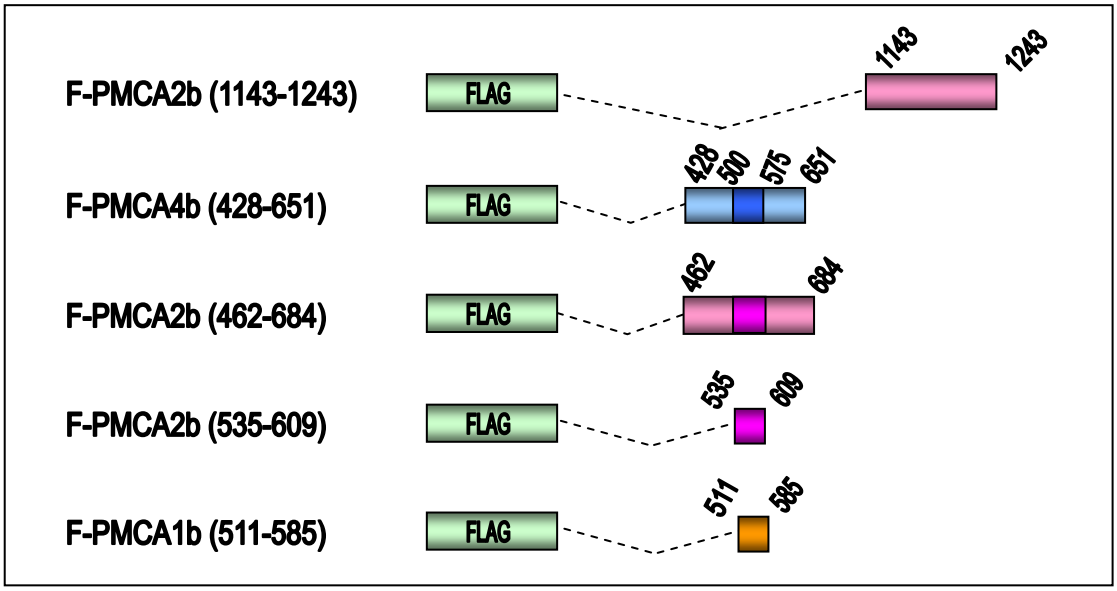

Fig. 3.3.1 Schematic overview of the constructs used for determination of the calcineurin interaction domain of PMCA2.

3.3.1a(i) Construction of p3xFlag-PMCA2b(1143-1243) plasmid

To generate construct p3xFlag-PMCA2b(1143-1243) the fragment encoding amino acids 1143-1243 of human PMCA2b (numbering according to gene bank accession number: NM_001683) was amplified by PCR following the standard conditions described in section 2.2.1 and using primers;

sense 5'-TCTTCCCAAgCTTgTCgTgAAggCgTTCCgTAg-3' and

antisense 5'-CTTCgCggATCCTCAAAgCgACgTCTCCAggCTgT-3'.

The optimum annealing temperature for both primers was 64°C.

The PCR product was digested with restriction enzymes EcoRI and BamHI, and cloned into the EcoRI-BamHI sites of plasmid p3xFlag-cmv7.1 (Fig. 3.3.2).

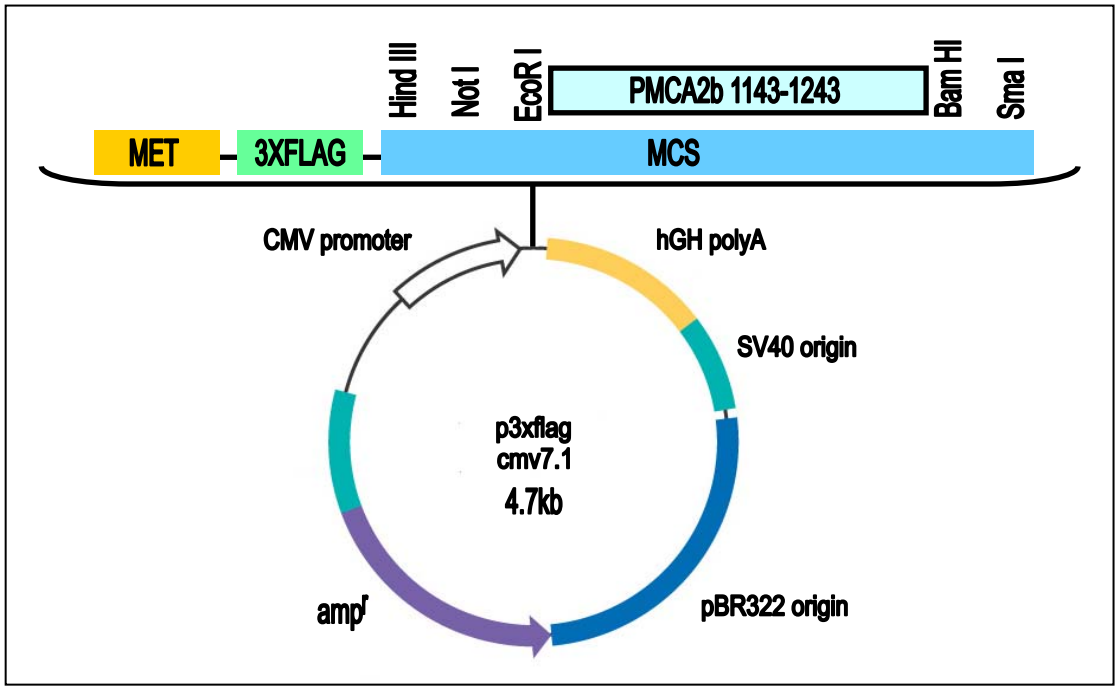

Fig. 3.3.2 Schematic diagram for the cloning strategy of p3xflag-PMCA2b(1143-1243).

To confirm the successful generation of the recombinant expression vector p3xFlag-PMCA2b(1143-1243), the plasmid was digested with restriction enzymes EcoRI and BamHI. DNA electrophoresis of the digestion reactions in agarose gels showed two DNA fragments corresponding to insert (300bp) and plasmid (4700bp) as expected (Fig. 3.3.3).

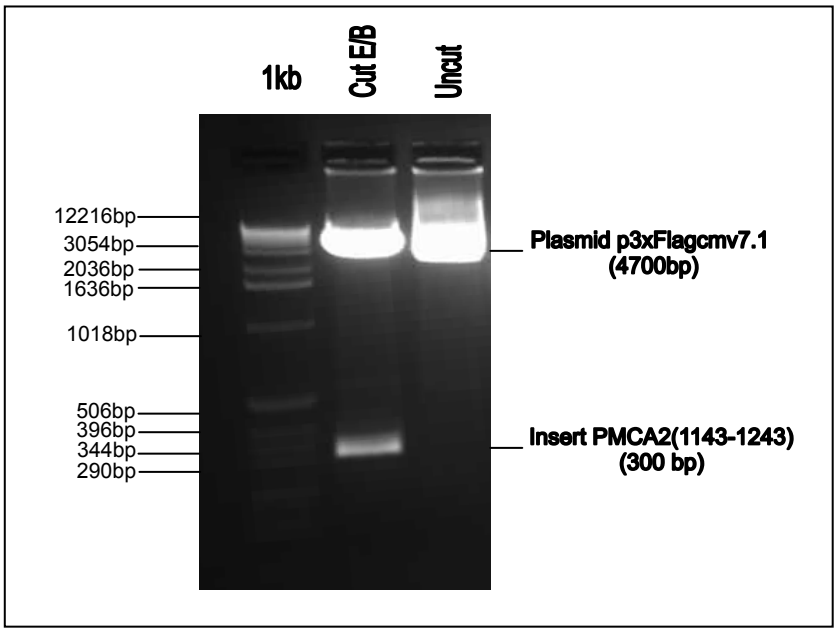

Fig. 3.3.3 Agarose gel electrophoresis of restriction enzyme digested p3xFlag-PMCA2(1143-1243). Digestion released a fragment of DNA 300bp in size relating to amino acids 1143-1243 of human PMCA2 and a larger fragment of 4.7 relating to the expression plasmid p3xflagcmv7.1.

3.3.1a(ii) Construction of p3xFlag-PMCA2b(462-684) plasmid

To generate construct p3xFlag-PMCA2b(462-684) the fragment encoding amino acids 462-684 of human PMCA2b (numbering according to gene bank accession number: NM_001683) was amplified by PCR following the standard conditions described in section 2.2.1 and using primers;

sense 5'-TCTTCCggAATTCTgCCGTCACCATCTCgTTgg-3' and

antisense 5'-CTTCgCggATCCTCACTCCgggCTgCTggggAAgTCg-3'.

The optimum annealing temperature for both primers was 62°C.

The PCR product was digested with restriction enzymes EcoRI and BamHI, and cloned into the EcoRI-BamHI sites of plasmid p3xFlagcmv7.1 (Fig. 3.3.4).

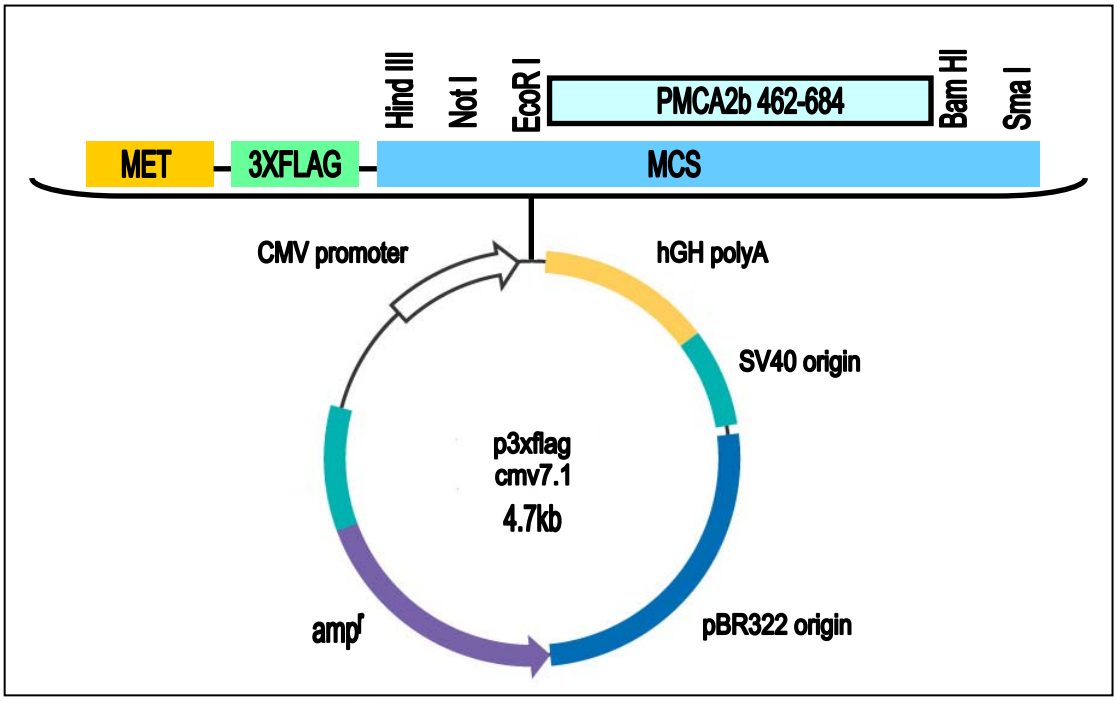

Fig. 3.3.4 Schematic diagram for the cloning strategy of p3xflag-PMCA2b(462-684).

To confirm the successful generation of the recombinant expression vector p3xFlag-PMCA2b(462-684), the plasmid was digested with restriction enzymes EcoRI and BamHI. DNA electrophoresis of the digestion reactions in agarose gels showed two DNA fragments corresponding to insert (666bp) and plasmid (4700bp) as expected (Fig. 3.3.5).

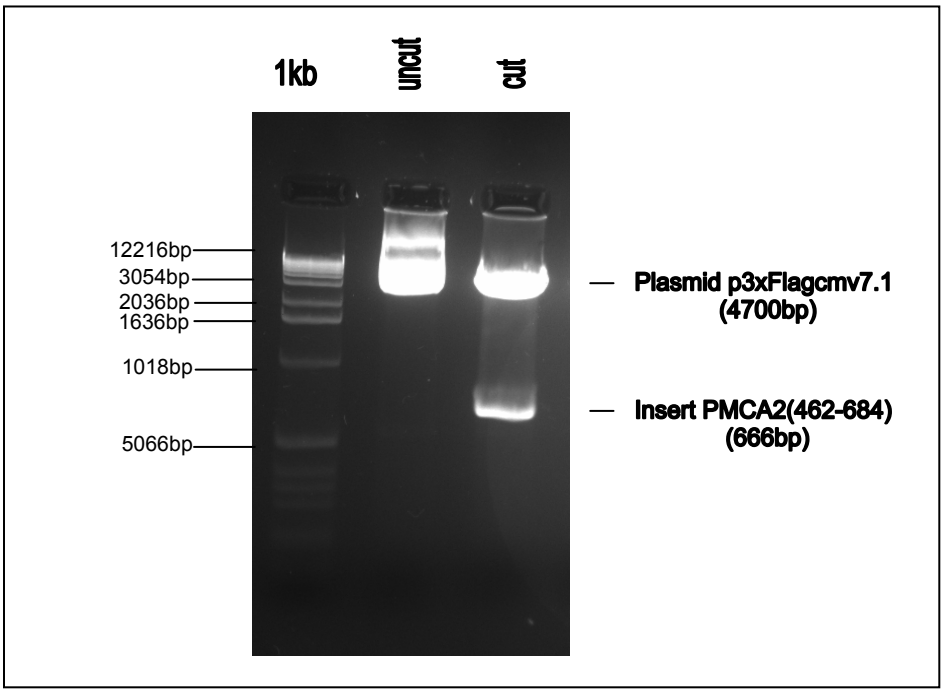

Fig. 3.3.5 Agarose gel electrophoresis of restriction enzyme digested p3xFlag-PMCA2(462-684). Digestion released a fragment of DNA 666bp in size relating to amino acids 462-684 of human PMCA2 and a larger fragment of 4.7 relating to the expression plasmid p3xflagcmv7.1

3.3.1a(iii) Construction of p3xFlag-PMCA2b(535-609)

To generate construct p3xFlag-PMCA2b(535-609) the fragment encoding amino acids 535-609 of human PMCA2b (numbering according to gene bank accession number: NM_001683) was amplified by PCR following the standard conditions described in section 2.2.1 and using primers;

sense 5'-TCTTCCCAAgCTATggAgCTgCTgATCAATgCC-3'and

antisense 5'-CTTCgCggATCCTCACATggACTTgCgCACggAgTTg-3'.

The optimum annealing temperature for both primers was 62°C.

The PCR product was digested with restriction enzymes HindIII and BamHI, and cloned into the HindIII-BamHI sites of plasmid p3xFlagcmv7.1 (Fig. 3.3.6).

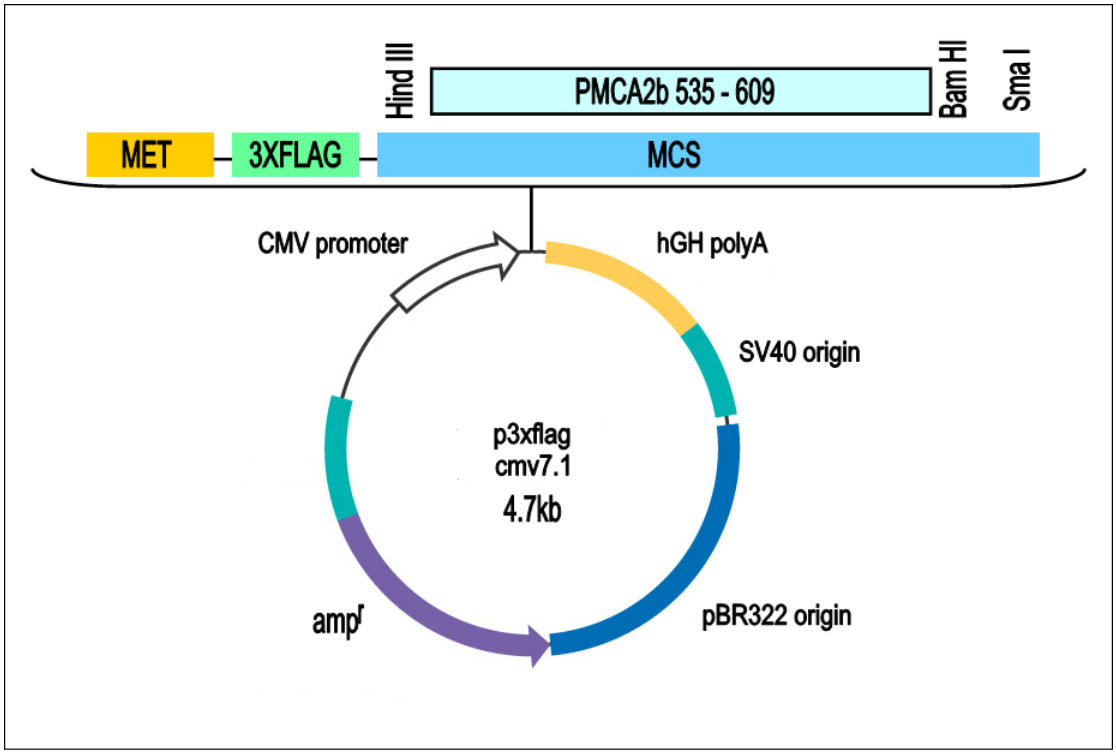

Fig. 3.3.6 Schematic diagram for the cloning strategy of p3xflag-PMCA2b(535-609).

To confirm the successful generation of the recombinant expression vector p3xFlag-PMCA2(535-609), the plasmid was digested with restriction enzymes HindIII and BamHI. DNA electrophoresis of the digestion reactions in agarose gels showed two DNA fragments corresponding to insert (222bp) and plasmid (4700bp) as expected (Fig. 3.3.7).

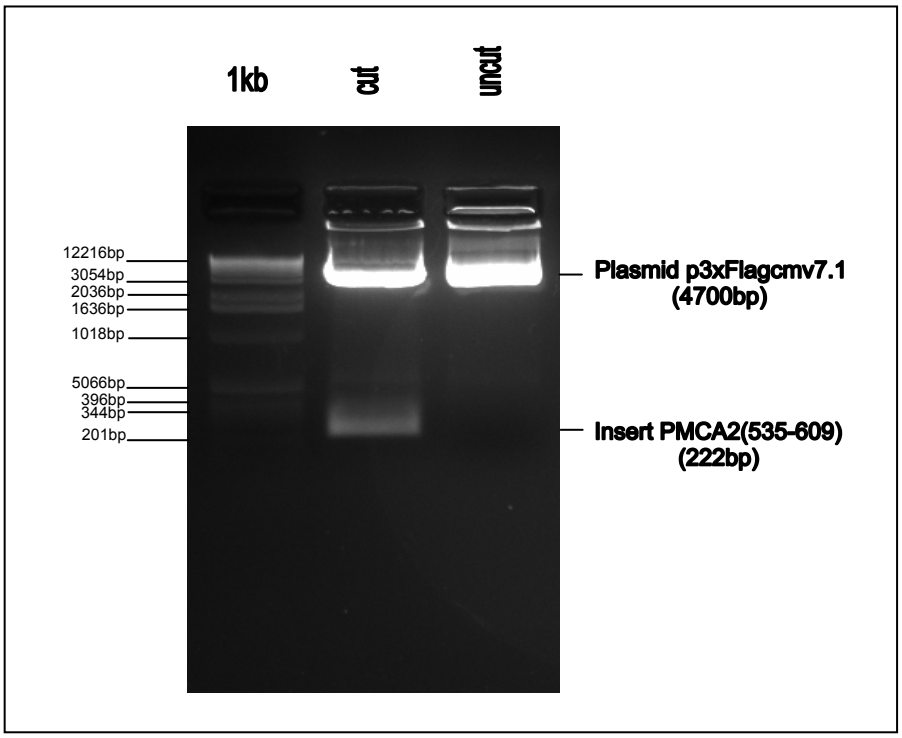

Fig. 3.3.7 Agarose gel electrophoresis of restriction enzyme digested p3xFlag-PMCA2(535-609). Digestion released a fragment of DNA 222bp in size relating to amino acids 535-609 of human PMCA2 and a larger fragment of 4.7 relating to the expression plasmid p3xflagcmv7.1

3.3.1a(iv) Construction of p3xFlag-PMCA1b(511-585)

To generate construct p3xFlag-PMCA1b(511-585) the fragment encoding amino acids 511-585 of human PMCA1b (numbering according to gene bank accession number: NM_001682) was amplified by PCR following the standard conditions described in section 2.2.1 and using primers;

sense 5'-TCTTCCggAATTCTTTgTCCTATCTTgTAACAggAA-3'and

antisense 5'-CTTCgCggATCCTCACATggACTTCCTAACAgAATTG-3'.

The optimum annealing temperature for both primers was 56°C.

The PCR product was digested with restriction enzymes EcoRI and BamHI, and cloned into the EcoRI-BamHI sites of plasmid p3xFlagcmv7.1 (Fig. 3.3.8).

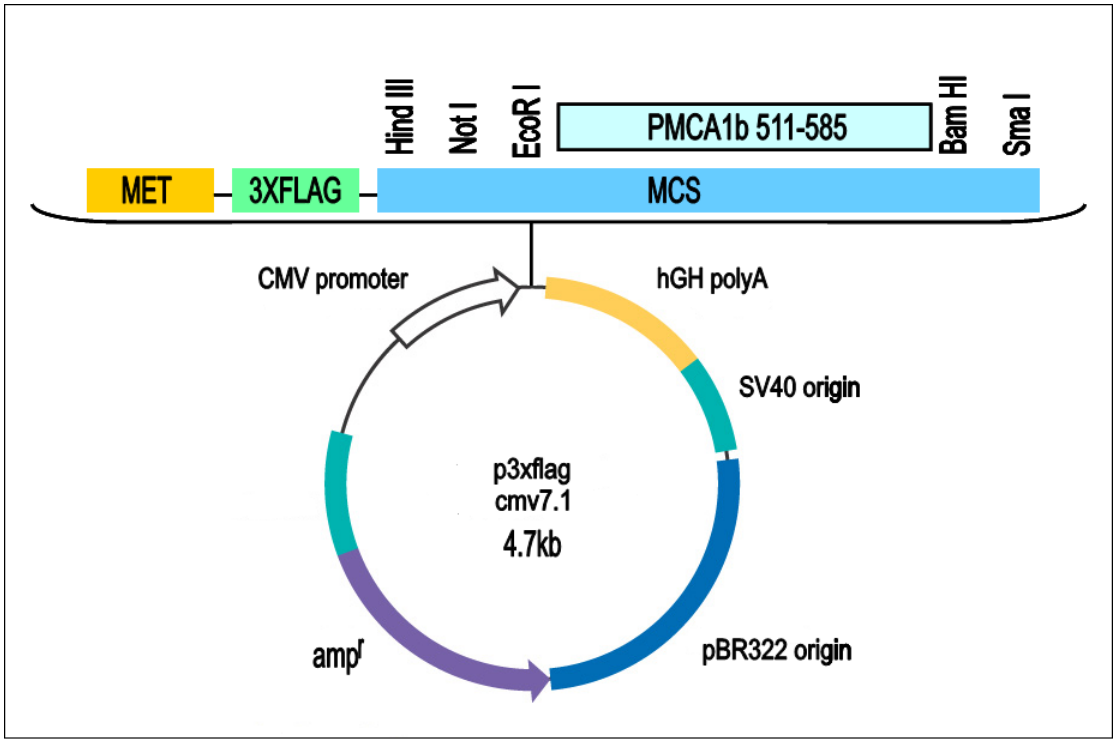

Fig.3.3.8 Schematic diagram for the cloning strategy of p3xflag-PMCA1b(511-585).

To confirm the successful generation of the recombinant expression vector p3xFlag-PMCA1(511-585), the plasmid was digested with restriction enzymes EcoRI and BamHI. DNA electrophoresis of the digestion reactions in agarose gels showed two DNA fragments corresponding to insert (222bp) and plasmid (4700bp) as expected (Fig.3.3.9).

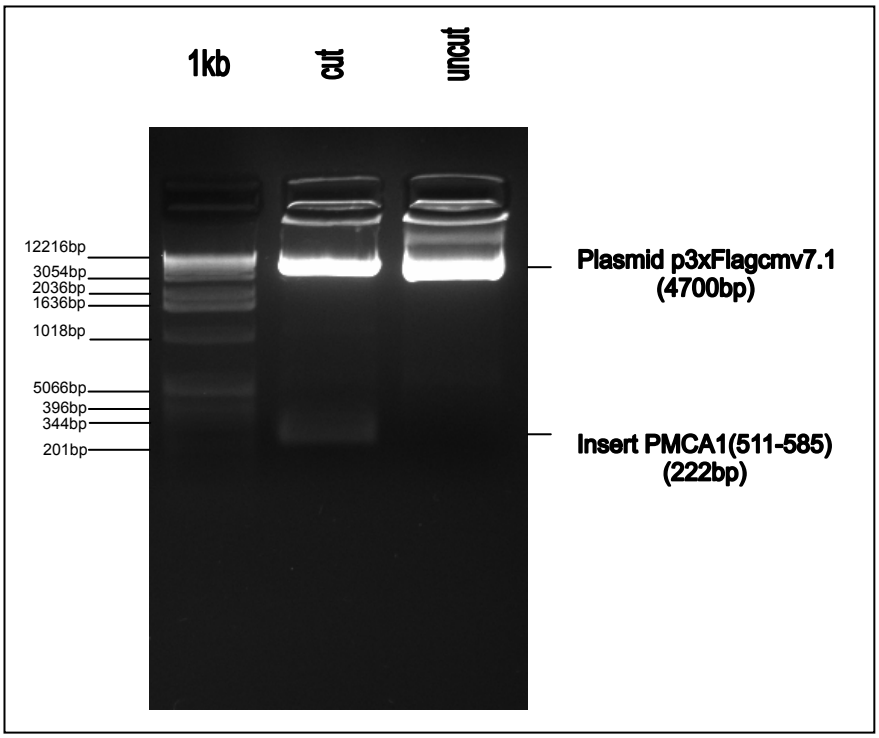

Fig. 3.3.9 Agarose gel electrophoresis of restriction enzyme digested p3xFlag-PMCA1(511-585). Digestion released a fragment of DNA 222bp in size relating to amino acids 511-585 of human PMCA1 and a larger fragment of 4.7 relating to the expression plasmid p3xflagcmv7.1.

3.3.2 Analysis of the interaction between calcineurin and recombinant Flag-tagged PMCA2 truncated proteins

Flag-tagged fusion proteins containing the amino acid regions 462-684, or 1143-1243 of PMCA2b were used to investigate if the region 462-684 of PMCA2 may interact with calcineurin. Plasmids p3xFlag-PMCA2b(462-684) or p3xFlag-PMCA2b(1143-1243) were transfected into HEK293 cells. Protein lysates of transfected cells were incubated with commercially available calcineurin (40 nM final concentration) (Sigma), immunoprecipitated with an anti-calcineurin A monoclonal antibody and, subsequently, immunoprecipitated proteins were detected by western blot using an anti-Flag monoclonal antibody (Sigma). Flag-

PMCA2b(462-684) co-precipitated with calcineurin (Fig. 3.3.10, upper panel) whereas no precipitation was observed for Flag-PMCA2b(1143-1243) (Fig. 3.3.10, upper panel) demonstrating the selectivity of the interaction. HEK293 cells transfected with the vector p3xFlag-PMCA4b(428-651), encoding the region of PMCA4b reported to interact with calcineurin, were used as a positive control (Fig. 3.3.10,upper panel). These results demonstrated that the region 462-684 of the catalytic, big intracellular loop of human PMCA2b is involved in the interaction with calcineurin A.

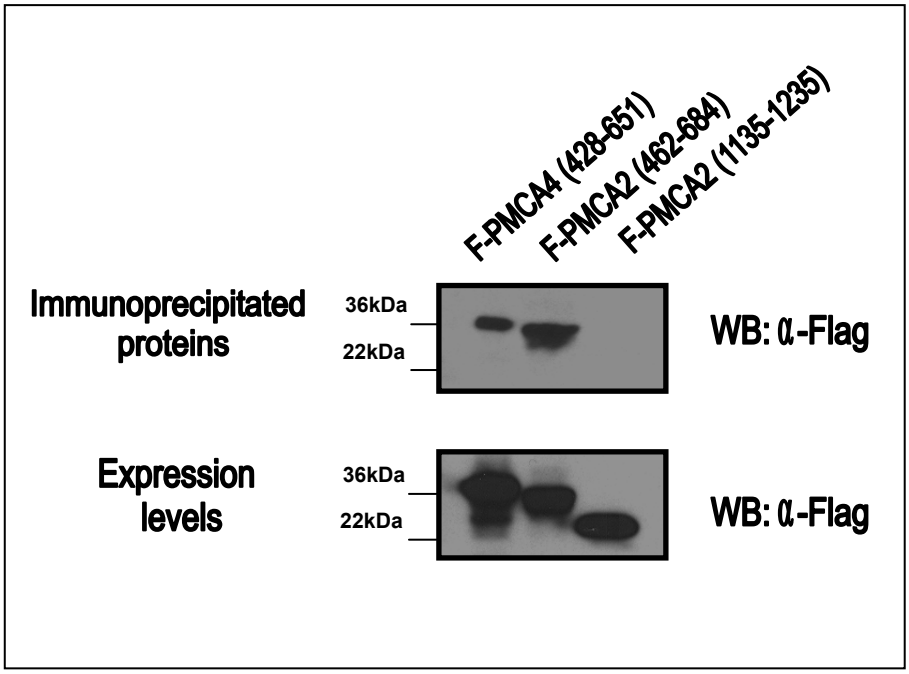

Fig. 3.3.10 Amino acid region 462-684 of human PMCA2b interacts with calcineurin A. Western blot and Immunoprecipitation analysis of the interaction between calcineurin A and Flag-tagged proteins described in chapter 3 section 3.3. Amino acid region 462-684 of PMCA2 specifically interacts with calcineurin A. A representative result from three independent experiments is shown.

Dr. Armesilla's groups previous data has shown that the region 501-575 (located within the interaction domain of PMCA4b, region 428-651) is essential for the interaction of this protein with calcineurin A (Buch et al., 2005). Therefore, we next tested the ability of the equivalent region of PMCA2b (fragment 535-609) to interact with calcineurin A. Following the same strategy as described above, we determined that Flag-PMCA2b(535-609) co-precipitated with calcineurin A (Fig. 3.3.11, upper panel), however precipitation occurred to a lesser degree than that

observed for the whole interaction domain (462-684), suggesting that the region 535-609 of PMCA2b interacts with calcineurin, although some adjacent sequences might be essential for a full interaction. To confirm the lack of interaction between PMCA1 and calcineurin A we generated an expression vector encoding Flag-PMCA1b(511-585), a Flag-tagged fusion protein containing the region of human PMCA1b equivalent to the region 535-609 of PMCA2b. Precipitation of calcineurin A failed to co-precipitate Flag-PMCA1b(511-585) (Fig. 3.3.11, upper panel) confirming the lack of interaction observed between endogenous PMCA1 and calcineurin.

Western blot, with a monoclonal antibody raised against the Flag epitope, of the protein extracts prior to immunoprecipitation showed similar expression of the Flag-tagged fusion proteins in the different transfections (Fig 3.3.10 and Fig. 3.3.11, lower panels). Poor expression was ruled out for the lack of interaction as the Flag-tagged proteins were expressed at equivalent levels.

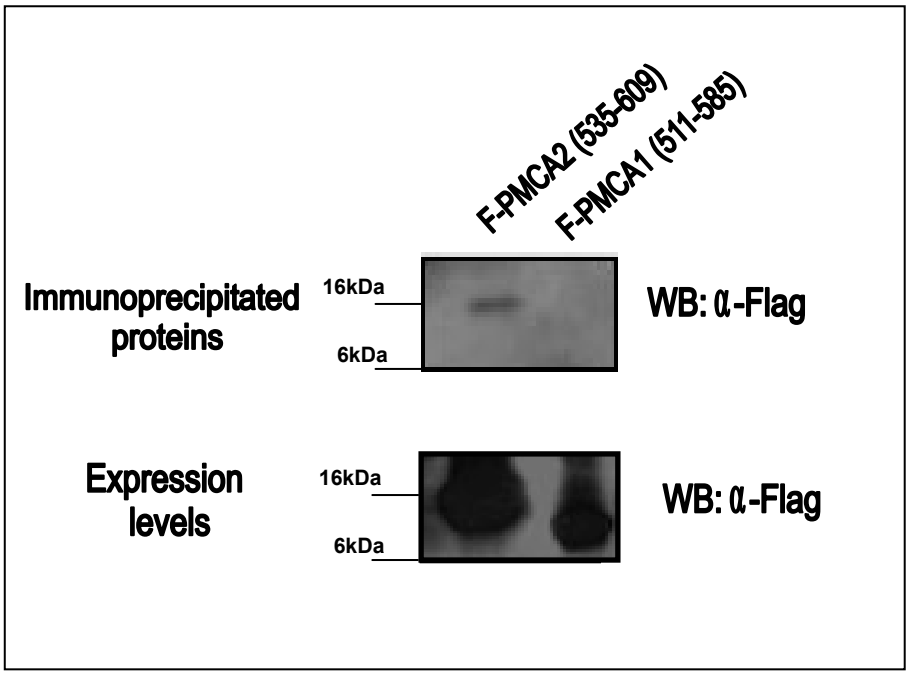

Fig. 3.3.11 The region 535-609 interacts with calcineurin. Immunoprecipitation and western blot analysis of Flag recombinant protein containing the region 535-609 of human PMCA2 showed a weak interaction between this domain of PMCA2 and calcineurin A. However the Flag-tagged protein containing the equivalent region of PMCA1 did not interact at all with calcineurin A. A representative result from three independent experiments is shown.

3.4 Functional analysis of the interaction between PMCA2 and calcineurin

To determine the functionality of the interaction between PMCA2 and calcineurin, an NFAT-dependent luciferase reporter assay was performed as an indicator of the effect of PMCA2 overexpression on calcineurin activity. Calcineurin dephosphorylates NFAT allowing it to translocate to the nucleus of the cells and activate NFAT-dependent genes. To monitor the activity of the calcineurin/NFAT pathway we transfected HEK293 cells with a pNFAT-TA-luc plasmid (Clontech) which when activated by NFAT promotes the expression of luciferase. A luciferase substrate is then added to the lysed cells and the relative luciferase units (RLU) are a direct representation of NFAT activation and consequently calcineurin activity.

Plasmids pcDNA3-PMCA2, pcDNA3-PMCA1 or pcDNA3-empty were transfected into HEK293 cells along with pNFAT-luc. Cells were stimulated with PMA and calcium ionophore for 16 hours to activate the calcineurin/NFAT pathway. Cell lysates of the transfected cells were incubated with luciferase substrate and the RLU was measured for each experimental condition.

Ectopic expression of PMCA2 in HEK293 cells resulted in a 32% reduction in NFAT-dependent luciferase (and therefore calcineurin/NFAT) activity. However, the transfection of an expression plasmid encoding PMCA1 did not reduce NFAT-dependent luciferase production, confirming the lack of an interaction between PMCA1 and calcineurin observed previously (fig. 3.4.1).

These results indicate that PMCA2 inhibits the calcineurin/NFAT pathway in mammalian cells when overexpressed and that this interaction is specific to PMCA2, as PMCA1 overexpression had no effect on calcineurin activity.

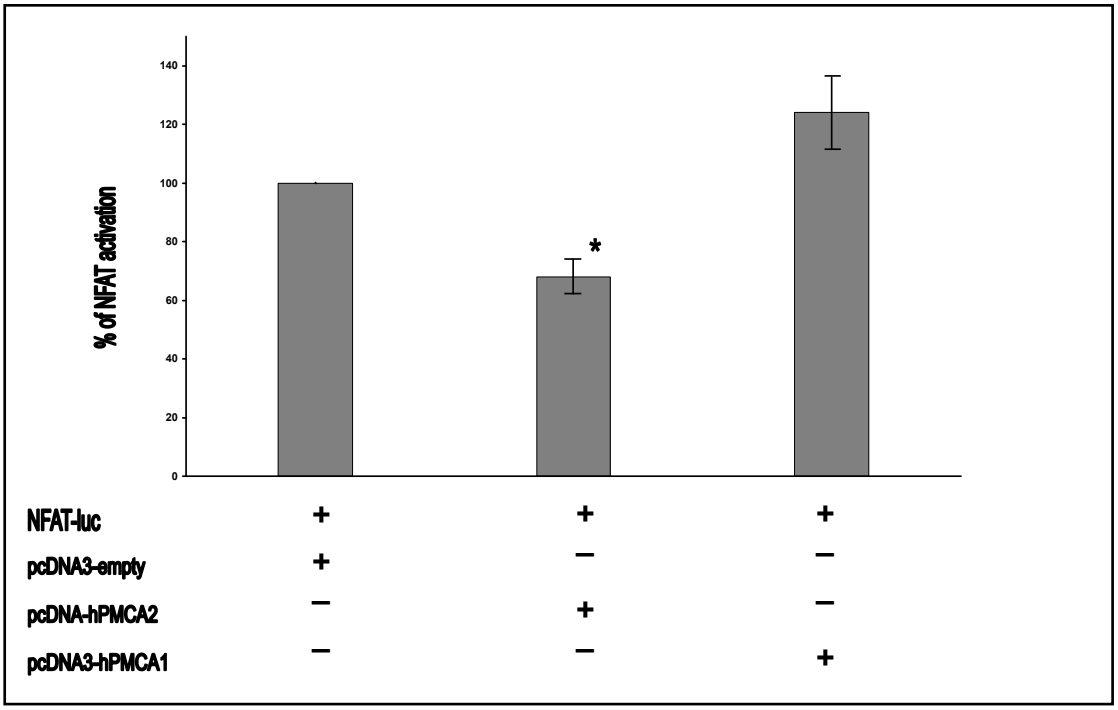

Fig. 3.4.1 NFAT transcriptional activity is significantly inhibited by PMCA2**.** PMCA2 inhibits the activation of an NFAT-dependent luciferase reporter vector in response to PMA plus Calcium ionophore (Io) A23187 treatment of cells. HEK293 cells were co-transfected with 10μg of pcDNA3-hPMCA2b or control vector pcDNA3, and 5μg of the NFAT –dependent luciferase reporter vector pNFAT-TA-Luc. PMA (20ng/ml) plus Io (1μM) was used to stimulate the cells for 16h. Induction after PMA/Io stimulation of reporter vector luciferase activity in the presence of co-transfected control plasmid pcDNA3 was taken as reference (100%), this control experiment was perfomed independently for each of the different conditions but combined to allow the data presentation in one graph. A significant reduction (32% inhibition) of luciferase activity was observed after co-expression of human PMCA2b. ∗, statistically significant ($p \leq 0.05$, according to an unpaired Student's t test) when compared to NFAT-luc/pcDNA3-empty controls. Means ± S.E. of six independent experiments are shown.

3.5 Disruption of the interaction between endogenous PMCA2 and calcineurin by Flag-PMCA2(462-684) activates the calcineurin/NFAT pathway.

Region 462-684 of PMCA2 encompasses the interaction domain of PMCA2 with calcineurin A. We hypothesised that by ectopically overexpressing this region in HEK293 cells the interaction between endogenous PMCA2 and calcineurin A could be disrupted. To demonstrate this disruption HEK293 cells were co-transfected with pcDNA3, pcDNA3-PMCA2 or pcDNA3-PMCA2(462-684) and the

NFAT-dependent luciferase reporter vector pNFAT-TA-Luc (Clontech). Cells were stimulated with PMA/Io for 16 hours induce calcineurin A activation and consequent NFAT transcriptional activity. Cell lysates were incubated with luciferase substrate and the RLU for each experimental condition was recorded.

A significant increase (55%) was detected in the PMCA/Io-dependent luciferase activity of samples expressing F-PMCA2(462-684) protein (Fig. 3.5.1). Upon the addition of pcDNA2-PMCA2b the effect caused by F-PMCA2b(462-684) on the activation of the luciferase reporter vector was reversed. A control experiment was performed with pcDNA3-empty and pNFAT-luc, this was used as the value for 100% activation of NFAT.

These results suggest that F-PMCA2b(462-684) significantly disrupts the interaction between endogenous PMCA2 and calcineurin A in mammalian cells. This disruption can be overcome by expressing an excess of the whole PMCA2 molecule. Disruption of the interaction PMCA2/calcineurin results in a significant increment in the activity of the calcineurin/NFAT pathway, confirming our hypothesis of a role for the interaction PMCA/calcineurin as an endogenous inhibitor of the calcineurin/NFAT pathway.

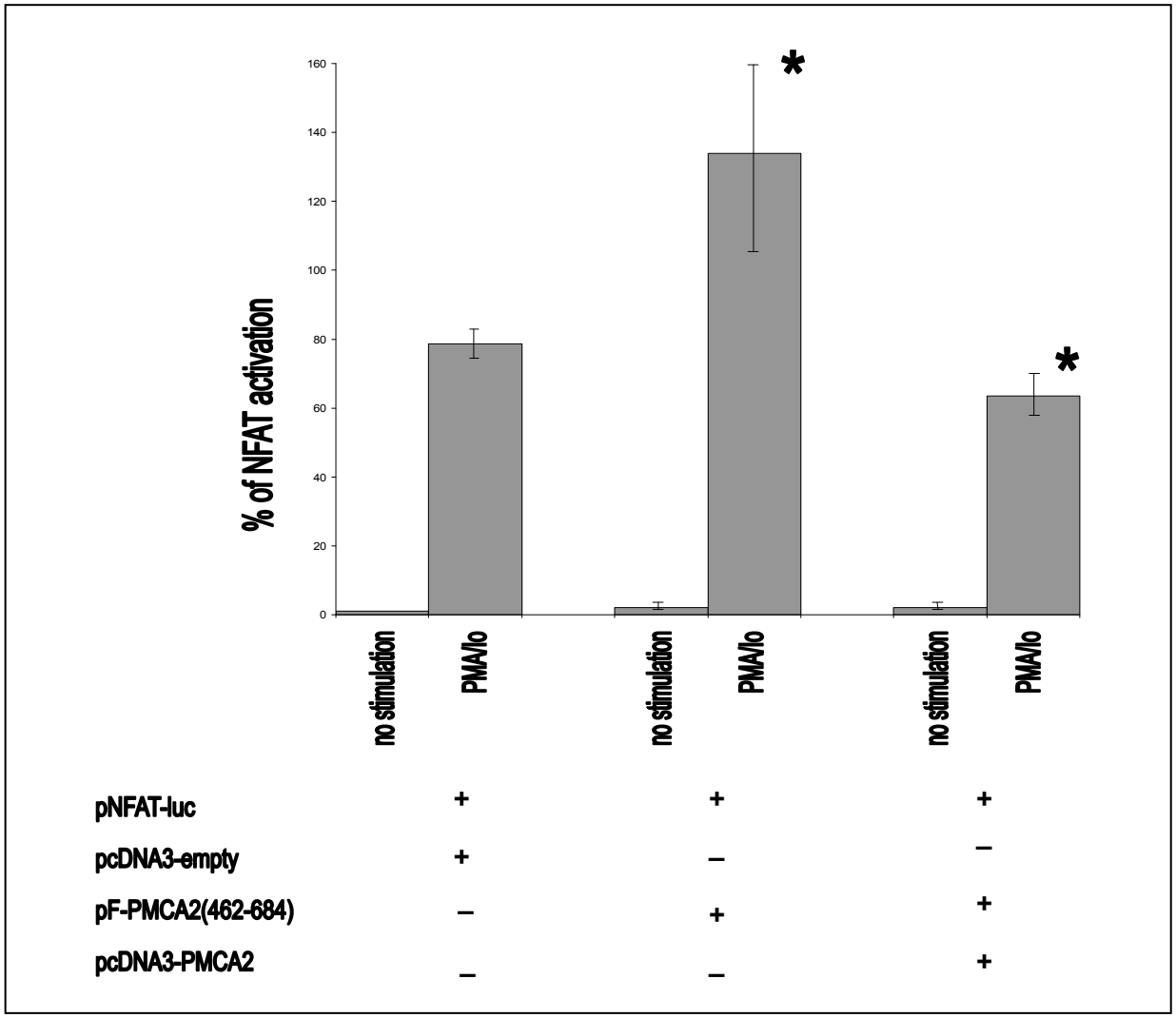

Fig. 3.5.1 Ectopic expression of F-PMCA2(462-684) significantly activates NFAT transcriptional activity and further introduction of PMCA2 reverses this effect**.** F-PMCA2(462-684) activates the activity of an NFAT-dependent luciferase reporter vector in response to PMA plus calcium ionophore (Io) A23187. 5μg of pF-PMCA2(462-684) or control vector pcDNA3-empty, 2.5μg of pcDNA3-PMCA2 and 7.5μg of the NFAT-dependent luciferase reporter vector pNFAT-TA-Luc were co-transfected in HEK293 cells. Cells were stimulated with PMA (20ng/mL) plus Io (1μM) for 16 h. Induction after PMA/Io stimulation of reporter vector luciferase activity in the presence of co-transfected control plasmid pcDNA3-empty was taken as reference, this control experiment was perfomed independently for each of the different conditions but combined to allow the data presentation in one graph. Co-expression of F-PMCA2(462-684) resulted in a significant increase (55%) of luciferase induction. Co-expression of F-PMCA2(462-684) and human PMCA2 resulted in a significant reversal (70%) of this effect. *, statistically significant ($p \leq 0.05$, according to an unpaired Student's T-test) when compared to NFAT-luc/pcDNA3-empty controls. Mean ± S.E. of six independent experiments are shown.

3.6 PMCA-calcineurin interaction in primary endothelial cells, HUVEC.

It has been previously reported that the calcineurin/NFAT pathway plays a major role in the progression of angiogenesis in response to VEGF stimulation of endothelial cells. The expression of PMCAs in endothelial cells prompted us to analyse the possibility that endogenous PMCA and calcineurin interact in endothelial cells. To test this hypothesis, Human Umbilical vein endothelial cells (HUVEC) were used as a model for primary endothelial cells.

To determine any interaction between PMCA and calcineurin in HUVEC cells, protein extracts were immunoprecipitated with a monoclonal anti-calcineurin A antibody (Sigma). Precipitated proteins were probed by western blot using isoform-specific anti-PMCA antibodies (Swant).

A very faint, reproducible band corresponding to PMCA1 was detected in samples probed with an anti-PMCA1 antibody (Fig. 3.6.1, lower panel). This suggests a very weak interaction between endogenous calcineurin A and human PMCA1 in HUVEC cells. High levels of co-precipitated PMCA2 and 4 were revealed by performing a western blot with anti-PMCA2 and –PMCA4 antibodies respectively (Fig. 3.6.1, top and middle panels). This data suggests a strong interaction between both human PMCA2 and 4 and endogenous calcineurin A in HUVEC cells. Immunoprecipitations carried out with the 5F10 anti-PMCA monoclonal antibody were performed as a control and demonstrated high expression levels of PMCA1, 2 and 4 isoforms in HUVEC cells (Fig. 3.6.1), indicating that low expression levels of PMCA1 or low affinity of the antibody were not the cause of the weak band detected.

Confirmation on the selectivity of the interactions was determined by immunoprecipitations carried out with an irrelevant antibody (anti-Luciferase) which precipitated no protein in all cases (Fig. 3.6.1).

To summarise these results show that PMCA interacts with calcineurin in HUVEC endothelial cells and suggests that the strongest interactions are detected between PMCA2 or 4 and calcineurin.

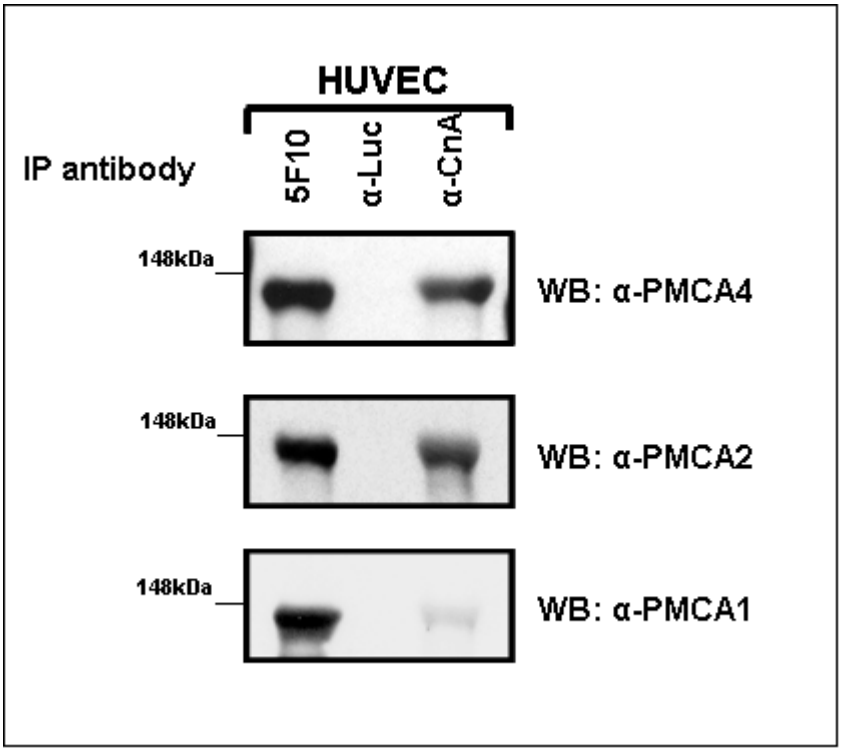

Fig. 3.6.1 Endogenous PMCA and calcineurin interact in HUVEC endothelial cells. Calcineurin A and PMCA 1, 2 and 4 co-precipitation demonstrates a strong interaction between calcineurin A and PMCA2 or 4. (5F10) anti-PMCA monoclonal antibody, (α-luc) anti-luciferase antibody and (α-cal) anti-calcineurin A monoclonal antibody. Western blots of immunoprecipitated proteins were probed with antibodies specific for PMCA1, PMCA2 or PMCA4 (Swant) to detect PMCA1 (WB: PMCA1), PMCA2 (WB: PMCA2) and (WB: PMCA4) respectively. A representative result from three independent experiments is shown.

3.7 Discussion

Previous findings by Dr Armesilla's group have shown that PMCA interacts with calcineurin and inhibits its activity and subsequent signalling pathways (Buch et al., 2005). It was the aim of this research to further define this interaction in an isoform- and tissue-specific manner.

This investigation has demonstrated that endogenous PMCA and calcineurin interact in an isoform-specific manner in MCF-7 breast adenocarcinoma cells and primary endothelial cells. The strongest interaction was detected between PMCA2 and calcineurin A and the calcineurin/NFAT pathway was inhibited by overexpression of PMCA2. Overexpression of the flag-tagged PMCA2 interaction domain, amino acids (462-684), resulted in the functional disruption of the interaction between endogenous PMCA2 and calcineurin.

This investigation also found that PMCA2b, 1b and 4b proteins are expressed in endothelial and breast cancer cells. The finding that PMCA2 was the important isoform for the interaction with calcineurin in endothelial and breast cancer cells is interesting as PMCA2 has been described as the isoform of PMCA with the highest calmodulin affinity (Elwess et al., 1997), it has been shown to be 2-3 times more active than PMCA1 and 4, the ubiquitously expressed PMCA isoforms (Hill et al., 2006) and maintains high basal activity in the absence of activators (Hilfiker et al., 1994).

3.7.1 PMCA2/calcineurin interaction and mammary tissue

PMCA2 has been implicated to play a major role in mammary gland physiology. PMCA2b has been found to be highly expressed in lactating mammary tissues (Reinhardt et al., 2000) and mice homozygous for a null-mutation of the *pmca2* gene produced milk with 60% less calcium than wild type animals (Reinhardt et al., 2004). It is possible that PMCA2 is localised to certain cells within that lactating mammary gland where it may also play an essential role in inhibiting calcineurin-mediated apoptosis by sequestering and inhibiting calcineurin activity allowing these cells to evade apoptosis and continue to produce high levels of calcium which would normally trigger apoptosis.

3.7.2 PMCA2/calcineurin interaction and cancer

Elements of signal transduction pathways are often deregulated in cancer cells contributing to a malignant phenotype. There have been a number of reports where calcineurin inhibitors used during immunosuppression have resulted in increased risk of cancer development (reviewed in Weischer et al., 2007), indicating that calcineurin-mediated apoptosis may be an important mechanism in reducing cancer development. In support of this theory Rivera et al. (2005) have demonstrated that the p53 tumour suppressor plays an important role in the activation of calcineurin-mediated apoptosis, demonstrating the importance of calcineurin regulation in cancer progression.

PMCA2 expression has been found to be up-regulated in breast cancer cell (Lee et al., 2005). These findings suggest that deregulation of PMCA might contribute to breast tumorigenesis. The work conducted during this project has demonstrated that PMCA2 inhibits the activity of calcineurin and the subsequent calcineurin/NFAT pathway. Increased PMCA2 expression in breast cancer cells would therefore be expected to inhibit the calcineurin/NFAT pathway and subsequent calcineurin-mediated apoptosis (Asai et al., 1999). PMCA2-mediated down-regulation of calcineurin activity might result in inhibition of apoptosis and promotion of tumorigenesis in cancer cells. In agreement with this hypothesis, Padma, et al. (2005) have shown that calcineurin activity is down-regulated in cervical carcinoma. It would be interesting to analyse the level of PMCA2 expression in these cells and determine if it regulates calcineurin activity and apoptosis in cervical carcinomas. Also PMCA2 expression is elevated in neuroblastoma cells (Usachev et al., 2001) determination of the effect of this on calcineurin activity would be an interesting avenue to pursue.

Over-expression of other PMCA isoforms has also been recorded in certain cancers. Aung et al. (2007) and Ribiczey et al. (2007) have found that PMCA4 may play a role in colon cancer and gastric cancers, indicating that the isoform of PMCA which is up-regulated is dependent on the cancerous cell type.

3.7.3 PMCA2/calcineurin interaction and hair cells

The finding that PMCA2 interacts with and inhibits calcineurin activity and that this may hypothetically regulate calcineurin-mediated apoptosis might have some relevance to the hearing and balance deficits observed in PMCA2 knockout mice models (Kozel et al., 1998).

It has been observed that PMCA2 is expressed at high levels in the outer hair cells of the cochlea (Furuta et al., 1998). Dodson et al. (2001) have demonstrated that in mice with mutated PMCA2 function (deafwaddler) apoptotic hair cell death is increased. Hypothetically the lack of functional PMCA2 in the deafwaddler mouse might lead to an increase in calcineurin-mediated apoptosis in outer hair cells resulting from increased calcineurin activity. Vincente-Torres and Schacht (2006) have demonstrated that increased activity of calcineurin promotes cochlear acoustic injury by outer hair cell death. To support this hypothesis Minami et al. (2004) and Uemaetomari et al. (2005) in two separate experiments have shown that calcineurin inhibitors, cyclosporine A and FK506, can decrease noise-induced hearing loss. All this evidence suggests that the PMCA and calcineurin interaction may play a major role in the regulation of apoptosis in hair cells. Potentially PMCA2 mutations and their effect on calcineurin-mediated apoptosis might be important in some hearing disorders displayed in humans. Evidence to support this has been provided by Schultz et al. (2005) where they demonstrated that certain mutations in PMCA2 can result in modifications of human hearing-loss phenotypes, specifically noise induced sensorineural hearing loss.

3.7.4 PMCA2/calcineurin interaction and neuronal degeneration

As well as being implicated in hearing loss and cancer the PMCA2-calcineurin interaction may also regulate apoptosis in neuronal cells. PMCA2 is involved in neuronal development and is highly expressed in fully differentiated neurons (Guerini et al., 1999). In diseases such as multiple sclerosis, where neuronal damage is a key feature, the expression of PMCA2 has been shown to be down-regulated (Nicot et al., 2003) potentially increasing the rate of calcineurin-mediated apoptosis in neuronal cells and augmenting the disease.

As well as having an involvement in neuronal disease the PMCA-calcineurin interaction may be relevant in spinal cord injuries involving ischemia of neuronal cells. Certain areas of the brain more susceptible to ischemia also contain

relatively high concentration of calcineurin (Goto et al., 1986). Asai et al. (1999) suggest that it is this high level of calcineurin that predisposes these cells to apoptosis.

Preliminary research in rat neurons, used for ischemia therapy, by Castilho et al. (2000) has demonstrated that treatment of grafted neurons with cyclosporine A increased their survival, possibly due to the inhibition of calcineurin-mediated apoptosis (Canellada et al., 2006). This has also been shown to be the case in human neurons where treatment with cyclosporin A protected neurons against ischemia induced programmed cell death, implicating calcineurin-mediated apoptosis (Sheehan et al., 2006).

This is encouraging evidence for the potential of PMCA2 overexpression in neurons as a specific therapy for neuronal degeneration seen in spinal cord injuries and disease, by the inhibition of calcineurin-mediated apoptosis.

3.7.5 PMCA2/calcineurin interaction and pathological cardiac hypertrophy

The regulation of calcineurin by PMCA2 may also have implications in pathological cardiac hypertrophy. The calcineurin/NFAT pathway is known as a key signal transduction pathway in mediating cardiac hypertrophy (Molkentin et al., 1998). Although the predominant forms of PMCAs in the heart are isoforms 1 and 4, PMCA2 has also been shown to be expressed to a lesser degree (Guerini et al., 1999) and since this isoform is 2-3 times more active and with a higher basal activity (Hill et al., 2006 and Hilfiker et al., 1994), its effects may be more wide-spread than that of the ubiquitously expressed PMCA isoforms. Zwadlo et al. (2005) have shown that PMCA2 gene expression is elevated in a spontaneous hypertensive rat model (SHR), supporting the hypothesis that PMCA2 may inhibit calcineurin and calcineurin-mediated cell cycle regulation (reviewed in Vega et al., 2003) in aberrant cardiac cells, leading to hypertension and hypertrophy. Cyclosporine A treatment of cardiac hypertrophy in mouse and rat models often resulted in inhibition or complete reversal of the effects, however there were obvious adverse side-effects caused by the wide-ranging effects of cyclosporine A. Research into specific calcineurin inhibitors is important in this field, as cardiomyopathy and heart failure often occur from sustained hypertrophy, (Schulz and Yutzey, 2004).

3.7.6 PMCA2/calcineurin interaction and Diabetes

PMCA2 has been shown to be expressed in pancreatic β cells (Kamagate et al., 2000). The expression level of PMCA2 in diabetic β cells has yet to be established, but theoretically if the level of PMCA2 were to be increased in the diseased state, calcineurin-mediated cell cycle regulation would halt resulting in a lack of β cell proliferation and growth. In support of this hypothesis, upon glucose stimulation in the healthy β cell, a switch occurs from the low efficiency PMCA2 to a higher affinity calcium pump (Herchuelz et al., 2007). It is perhaps the case that this switch does not occur in diabetic β cells resulting in increased calcineurin inhibition by PMCA2. Sustained high glucose levels have been found to inhibit the expression and activity of PMCA2 (Reviewed in Gagliardino et al., 1994) in which case calcineurin would be released from inhibition and may perhaps instigate expression of genes related to calcineurin-mediated apoptosis triggered by different signals than those that promote cell cycle progression.

3.7.7 PMCA2/calcineurin interaction and Angiogenesis

Armesilla et al. (1999) have demonstrated that upon VEGF stimulation of primary endothelial cells, NFAT, the primary substrate of calcineurin, is dephosphorylated and may play a role in the expression of tissue factor, an important glycoprotein involved in the regulation of endothelial cell sprouting during angiogenesis (Senger 1996). Further support for the essential role of calcineurin in angiogenesis has been demonstrated by Hernandez et al. (2001). They have shown that VEGF-mediated angiogenesis is inhibited by the specific calcineurin inhibitor, cyclosporine A. Graef et al. (2001) have shown that a lack of calcineurin/NFAT signalling can result in the inhibition of vessel growth and that vessels tend to grow into regions with high NFAT expression.

This project has demonstrated that PMCA2 is expressed in endothelial cells and strongly interacts with calcineurin. It is possible to consider the overexpression of PMCA2 as a way to regulate vessel growth as a therapy in conditions where aberrant angiogenesis occurs, such as diabetic retinopathy and cancer.

Conversely by using a small interacting peptide, such as the pFlag-PMCA2(462-684) created during this project, the inhibitory interaction between PMCA2 and calcineurin could be overcome resulting in increased calcineurin/NFAT signaling

and angiogenesis, as a therapy for prevention of conditions such as atherosclerosis or for increasing wound healing capabilities.

3.8 Conclusion

In conclusion, our results demonstrate that the interaction between endogenous PMCA and calcineurin is isoform-specific in MCF-7 adenocarcinoma cells and endothelial cells suggesting that this interaction might play an important role in the regulation of many important physiological and pathological conditions.

This work has also demonstrated that PMCA2 can inhibit the calcineurin/NFAT pathway and that this inhibition can be disrupted by the overexpression of the small interacting protein pFlag-PMCA2(462-684) which expresses the region of PMCA2 primarily involved in the interaction with calcineurin. This discovery sheds new light on PMCA as a regulator of signal transduction pathways and may provide potential avenues for the development of effective inhibitors and activators of the calcineurin/NFAT pathway that may have fewer side-effects than treatments currently available, due to their endogenous and specific nature.

4. CHAPTER FOUR RESULTS

PMCA AS AN INHIBITOR OF THE NITRIC OXIDE SIGNAL TRANSDUCTION PATHWAY.

4.1 Introduction

Schuh et al. (2001) have recently demonstrated that PMCA4 interacts with neuronal NOS, nNOS, in mammalian cells and this interaction has an inhibitory effect on the production of NO by nNOS in cardiac cells (Oceandy et al., 2007). In this thesis the interaction between PMCA isoforms and the endothelial isoform of NOS, eNOS, has been investigated in endothelial cells to extend the current knowledge on PMCA interactions with NOS isoforms.

4.2 Analysis of the interaction PMCA-eNOS in HUVEC primary endothelial cells

To analyse the interaction between endogenous PMCA 1, 2 and 4 and eNOS in endothelial cell line EAhy926 and HUVEC cells, 5F10 anti-PMCA monoclonal antibody (abcam) was used to immunoprecipitate protein extracts isolated from EAhy926 and HUVEC cells. A western blot was then performed on immunoprecipitated proteins using a rabbit anti-eNOS antibody (Sigma) (Fig. 4.2.1, A). A strong and reproducible band corresponding to eNOS was visible in western blots for both EAhy926 and HUVEC cells indicating that PMCA and eNOS interact strongly in both endothelial cells types.

The 5F10 monoclonal antibody recognizes all PMCA isoforms. As a first step to evaluate the isoform/s of PMCA involved in the interaction with eNOS, the expression of PMCA isoforms in HUVEC primary cells was assessed. For this purpose 5F10 anti-PMCA monoclonal antibody was used to immunoprecipitate protein extracts from HUVEC cells. A western blot was then performed on immunoprecipitated proteins using isoform-specific anti-PMCA antibodies (Swant). Strong and reproducible bands for PMCA isoforms 1, 2 and 4 were visible in western blots indicating that they are expressed in HUVEC cells (Fig. 4.2.1, B).

The determination of the isoform involved in the interaction of PMCA with eNOS was performed using isoform-specific PMCA antibodies (Swant) against PMCA1, 2 or 4, to immunoprecitate protein extracts from HUVEC cells. Western blot was then performed with a monoclonal anti-eNOS antibody (Zymed).

Reproducible bands for each PMCA isoform were visible, however, the bands for PMCA 1 and 4 were weak compared to the band for PMCA2 which was stronger (Fig. 4.2.1, C).

The selectivity of the interactions was confirmed by performing control immunoprecipitations with an irrelevant antibody (anti-luciferase) (Promega) in which no protein was precipitated (Fig. 4.2.1, C).
These results demonstrate the interaction between endogenous PMCAs and eNOS in endothelial cells. They suggest that PMCA2 is the predominant isoform interacting with eNOS, although a very weak interaction is also visible between eNOS and PMCAs 1 and 4.

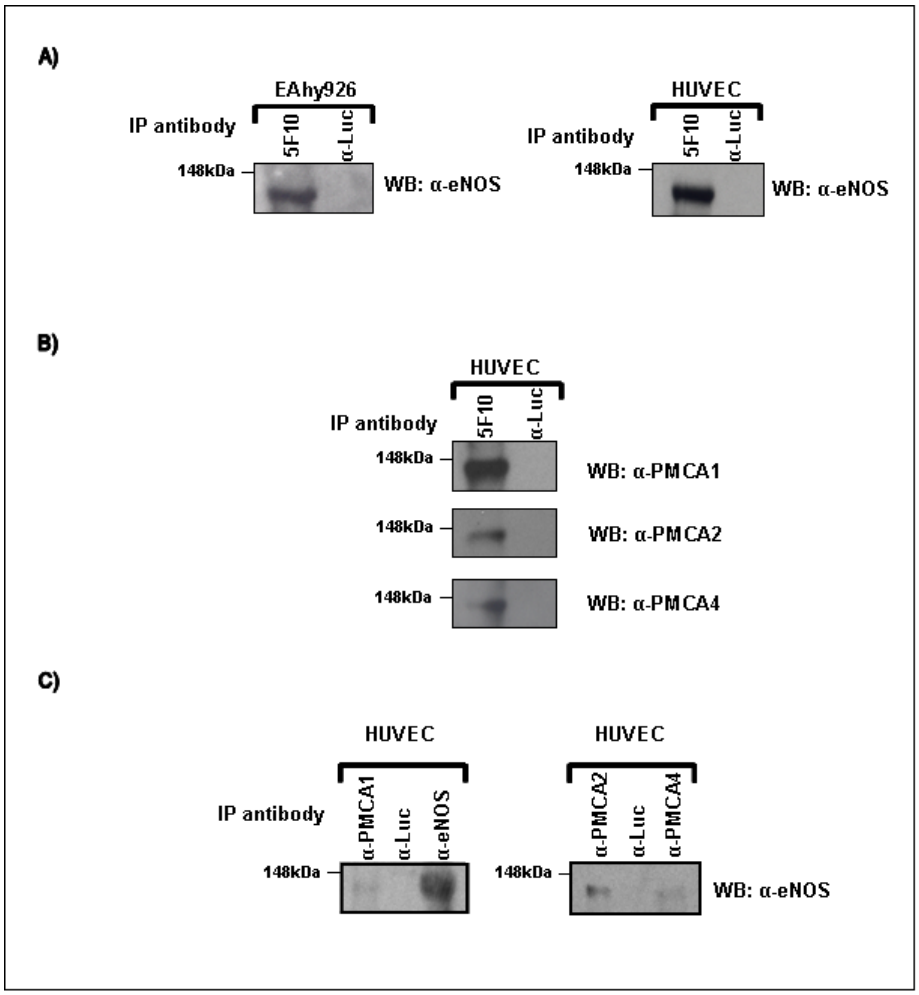

Fig. 4.2.1 Endogenous PMCA and eNOS interact in human endothelial cells*. A*, PMCA and eNOS co-precipitate in endothelial cells. Primary HUVEC endothelial cells (right panel) or the HUVEC-derived endothelial cell line EAhy926 (left panel) protein lysates were incubated with an anti-PMCA monoclonal antibody (5F10), or an irrelevant antibody raised against firefly luciferase (α-Luc), and Western blot of the immunoprecipitated proteins was performed with an anti-eNOS rabbit polyclonal antibody. *B*, Isoforms 1, 2 and 4 of PMCA are expressed in HUVEC cells. HUVEC cell protein lysates were precipitated with an anti-PMCA monoclonal antibody (5F10), or an irrelevant antibody raised against firefly luciferase (α-Luc). Western blot of the precipitated proteins was performed with rabbit polyclonal antibodies specific for PMCA isoforms 1, 2, or 4 (Swant). PMCA isoforms 1, 2, and 4 were expressed in HUVEC cells. *C*, The main isoform interacting with eNOS in endothelial cells is PMCA2. Protein extracts isolated from HUVEC cells were immunoprecipitated with polyclonal antibodies recognising specifically the isoforms 1, 2, or 4 of PMCA (Swant). Immunoprecipitated proteins were subjected to western blot analysis with an anti-eNOS mouse antibody (Zymed). Endogenous PMCA1 and 4 co-precipitated weakly with eNOS, whereas, PMCA2 and eNOS co-precipitated strongly, suggesting that in endothelial cells PMCA2 is the major isoform interacting with eNOS. An irrelevant antibody against firefly luciferase (α-Luc) was used during immunoprecipitation as a negative control and an antibody against eNOS (α-eNOS) was used as a positive control. A representative result from three independent experiments is shown.

As PMCA2 seems to be the predominant PMCA isoform interacting with eNOS the project focused on the interaction between these two proteins from this point onwards.

4.3 Analysis of the ectopic interaction between PMCA2 and eNOS in mammalian cells

In order to further confirm the interaction of PMCA2 with eNOS, human PMCA2 and eNOS were ectopically expressed in mammalian HEK293 cells.

To analyse the interaction between ectopic PMCA2 and eNOS in HEK293 cells, pcDNA3-PMCA2 (encoding human PMCA2) and pcDNA3-eNOSHumanA (encoding human eNOS) plasmids were transfected into HEK293 cells. Protein lysates of transfected cells were immunoprecipitated with 5F10, anti-PMCA monoclonal antibody. A western blot was then performed on the immunoprecipitated proteins using a rabbit anti-eNOS antibody (Sigma). As a negative control HEK293 cells were also transfected with pcDNA3-empty and pcDNA3-eNOS plasmids in the absence of ectopic pcDNA3-PMCA2.

A strong, reproducible band representing ectopic eNOS was only detected in samples expressing both ectopic PMCA2 and eNOS (Fig. 4.3.1, upper panel). In samples expressing only eNOS no band was visible, as expected. Ectopic expression of PMCA2 had no effect on the expression of the recombinant eNOS protein (Fig. 4.3.1, lower panel). The selectivity of the interaction was confirmed by performing control immunoprecipitations using an irrelevant antibody (anti-luciferase) (Promega) in which no protein was precipitated (Fig. 4.3.1).

To summarise, these results demonstrate that ectopically expressed PMCA2 and eNOS interact strongly and specifically in HEK293 mammalian cells.

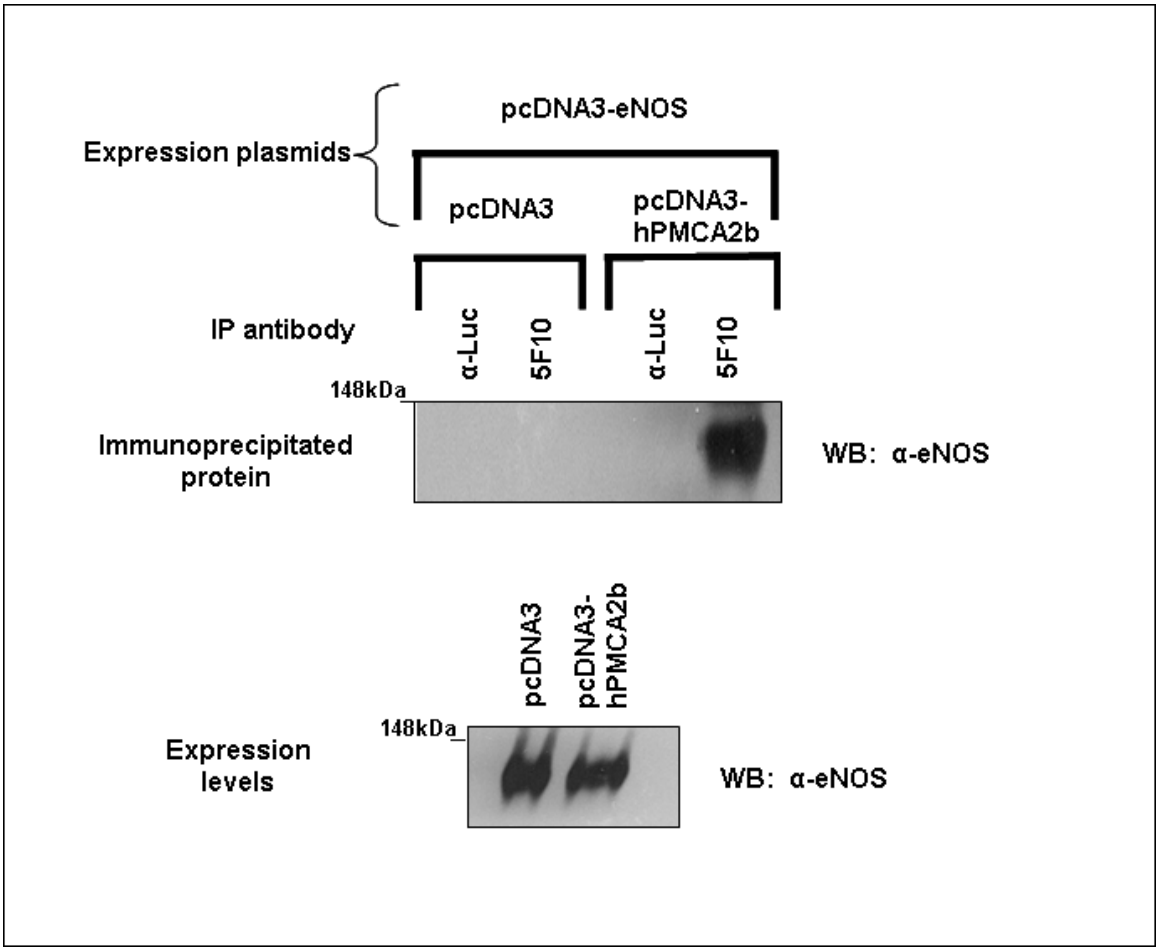

Fig. 4.3.1 Ectopically expressed PMCA2b and eNOS interact in HEK293 cells. Co-transfection of HEK 293 cells with expression vector pcDNA3-eNOS (encoding human eNOS) together with either pcDNA3-empty plasmid (negative control) or pcDNA3-hPMCA2b (encoding human PMCA2b). An antibody against PMCA (5F10) or an irrelevant antibody raised against firefly luciferase (α-Luc) were used to immunoprecipitate protein lysates from transfected cells. An anti-eNOS rabbit antibody (Sigma) was used during Western blot analysis to detect immunoprecipitated proteins or expression levels of eNOS. A representative result from three independent experiments is shown.

4.4 Analysis of the interaction between eNOS and recombinant Flag-tagged PMCA2 truncated proteins.

As shown in Chapter three region 462-684 of PMCA2 interactys with calcineurin, a calcium/calmodulin-dependent protein. This highlighted the possibility that this domain of PMCA2 may also interact with eNOS, as it too is regulated by calcium/calmodulin. Another domain of PMCA2 potentially implicated in the interaction with eNOS is the region 1143-1243, corresponding to the C-terminal tail of PMCA2. The regions of PMCA1 and 4 equivalent to this have been described to interact with nNOS in mammalian cells (Schuh et al., 2001).

The involvement of these two domains of PMCA2 in the interaction with eNOS was investigated in this work using flag-tagged fusion proteins containing the amino acid regions 462-684, or 1143-1243 of PMCA2b to investigate if the region 462-684 of PMCA2 may interact with eNOS. Plasmids p3xFlag-PMCA2b(462-684) or p3xFlag-PMCA2b(1143-1243) were transfected into HEK293 cells. The generation of expression plasmids p3xFlag-PMCA2(462-684) and p3xFlag-PMCA2(1143-1243) have been described previously (section 3.2.1).
Protein lysates of transfected cells were incubated with commercially available recombinant human eNOS (10 μM final concentration) (Sigma), immunoprecipitated with an anti-eNOS monoclonal antibody and, subsequently, immunoprecipitated proteins were detected by Western blot using an anti-Flag monoclonal antibody (Sigma). Flag-PMCA2b(462-684) co-precipitated with eNOS (Fig. 4.4.1, upper panel) whereas no precipitation was observed for Flag-PMCA2b(1143-1243) or the negative control pcDNA3-empty (Fig. 4.4.1, upper panel) demonstrating the selectivity of the interaction. Both recombinant proteins were expressed at equal levels (Fig. 4.4.1, lower panel). These results demonstrate that the region 462-684 of the catalytic, big intracellular loop of human PMCA2b is involved in the interaction with eNOS

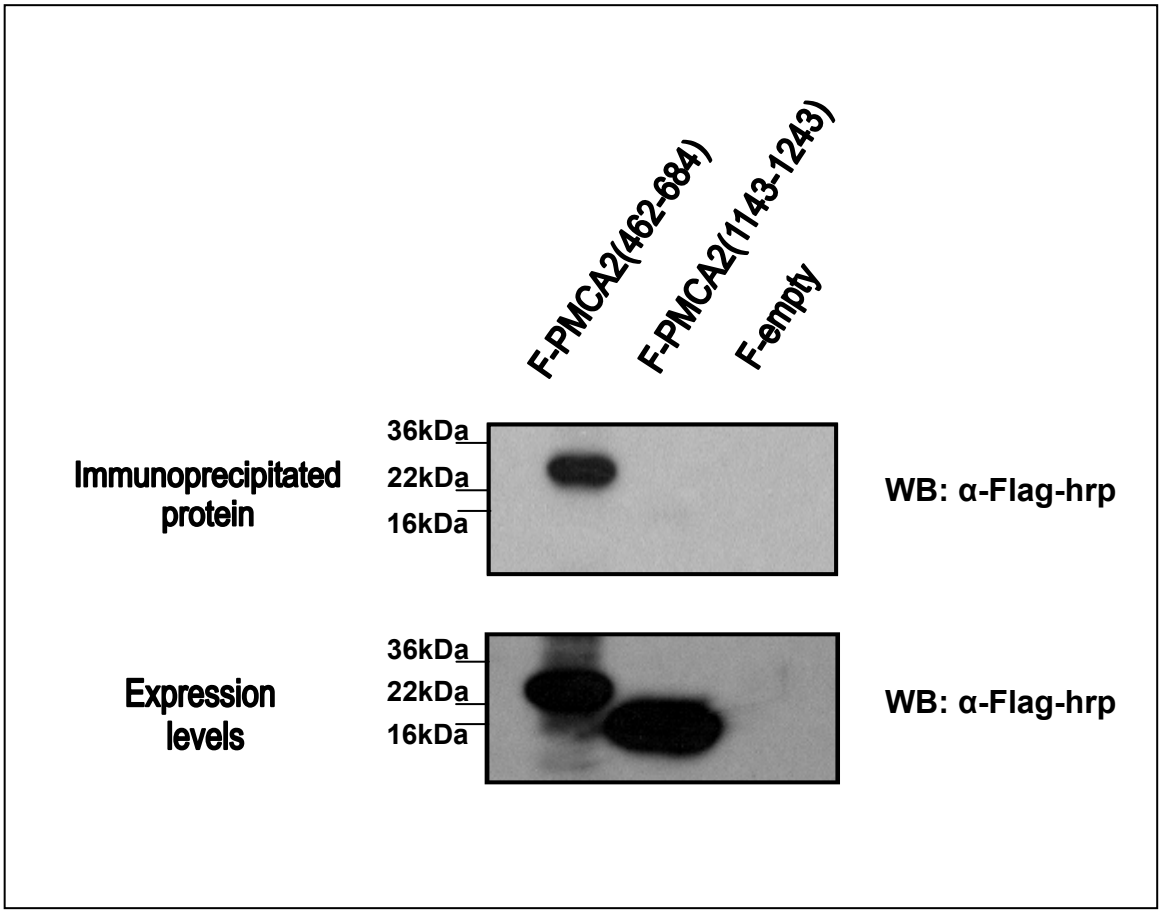

Fig. 4.4.1 eNOS interacts with region 462-684 of PMCA2b. HEK293 cells were transfected with Flag-tagged PMCA2(426-684) or PMCA2(1143-1243). As a negative control pF-CMV7.1 empty vector (Sigma) (F-empty) was used. Commercial recombinant eNOS (Sigma) (10 µM, final concentration) was incubated with protein lysates and precipitated with an anti-eNOS polyclonal antibody (Sigma). M2 anti-Flag peroxidase monoclonal antibody (upper panel) was used to detect immunoprecipitated proteins during Western blot analysis. Prior to immunoprecipitation expression levels were analysed by Western blot using the same antibody (lower panel). The region 426-684 of PMCA2 was found to specifically interact with eNOS. A representative result from three independent experiments is shown.

4.5 Determination of the domain of eNOS interacting with PMCA2

4.5.1 Generation of the Flag-tagged expression plasmids for identification of the eNOS domain interacting with PMCA2

To further characterize the interaction between PMCA2 and eNOS the domain of eNOS interacting with PMCA2 was also defined. Flag-tagged recombinant proteins were constructed encoding various small regions of eNOS spanning the whole length of the eNOS protein to determine the domain involved in the interaction with PMCA2.

4.5.1a Cloning strategy

In order to generate expression vectors encoding Flag-tagged truncated proteins of human eNOS, the corresponding fragments of human eNOS cDNA (gene bank accession number: NM_000603) were amplified by PCR and cloned into the expression vector p3xFlagcmv7.1 (Sigma). The fidelity of the amplified fragment was confirmed by sequencing.

Prior to primer generation, the sequence of the fragments of eNOS cDNA encoding the relevant regions of the eNOS protein were analysed for restriction site mapping. Primers for PCR reactions were designed containing appropriate restriction enzymes for cloning, not found within the fragment of eNOS cDNA to be amplified.

An overview of the Flag-tagged eNOS truncated proteins used in this study is shown in Fig. 4.5.1.

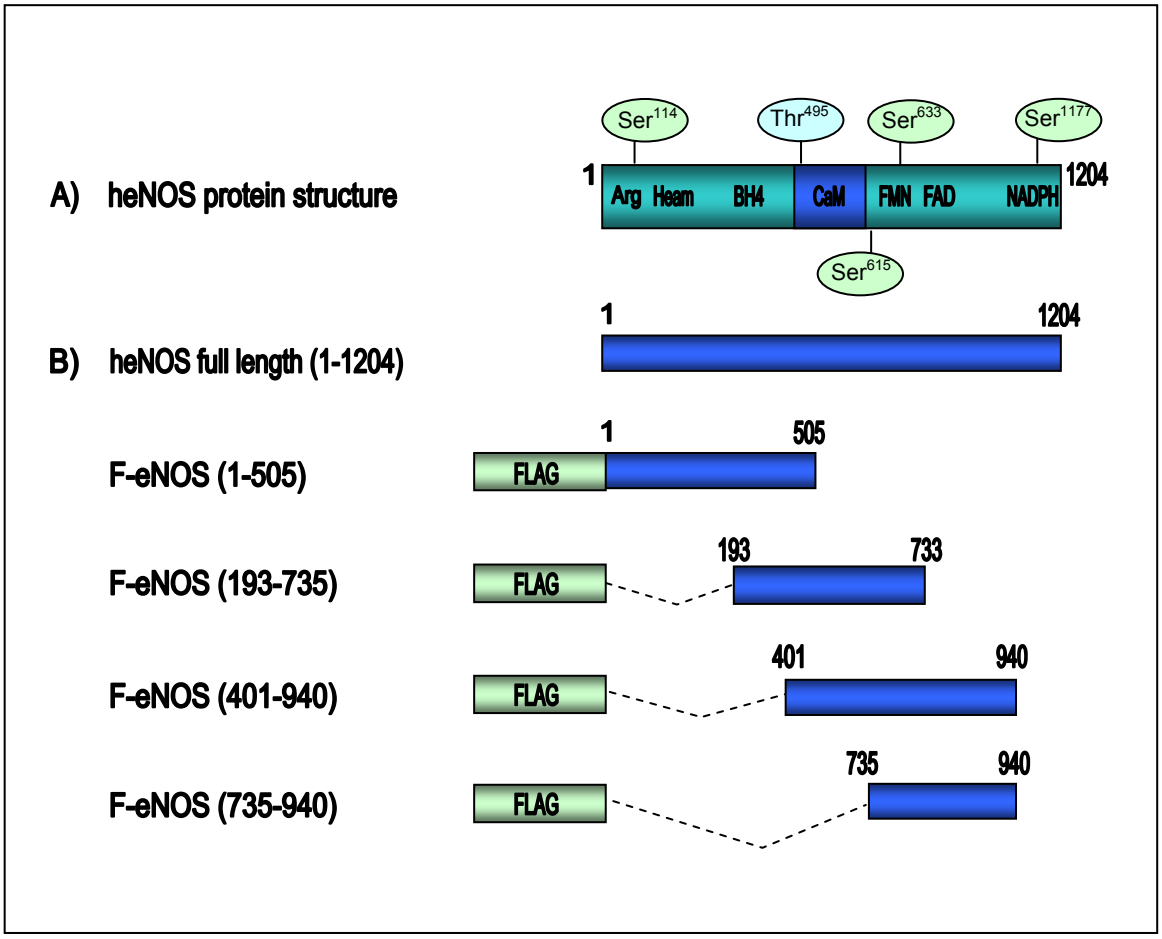

Fig. 4.5.1 Schematic overview of the constructs used for determination of the PMCA2 interaction domain of eNOS. A) Full length eNOS including phosphorylation sites and domains as a reference. B) Structure of eNOS flag-tagged fusion proteins.

4.5.1a(i) Construction of pFlag-eNOS(1-505) plasmid

To generate construct pFlag-eNOS(1-505) naturally occurring restriction enzyme sites HindIII and BglII of human eNOS were utilized. The pcDNA3-eNOSHumanA plasmid was digested with restriction enzymes HindIII-BglII and cloned into the HindIII-BglII sites of plasmid pFlag-cmv5b (Fig. 4.5.2).

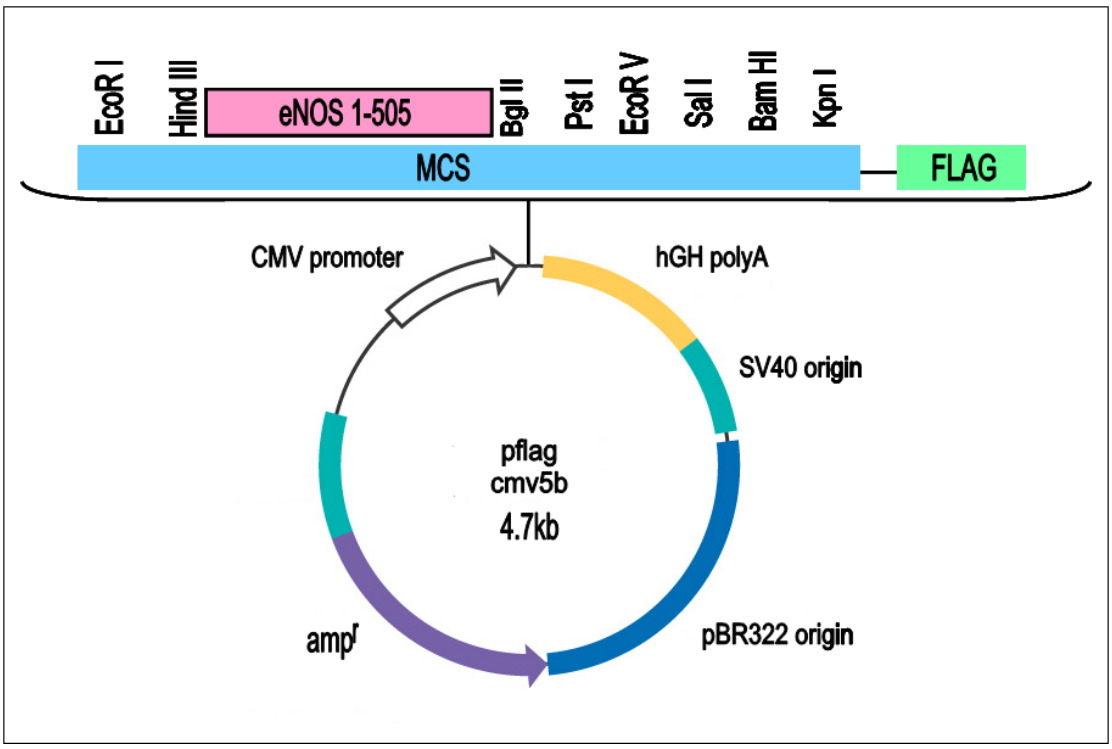

Fig. 4.5.2 Schematic diagram of the cloning strategy for pflagcmv5b-eNOS (1-505).

To confirm the successful generation of the recombinant expression vector pFlag-eNOS(1-505), the plasmid was digested with restriction enzymes HindIII-BglII. DNA electrophoresis of the digestion reaction in agarose gels showed two DNA fragments corresponding to insert (1515bp) and plasmid (4700bp) as expected (Fig. 4.5.3)

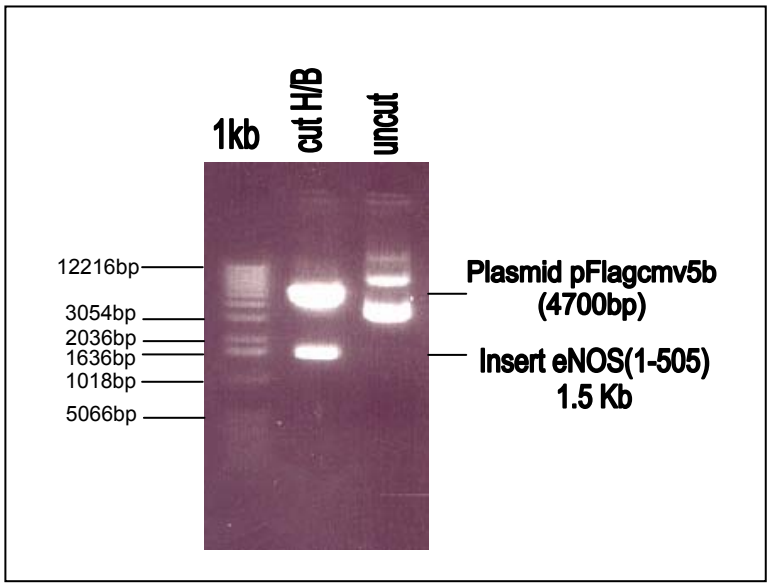

Fig. 4.5.3 Agarose gel electrophoresis of restriction enzyme digested pflagcmv5b-eNOS (1-505). Digestion released a fragment of DNA 1515bp in size relating to amino acids 1-505 of human eNOS and a larger fragment of 4.7 relating to the expression plasmid pflagcmv5b.

4.5.1a(ii) Construction of p3xFlag-eNOS(193-735) plasmid

To generate construct p3xFlag-eNOS(193-735) the fragment encoding amino acids 193-735 of human eNOS (numbering according to gene bank accession number: NM_000603) was amplified by PCR following the standard conditions described in section 2.2.1 and using primers;

sense 5'-TCTTCCggAATTCTCTgCAggTgTTCgATgCCCg -3' and

antisense 5'- CTTCCCTCTAgATCACTggCgCTTCCAgCTCCg -3'.

The optimum annealing temperature for both primers was 62°C.

The PCR product was digested with restriction enzymes EcoRI and XbaI, and cloned into the EcoRI-XbaI sites of plasmid p3xflag-cmv7.1 (Fig. 4.5.4).

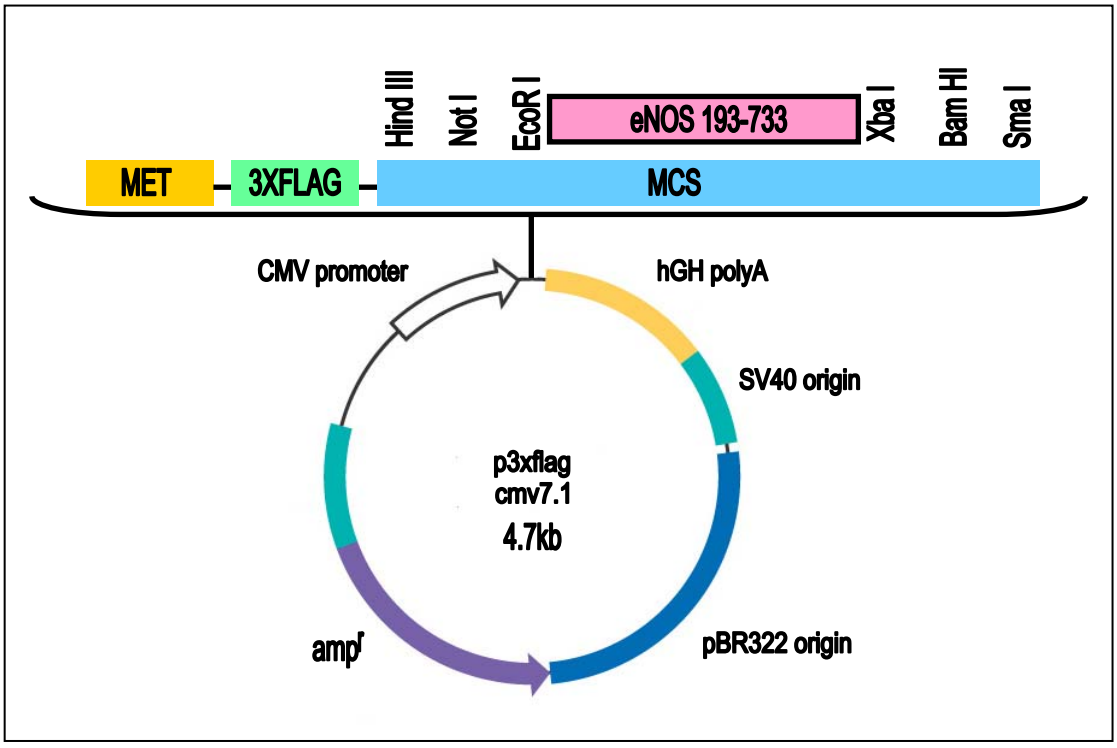

Fig. 4.5.4 Schematic diagram of the cloning strategy for p3xFlag-eNOS(193-733)

To confirm the successful generation of the recombinant expression vector p3xFlag-eNOS(193-735), the plasmid was digested with restriction enzymes EcoRI and XbaI. DNA electrophoresis of the digestion reactions in agarose gels showed two DNA fragments corresponding to insert (1626bp) and plasmid (4700bp) as expected (Fig. 4.5.5)

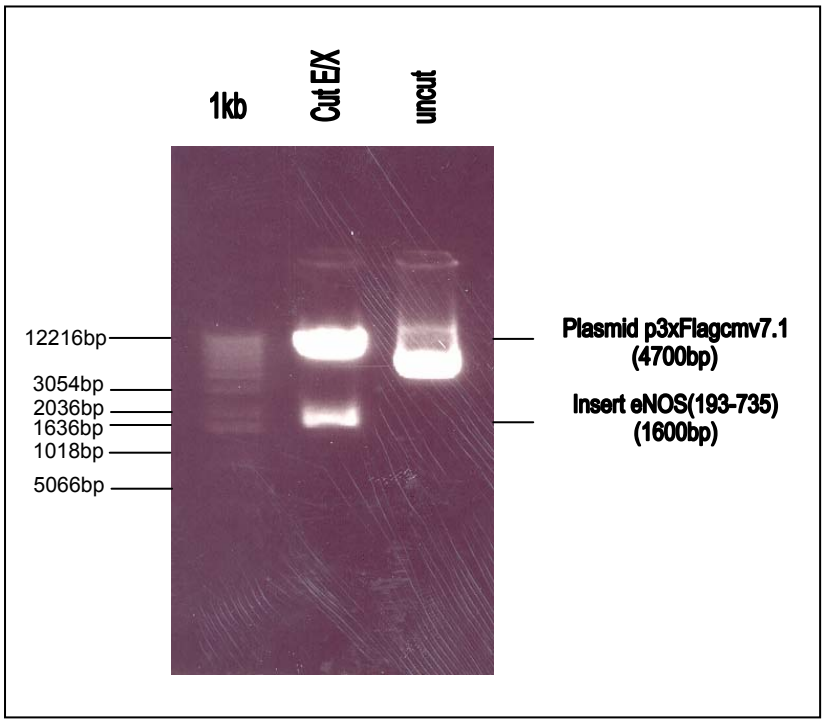

Fig. 4.5.5 Agarose gel electrophoresis of restriction enzyme digested p3xFlag-eNOS(193-733). Digestion released a fragment of DNA 1.6kb in size relating to amino acids 193-733 of human eNOS and a larger fragment of 4.7 relating to the expression plasmid p3xflagcmv7.1.

4.5.1a(iii) Construction of p3xFlag-eNOS(401-940) plasmid

To generate construct p3xFlag-eNOS(401-940) the fragment encoding amino acids 401-940 of human eNOS (numbering according to gene bank accession number: NM_000603) was amplified by PCR following the standard conditions described in section 2.2.1 and using primers;

sense 5'-TCTTCCggAATTCTCTgCAggTgTTCgATgCCCg -3' and

antisense 5'-CTTCCCTCTAgATCACTggCgCTTCCAgCTCCg -3'.

The optimum annealing temperature for both primers was 62°C.

The PCR product was digested with restriction enzymes EcoRI and XbaI, and cloned into the EcoRI-XbaI sites of plasmid p3xflag-cmv7.1 (Fig. 4.5.6).

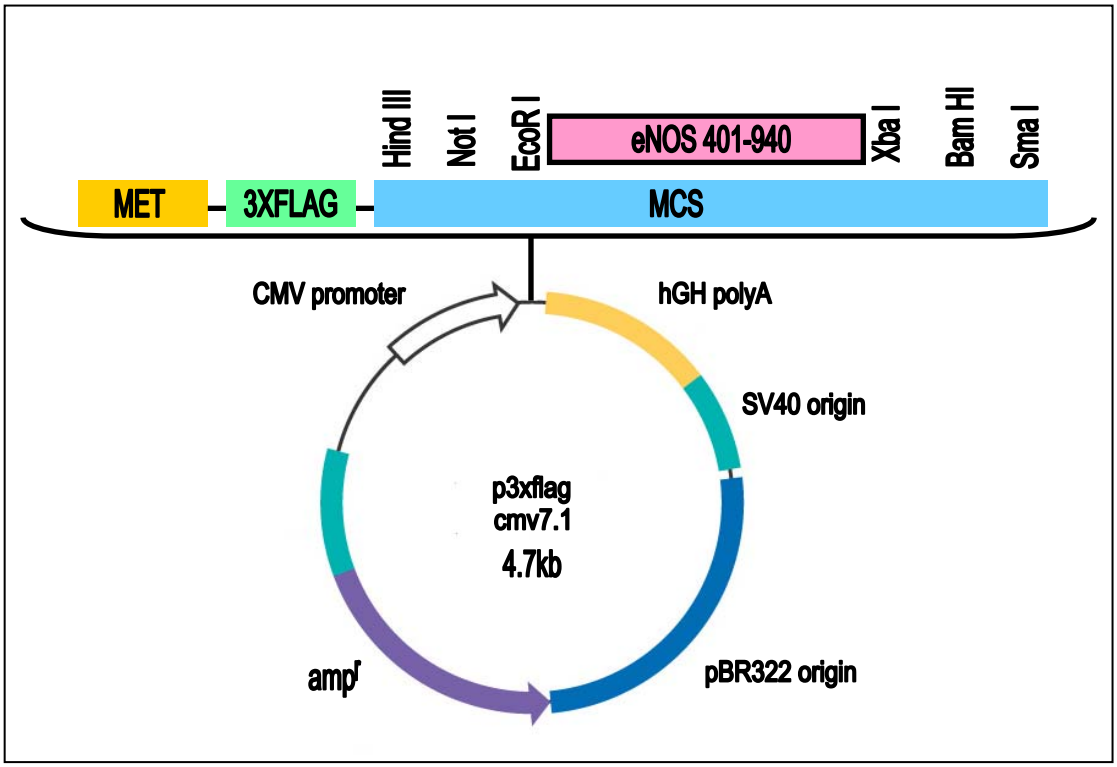

Fig. 4.5.6 Schematic diagram of the cloning strategy for p3xflag-eNOS(401-940).

To confirm the successful generation of the recombinant expression vector p3xFlag-eNOS(401-940), the plasmid was digested with restriction enzymes EcoRI and XbaI. DNA electrophoresis of the digestion reactions in agarose gels showed two DNA fragments corresponding to insert (1617bp) and plasmid (4700bp) as expected (Fig. 4.5.7)

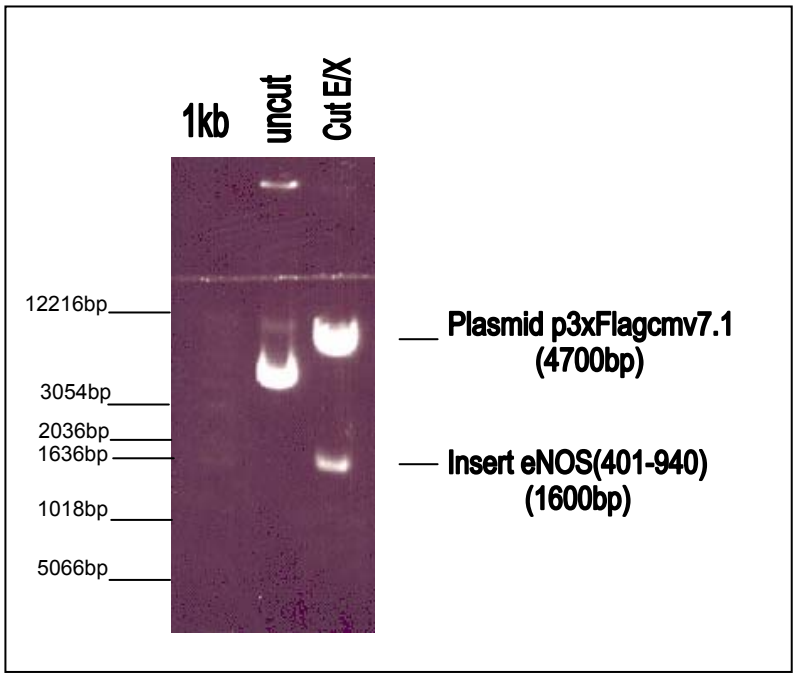

Fig. 4.5.7 Agarose gel electrophoresis of restriction enzyme digested p3xFlag-eNOS(401-940). Digestion released a fragment of DNA 1617bp in size relating to amino acids 401-940 of human eNOS and a larger fragment of 4.7 relating to the expression plasmid p3xflagcmv7.1.

4.5.1a(iv) Construction of p3xFlag-eNOS(735-940) plasmid

To generate construct p3xFlag-eNOS(735-940) the fragment encoding amino acids 735-940 of human eNOS (numbering according to gene bank accession number: NM_000603) was amplified by PCR following the standard conditions described in section 2.2.1 and using primers;

sense 5'- TCTTCCggAATTCTgAAATCAACgTggCCgTgCT -3' and

antisense 5'- CTTCCCTCTAgATCAgTAgTACCggggCTggAgCAg -3'.

The optimum annealing temperature for both primers was 66°C.

The PCR product was digested with restriction enzymes EcoRI and XbaI, and cloned into the EcoRI-XbaI sites of plasmid p3xflag-cmv7.1 (Fig. 4.5.8).

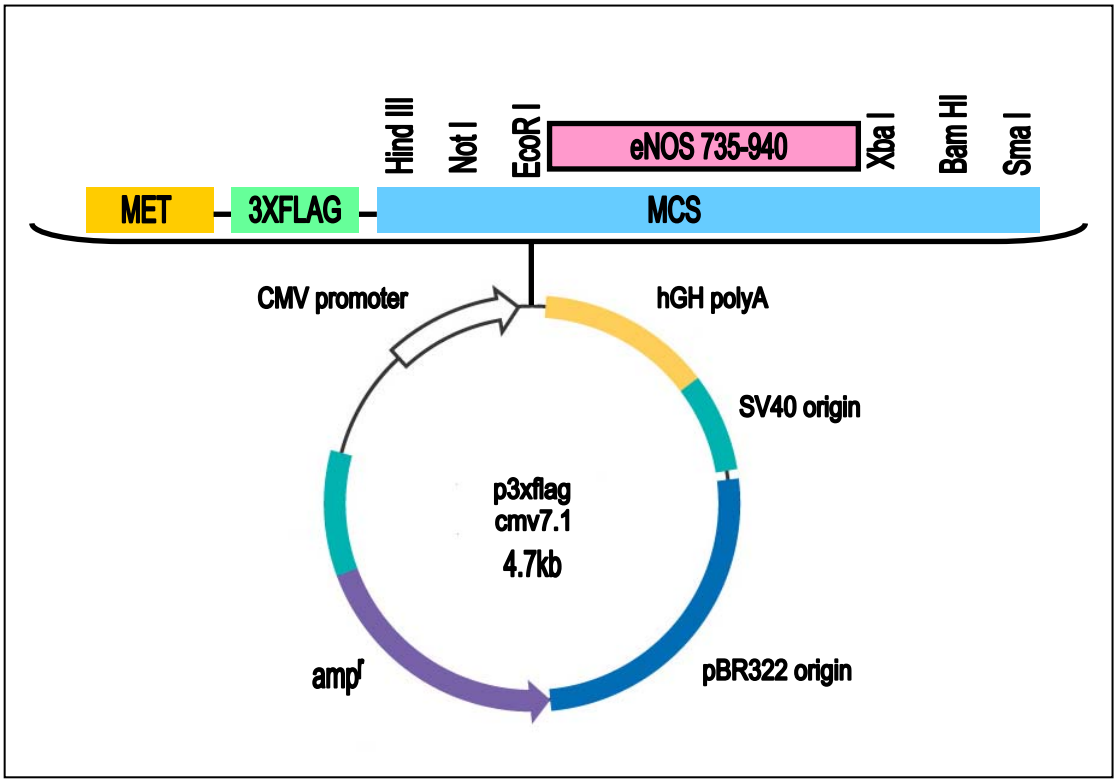

Fig. 4.5.8 Schematic diagram of the cloning strategy for p3xFlag-eNOS (735-940).

To confirm the successful generation of the recombinant expression vector p3xFlag-eNOS(735-940), the plasmid was digested with restriction enzymes EcoRI and XbaI. DNA electrophoresis of the digestion reactions in agarose gels showed two DNA fragments corresponding to insert (615bp) and plasmid (4700bp) as expected (Fig. 4.5.9).

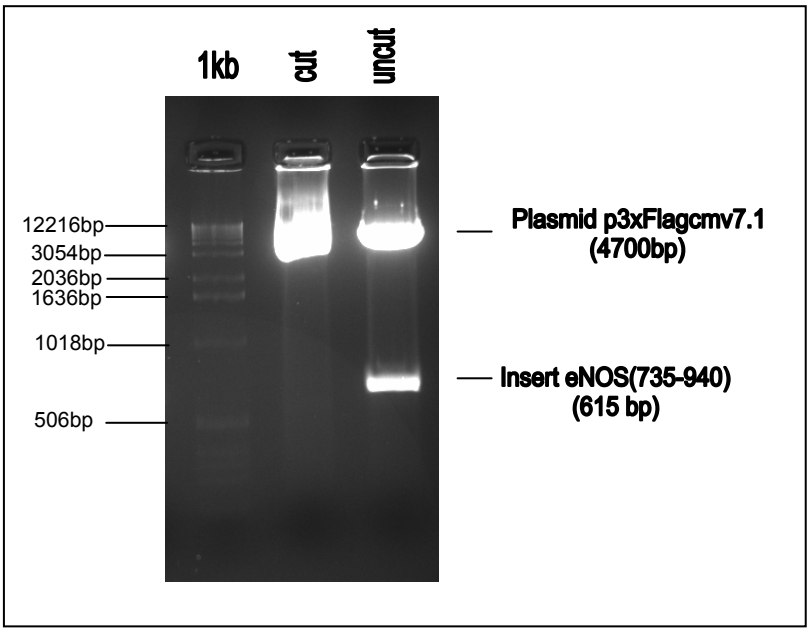

Fig. 4.5.9 Agarose gel electrophoresis of restriction enzyme digested p3xFlag-eNOS (735-940). Digestion released a fragment of DNA 615bp in size relating to amino acids 735-940 of human eNOS and a larger fragment of 4.7 relating to the expression plasmid p3xflagcmv7.1.

4.5.2 Analysis of the interaction between PMCA2 and recombinant Flag-tagged eNOS proteins

Flag-tagged fusion proteins containing the amino acid regions 1-505, 193-733, 401-940 and 735-940 of eNOS were used to investigate the region of eNOS involved in the interaction with PMCA2.

Plasmids pFlag-eNOS(1-505), p3xFlag-eNOS(193-733) and p3xFlag-eNOS(401-940) were co-transfected into HEK293 cells with pcDNA3-PMCA2. Protein lysates of transfected cells were immunoprecipitated with 5F10, anti-PMCA antibody. Western blot was performed on immunoprecipitated proteins using an anti-Flag monoclonal antibody.

Flag-eNOS(401-940) co-precipitated with PMCA2 (Fig. 4.5.10, A, lower panel) whereas no precipitation was observed for Flag-PMCA2(193-733) and (1-505). The expression levels for each recombinant protein were the same, indicating that lack of interaction was not due to lack of expression (Fig. 4.5.10, A, upper panel). These results indicate that the region 401-940 of human eNOS contains the domain responsible for the interaction with PMCA2.

To further define the region of eNOS involved in the interaction, plasmids p3xFlag-eNOS(735-940) and p3xFlag-eNOS(1-505) were co-transfected into HEK293 cells with pcDNA3-PMCA2. Protein lysates of transfected cells were immunoprecipitated with 5F10, anti-PMCA antibody. Western blot was performed on immunoprecipitated proteins using an anti-Flag monoclonal antibody.

Flag-eNOS(735-940) co-precipitated with PMCA2, whereas no precipitation was observed for Flag-eNOS(1-505) (Fig. 4.5.10, B, right panel). Both recombinant proteins were expressed at similar levels indicating that lack of interaction was not due to lack of expression (Fig. 4.5.10, B, left panel).

These results demonstrate that the region 735-940 of eNOS is involved in the interaction with PMCA2.

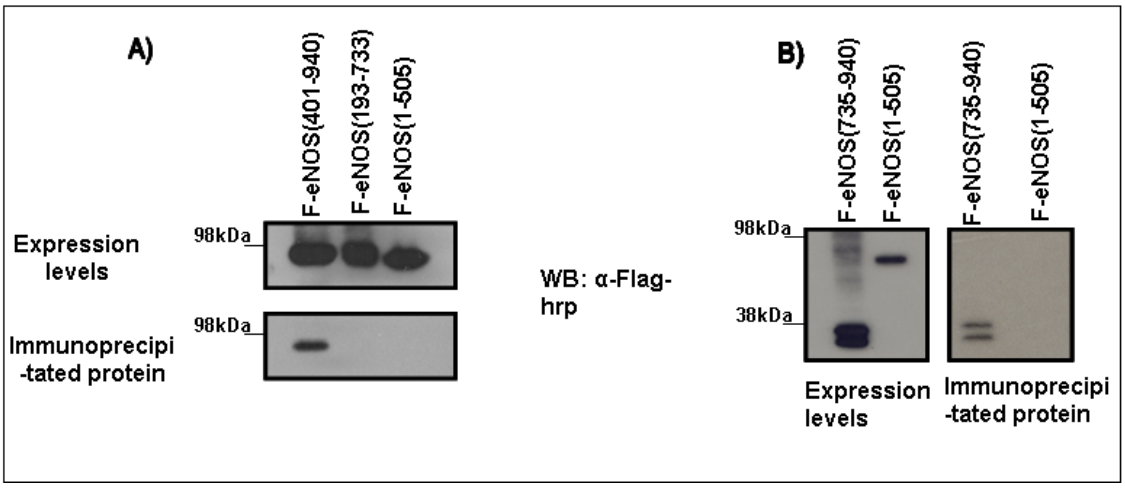

Fig. 4.5.10 PMCA2b interacts with the region 735-940 of eNOS *A*, HEK293 cells were co-transfected with human PMCA2b and Flag-tagged deletion mutants of eNOS encoding amino acids 401-940, 193-733, or 1-505. Protein lysates were immunoprecipitated with the 5F10 anti-PMCA monoclonal antibody and subjected to Western blot analysis with the M2 anti-Flag monoclonal antibody. PMCA2 interacted with F-eNOS(401-940) but F-eNOS-(193-733) or –(1-505) did not co-precipitate PMCA2b. This indicated that the region 733-940 of eNOS is essential for the interaction with PMCA2b. *B*, HEK293 cells were co-transfected with human PMCA2b and Flag-tagged deletion mutants encoding amino acids 735-940 or 1-505. Western blot was performed on immunoprecipitated protein lysates and revealed that F-eNOS(735-940) co-precipitated with PMCA2b but F-eNOS(1-505) failed to co-precipitate PMCA2. In summary the region 735-940 of eNOS is essential for eNOS interaction with PMCA2b. A representative result from three independent experiments is shown.

4.6 Functional analysis of the interaction between PMCA2 and eNOS in primary endothelial cells

To determine the functionality of the interaction between PMCA2 and eNOS the levels of cGMP were measured, using a kit from Amersham, in the presence or absence of ectopically expressed PMCA2. cGMP levels were used as a surrogate marker for the production of NO from eNOS. NO produced from eNOS activates guanylyl cyclases to convert GTP to cGMP, therefore, the levels of cGMP are directly proportional to NO production.

Plasmid pcDNA3-PMCA2 and pcDNA3-empty were transfected into HUVEC cells using the Amaxa method (described in section 2.1.6a(ii)). Cells were stimulated with the calcium Io A23187 and treated with superoxide dismutase, IBMX and L-Arginine prior to cGMP measurement. cGMP was measured in fmol/mL according to the manufacturer's instructions.

Samples expressing PMCA2 had a 43% reduction in NO-dependent cGMP production (Fig. 4.6.1). The control using pcDNA3-empty was used as the baseline for cGMP production and fold induction was calculated for the experimental results (Fig. 4.6.1).

These results indicate that PMCA2 inhibits eNOS NO production when overexpressed in endothelial cells.

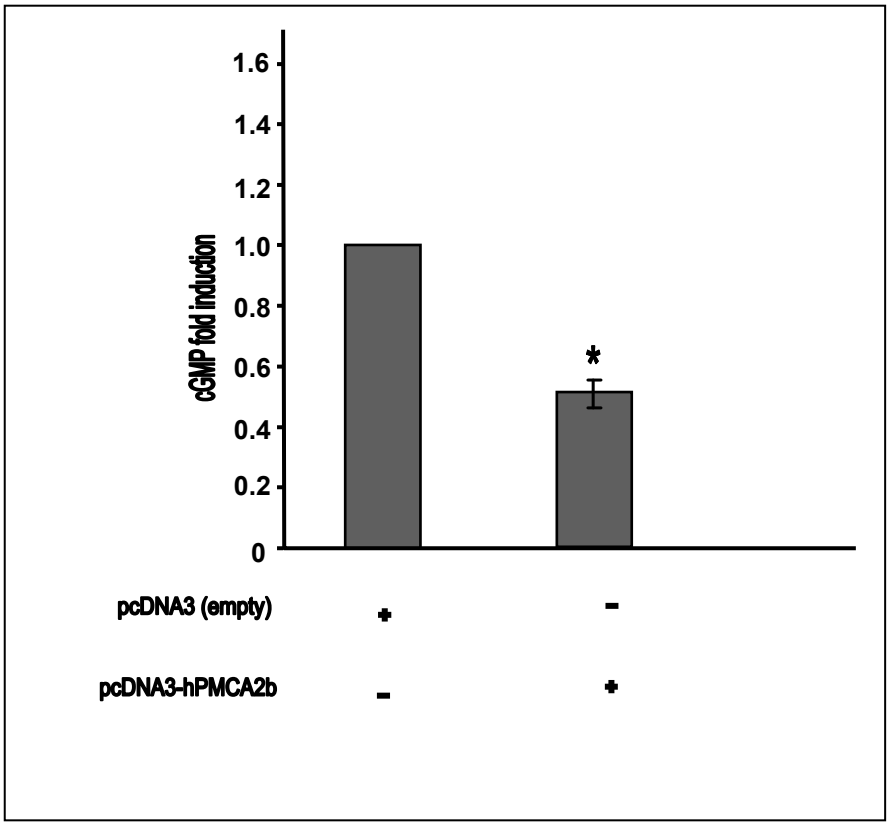

Fig. 4.6.1 Ectopic expression of human PMCA2 negatively regulates NO production in HUVEC endothelial cells. HUVEC cells were transfected with 5 µg of plasmids pcDNA3-hPMCA2b or pF-PMCA2b(462-684). A23187 ionophore (0.5 µM) was added 3 minutes before lysis to induce NO synthesis. NO-dependent cGMP production was calculated in relation to HUVEC cells transfected with 5 µg of pcDNA3 empty vector. Human PMCA2 overexpression resulted in significantly reduced (43%) NO-dependent cGMP production. (*) statistically significant ($P \leq 0.05$, according to an unparied Student's T-test) compared to the pcDNA3 control. Mean ±S.E of six independent experiments are shown.

4.7 Disruption of the interaction between PMCA2 and eNOS using Flag-PMCA2(462-684) in endothelial cells

4.7.1. Analysis of the effect of Flag-PMCA2(462-684) overexpression on the Interaction between PMCA2 and eNOS.

Region 462-684 of PMCA2 encompasses the interaction domain of PMCA2 with eNOS. We hypothesised that by introducing a high concentration of this region in endothelial cells the endogenous interaction between PMCA2 and eNOS could be disrupted.

To demonstrate this, endothelial cell lysates were immunoprecipitated with 5F10, anti-PMCA antibody. The immunoprecipitated proteins were incubated with various concentrations of Flag-PMCA2(462-684) recombinant protein, (purified as described in section 2.3.3), to determine if this could disrupt the endogenous interaction between PMCA2 and eNOS, Flag-empty protein was used as a control. Western blot analysis was performed on the final mixtures using polyclonal anti-eNOS (Sigma).

Addition of a high concentration of Flag-PMCA2(462-684) recombinant protein to the protein lysates resulted in a significant reduction of immunoprecipitated eNOS (Fig. 4.7.1), whereas protein lysates incubated with Flag-empty and low concentrations of Flag-PMCA2(462-684) demonstrated no effect on the immunoprecipitation of eNOS (Fig. 4.7.1).

These results demonstrate that region 462-684 of PMCA2 is capable of disrupting the interaction between endogenous PMCA2 and eNOS in endothelial cells.

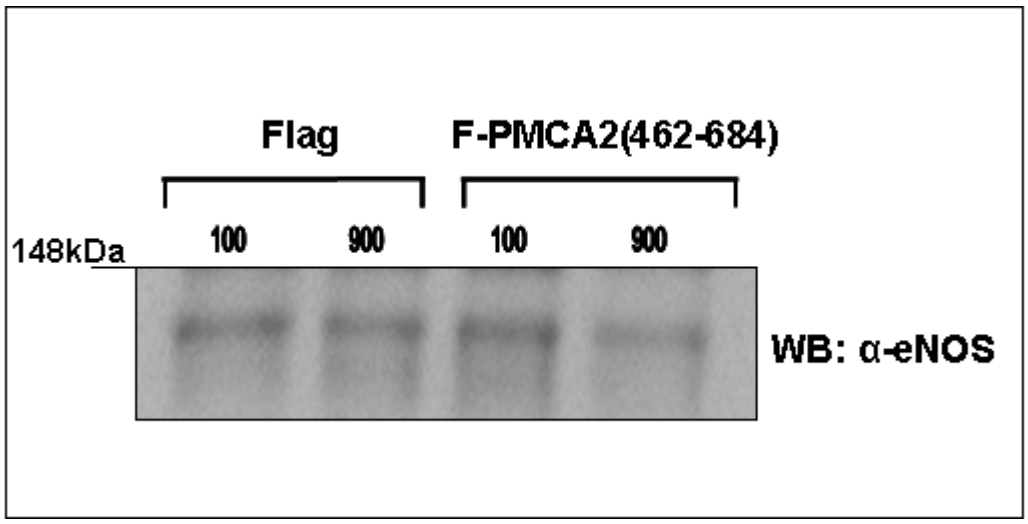

Fig. 4.7.1 The interaction between endogenous PMCA2 and eNOS in endothelial cells is disrupted by PMCA2 region 462-684. Endothelial cell protein extracts were immunoprecipitated with 5F10 anti-PMCA monoclonal antibody. Immunoprecipitation reactions were incubated with 100 or 900 µl of elution solution containing purified F-PMCA2(462-684) adjusted to a final volume of 1 ml with RIPA buffer. Western blot analysis with an anti-eNOS rabbit polyclonal antibody was used to determine eNOS protein co-precipitation. A significant decrease in the levels of co-precipitated eNOS was observed when excess recombinant F-PMCA2(462-684) was included. The levels of co-precipitation of eNOS were not altered when control immunoprecipitations were incubated with identical amounts of Flag elution solution obtained after transfection of HEK293 cells with pF-CMV7.1 empty vector. The region 462-684 of PMCA2 is capable of disrupting the interaction between endogenous PMCA2 and eNOS. A representative result from three independent results is shown.

4.7.2 Functional analysis of the disruption of the interaction between PMCA2 and eNOS by Flag-PMCA2(462-684) in endothelial cells.

To determine if the disruption of the interaction between PMCA2 and eNOS by Flag-PMCA2(462-684) had an effect on the inhibition of eNOS by PMCA2 a cGMP assay was performed.

Plasmid pFlag-PMCA2(462-684) and pcDNA3-empty were transfected into endothelial cells using the Amaxa method. Cells were stimulated with calcium Io A23187 and treated with superoxide dismutase, IBMX and L-Arginine prior to cGMP measurement. cGMP was measured in fmol/mL according to the manufacturer's instructions.

Samples expressing Flag-PMCA2(462-684) had a significant 49% increase in NO-dependent cGMP production, compared to the control pcDNA3 basal levels (Fig.

4.7.2). The control pcDNA3-empty was used as the baseline for HUVEC cGMP production (Fig. 4.7.2).

These results indicate that Flag-PMCA2(462-684) can disrupt the inhibitory effect of endogenous PMCA2 on eNOS resulting in an increase of total NO synthesis.

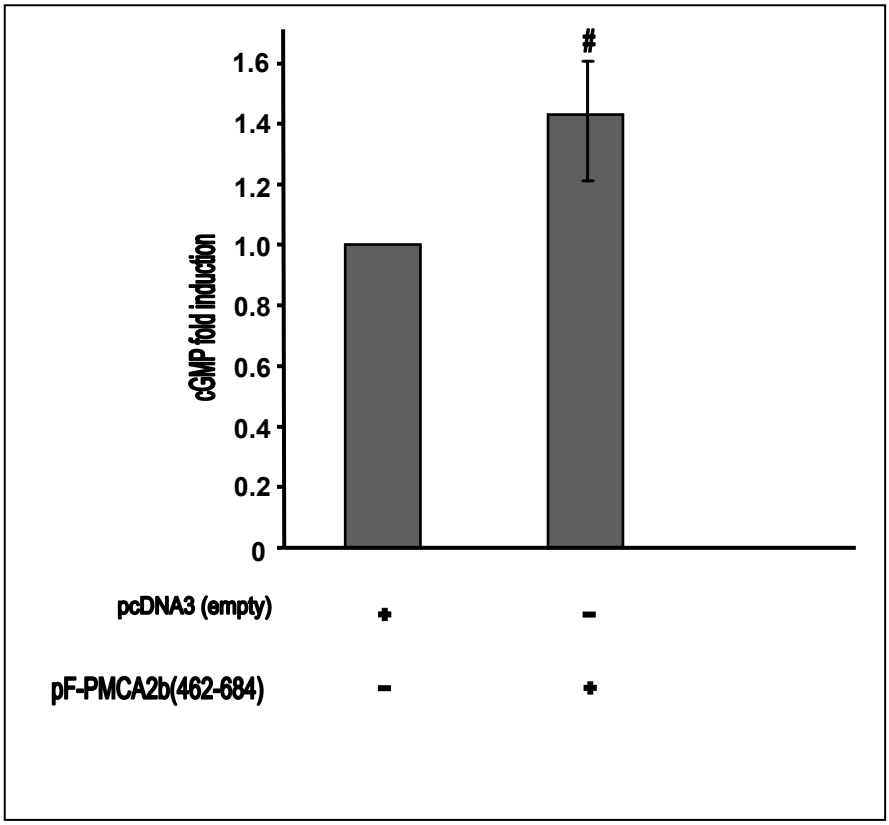

Fig. 4.7.2 Flag-PMCA2(462-684) can disrupt the inhibitory effect of PMCA2 on eNOS. The interaction between endogenous PMCA and eNOS was disrupted by overexpression of the region 462-684 of PMCA2 and resulted in a significantly increased (49%) NO-dependent cGMP production. (#) statistically significant ($P \leq 0.01$, according to an unpaired student's T-test) compared to the pcDNA3 control. Mean ± S.E. of six independent experiments are shown.

4.8 Discussion

It has been previously described that PMCA4 interacts with and inhibits the activity of the neuronal form of NOS (Schuh et al., 2001). It was the aim of this research to identify any isoform-specific interaction between PMCA and eNOS in endothelial cells and determine its functionality.

This investigation has demonstrated that endogenous eNOS and PMCA2 interact in endothelial cells, leading to a decrease in NO production by activated endothelial cells. In agreement with our observations on the functional relevance of the interaction PMCA2/eNOS as a novel mechanism for eNOS regulation, inhibitory interactions between eNOS and the intracellular domains of other plasma membrane proteins such us: caveolin-1 (Garcia-Cardena et al., 1997), the bradykinin B2 receptor (Ju et al., 1998) the endothelin-1 ETB receptor (Marrero et al., 1999) and the angiotensin II AT1 receptor (Marrero et al., 1999) have been previously reported.

This project also demonstrated that the interaction with eNOS maps to the region 462-684 of PMCA2, which is located in the large intracellular catalytic domain, defined by trans-membrane segments 4 and 5. As described in chapter 3 an inhibitory interaction between the same domain of PMCA2 and the calcium/calmodulin-dependent phosphatase calcineurin A (Holton et al., 2007). The interaction of another calcium/calmodulin-dependent protein with the same domain of PMCA2 suggests an important role for this region in the association with partner proteins that are regulated by calcium. We have also located the interaction domain with PMCA2 to the region 735-940 of eNOS. Overexpression of the PMCA2 interaction domain disrupts the interaction between endogenous PMCA2 and eNOS in endothelial cells.

4.8.1 eNOS and cancer growth

There is substantial evidence to suggest that tumour cells need eNOS to maintain their growth by the production of NO and subsequent activation of oncogenic signalling, this has been demonstrated by Lim et al. (2008) in human pancreatic cancer cells where eNOS initiates and maintains tumour growth by oncogenic Ras signaling.

The production of NO from NOS has been described to have a biphasic effect on the rate of apoptosis, with high levels of NO resulting in increased apoptosis and lower levels giving protection against apoptosis (Mortensen et al., 1999), however, the precise levels which induce the switch from pro-apoptosis to anti-apoptosis may differ depending in the cell type involved (Ridnour et al., 2008).
Lee et al. (2001) have shown that increased NO promotes apoptosis mediated by tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), an apoptosis-inducing cytokine, in colorectal carcinomas. In support of this melanoma cells (Qiu et al., 2003) and breast cancer cells (Mortentsen et al., 1999) have also been shown to enter apoptosis under conditions of high NO concentration. It has already been described that PMCA2 is highly expressed in breast cancer cells (Lee et al., 2005), possibly resulting in decreased NO production by eNOS. eNOS seems to be the predominant isoform of NOS expressed in breast cancer cells (Martin et al., 1999) therefore the introduction of the small interfering peptide F-PMCA2(462-684), may be useful in increasing NO production by eNOS and subsequent apoptosis. eNOS has been found to be expressed in colorectal (Yeh et al., 2009) and in melanoma cancers (Tu et al., 2006). It would be interesting to determine the expression profile of PMCA2 in these cell types and eNOS and PMCA2 expression in other cancer types which are also susceptible to NO-mediated apoptosis.

4.8.2 eNOS and angiogenesis in cancer

eNOS is the predominant isoform of NOS expressed in the endothelial cells which are important in the development of new blood vessels. PMCA has been reported, during this investigation, to be expressed in endothelial cells and the predominant isoform of PMCA involved in the interaction with eNOS in these cells is PMCA2.
Angiogenesis is an important process in the progression of cancer by providing the growing tumour with a structured vasculature supplying nutrients and oxygen, essential for cell survival as well as providing an avenue for tumour metastasis. For this reason specific inhibitors of pathological angiogenesis are of vital importance in cancer therapy. Endogenous inhibitors of angiogenesis are already in use (Reviewed in Dass et al., 2007). The mechanism of action of these inhibitors is in part by inhibition of VEGF, however, this has wide-ranging side-effects, such as hypertension, leading to coronary heart disease, stroke and heart

failure (Picirillo et al., 2004) and can also be bypassed by the tumour escape mechanisms which involve the induction of other pro-angiogenic growth factors such as FGF and TGF (reviewed in Drevs 2008). eNOS is often activated by VEGF, FGF and TGF signalling resulting in increased NO production and subsequently increased blood vessel formation (Bussolati et al., 2001) and heightened risk of metastasis (Shang and Li. 2005) in many tumour environments including human gastric cancer (Wang et al., 2005), human primary astrocytoma cells (Pan et al., 2005) and malignant melanomas (Tu et al., 2006). Ways to modulate eNOS activity in endothelial cells in a specific and controlled way may have lesser side-effects than found in current therapies and reduce the number of "escape avenues" for tumour progression. The use of PMCA2 as an endogenous angiogenesis inhibitor via eNOS inhibition has potential, perhaps as a combinational therapy with VEGF signalling inhibitors, if its expression can be localised to the endothelial cells of blood vessels surrounding tumours.

4.8.3 eNOS and diabetic retinopathy

Diabetic retinopathy is a serious side-effect of diabetes involving increased, abnormal angiogenesis. The eNOS signalling pathway has been shown to be upregulated in retinal endothelial cells under conditions of increased glucose (Huang and Sheibani 2008). The expression of PMCA2 in endothelial cells has been described during this work as has its involvement in the inhibition of eNOS activity. It is therefore reasonable to hypothesise that PMCA2 overexpression in endothelial cells in proximity to the retina would be a beneficial therapy for diabetic retinopathy by reducing aberrant blood vessel growth.

4.8.4 eNOS and arteriogenesis

Activation of arteriogenesis, development of small pre-existing arterioles into larger vessels, could play an important role as a therapy for heart disease such as ischemia induced by occluded arteries. Reports suggest that increased NO can stimulate vessel growth in ischemic limb models (Murohara et al., 1998 and Yu et al., 2005). Although shear stress significantly upregulates eNOS activity the use of a small disrupting peptide, such as Flag-PMCA(462-684) to release eNOS from endogenous PMCA2 inhibition and further enhance its activity may be a useful therapeutic tool for arteriogenesis after ischemia.

4.8.5 eNOS and cardiac hypertrophy

eNOS is a crucial regulator of cardiovascular physiology and consequently it is important in pathological conditions of the cardiovascular system due to its production of the important mediator chemical NO. A novel role for the plasma membrane calcium/calmodulin ATPase pump, PMCA4, as a negative regulator of NO signalling has been elucidated (Schuh et al., 2001) and demonstrated to have physiological relevance in the heart by Oceandy et al. (2007). This project has also shown that PMCA2 is an important negative regulator of eNOS in endothelial cells. NO from endothelial cells is important in cardiovascular physiology due to its involvement in angiogenesis, vasodilation and regulating smooth muscle contraction (reviewed in Feron et al., 2006). All these functions are crucial to maintaining a healthy cardiovascular system. PMCA is hypothesised to play a part in cardiac hypertrophy, the aberrant thickening of the ventricular wall (Hammes et al., 1998). It was discovered that overexpression of PMCA in cardiomyocytes resulted in increased growth rate in a hypertrophic model. Since NO is known to suppress abnormal proliferation it is possible that this overexpression of PMCA inhibited the NO signalling pathway resulting in hypertrophy. A therapeutic tool for this discovery would be to disrupt the interaction between PMCA and eNOS increasing NO production resulting in decreased growth rate and diminished hypertrophy. This could be achieved by using the F-PMCA2(462-684) peptide created during this work, known to disrupt the inhibitory effect of PMCA2 on eNOS.

4.8.6 eNOS and atherosclerosis

NO from eNOS is important in modulating vascular permeability and inflammation which are key components in the progression of atherosclerosis (reviewed in Frank et al., 2003). Levels of NO in the vascular endothelium are often diminished (reviewed in Napoli et al., 2006) contributing to atherosclerotic plaque formation. In order to enhance levels of NO in the vascular endothelium it would be beneficial to decrease the levels of inhibitory interactions upon eNOS, such as PMCA2. By overexpressing the F-PMCA2(462-684) peptide the endogenous eNOS/PMCA2 interaction could be disrupted resulting in increased NO production and reduced plaque formation.

4.8.7 PMCA, eNOS and calcineurin

An examination of the sequences corresponding to the interaction domains of eNOS (Chapter 4) and calcineurin A (Buch et al., 2005) with PMCA2 did not show any significant homology between the two regions, suggesting that, probably, eNOS and calcineurin interact with two different small fragments within the region 462-684 of PMCA2. This leads to the intriguing possibility that PMCA, eNOS and calcineurin might be present in endothelial cells as a ternary complex.

Multi-site phosphorylation represents another mechanism involved in the regulation of eNOS enzymatic activity (Mount et al., 2007). Dephosphorylation of Thr^{495} has been shown to promote activation of eNOS (Mount et al., 2007). Interestingly, Harris et al. (2001) reported that calcineurin mediates eNOS- Thr^{495} dephosphorylation (Harris et al., 2001). We have previously reported that PMCA2 inhibits calcineurin activity (Chapter 3) (Holton et al., 2007), thus, if PMCA2, calcineurin and eNOS are part of a macromolecular ternary complex, PMCA-dependent inhibition of calcineurin would result in a decrease in dephosphorylation of Thr^{495} contributing to eNOS inhibition. Our experiments do not rule out the possibility that part of the increase in eNOS activity observed after ectopic expression of Flag-PMCA2(462-684), is due to disruption of the interaction PMCA/calcineurin, leading to activation of calcineurin activity and subsequent dephosphorylation of eNOS-Thr^{495}. The precise mechanism responsible for this increase in eNOS activity requires further investigation.

The possibility that PMCA2, calcineurin and eNOS are forming a ternary macromolecular complex, introducing a new level of regulation in NO production by endothelial cells requires further investigation. Supporting this hypothesise PMCA has been reported to participate in the organisation of a macromolecular protein complex formed by endogenous PMCA, α-1 syntrophin and nNOS in cardiac cells (Williams et al., 2006). Evidence in support of this model is also given by a study by Yang et al., 2008 where they demonstrate that treatment of endothelial progenitor cells (EPC) with cyclosporine A, a known calcineurin inhibitor, results in increased eNOS mRNA and NO production.

4.9 Conclusion

In conclusion, this work shows an inhibitory interaction between endogenous PMCA2 and eNOS in human endothelial cells, and suggests PMCA2 as an important regulator of NO signalling in endothelial cells. Considering the relevant role of NO in cardiovascular physiology, apoptosis and angiogenesis, the implications of this interaction are far-reaching. Modulation of the interaction PMCA2/eNOS might, therefore, have an important significance as a potential therapeutic target to modulate NO in patients with cardiovascular disease, cancer and aberrant angiogenesis.

5. CHAPTER FIVE

FUTURE WORK

5.1 PMCA2 and calcineurin

This project has confirmed that PMCA2 interacts with calcineurin in an isoform-specific manner in breast cancer cells and that this interaction inhibits calcineurin activity. The importance of this discovery is clear from the number of physiological and pathological processes in which PMCA2 and calcineurin are involved. Further work into the characterisation of this interaction and its functional consequences in breast physiology and endothelial cell signalling are essential and ultimately very rewarding.

To further characterise the functionality of the interaction between PMCA2 and calcineurin the effect of PMCA2 overexpression on the rate of apoptosis in breast cancer cells should be assessed. It has been hypothesised during this work that calcineurin inhibition by PMCA2 would result in a reduction of calcineurin-mediated apoptosis.

It has been reported that paclitaxel treatment of MCF-7 breast adenocarcinoma cells results in FasL-mediated apoptosis of these cells. Expression of FasL in MCF-7 cells treated with paclitaxel is dependent on the full activation of the calcineurin/NFAT pathway, therefore, disruption of the interaction PMCA2/calcineurin by the overexpression of the region 462-684 should increase calcineurin activity in cancer cells and thus calcineurin-mediated apoptosis. Further work in Dr Armesilla's laboratory will focus on overexpressing the region 462-684 of PMCA2 in breast cancer cells and evaluating its effects on paclitaxel-induced apoptosis and FasL expression.

It would also be interesting to determine if the interaction between PMCA2 and calcineurin occurs in other cancer cells lines. Elucidating the PMCA2 expression profile in cancer cells would be beneficial in developing a therapy to exploit the apoptotic pathway involved in the PMCA2-calcineurin interaction. By creating a cell penetrating peptide that encodes the interaction domain of PMCA2 with calcineurin, its inhibition by PMCA2 could be released resulting in heightened calcineurin activity and subsequent apoptosis. Cell targeting to cancer cells could be achieved by creating a peptide that can only function in the presence of a cancer specific marker, such as reduced p53.

Another interesting avenue of research would be to look at overexpressing PMCA2 in systems where a lack of PMCA2 results in a pathological phenotype, such as in inner ear cells. Inner ear cells express high levels of PMCA2, however, when this level of PMCA2 is reduced or knocked down, there is a susceptibility to noise-induced hearing loss. By increasing the levels of PMCA2 this should result in less apoptosis in these cells and possibly the healthy development of the calcium rich otoconia, essential for balance. This could be achieved *In Vivo* by gene therapy at an early stage of development. Localised treatment of inner ear cells with a plasmid containing the *pmca2* gene under the control of a mammalian promoter using an efficient gene delivery system, such as recombinant lentivirus would allow for the expression of PMCA2 and possibly a reduction in noise induced hearing loss.

5.2 PMCA2 and eNOS

During this project the interaction between PMCA2 and eNOS has been identified and this interaction has been shown to reduce NO production in endothelial cells. The effects of PMCA and eNOS in physiological and pathological conditions are great, therefore this discovery has implications in the development of treatments for diseases where NO production is abnormal. Further characterisation of this interaction and its effects are of vital importance.

To further characterise the interaction between PMCA2 and eNOS, the exact mechanism used by PMCA2 to inhibit the activity of eNOS should be determined. It is thought that PMCA inhibits the activity of calcium/calmodulin-dependent enzymes by sequestering these proteins to a low calcium micro-domain generated by the calcium extrusion properties of PMCAs.
Currently being investigated in Dr Armesilla's lab is the possibility that PMCA2 inhibits the dephosphorylation of Thr^{495}, the negative regulatory serine of eNOS. Interestingly it has been reported that the dephosphorylation of eNOS Thr^{495} can be carried out by calcineurin. PMCA2 could be overexpressed in endothelial cells and using an antibody specific for eNOS phosphorylation site Thr^{495} the effect of this could be demonstrated. The work in this thesis (Chapter 3) has shown that PMCA2 is a negative regulator of calcineurin activity and, therefore, PMCA2-

mediated inhibition of calcineurin might lead to a lack of eNOS Thr^{495} dephosphorylation and result in inhibition of eNOS activity.

To confirm that the interaction between PMCA2 and eNOS is relevant in physiological terms the effect of overexpressing PMCA2 in an angiogenesis model could be performed. This could be achieved by growing endothelial cells on a collagen or fibrin matrix such as matrigel to assess the number of vessel-like tubes formed when PMCA2 is overexpressed. Hypothetically the number of tubes formed should reduce when PMCA2 is overexpressed as PMCA2 is inhibiting the activity of eNOS and NO production resulting in a lack of proangiogenic signalling. It would also be interesting to determine whether expression of the PMCA2 interaction domain protein could enhance tube formation by releasing eNOS from endogenous PMCA2 inhibition. This overexpression would also release calcineurin from the inhibitory effects of PMCA2 possibly resulting in increased NFAT-dependent gene activation and angiogenesis promotion.

It would also be interesting to investigate the PMCA2-eNOS interaction in other endothelial cells as there is evidence that there are significant differences between endothelial cells derived from large or small vessels and different organ sites (reviewed in Aubach et al., 2003). In particular microvascular endothelial cells are particularly relevant to angiogenesis in pathological diseases such as vascular dysfunction. This further characterisation would help in identifying the best treatment for angiogenic related diseases such as cancer metastasis.

5.3 PMCA2, eNOS and calcineurin

An interesting question arising from this work is that PMCA2, eNOS and calcineurin exist as a ternary complex in cells and that PMCA, as well as regulating their activity is involved in localising both proteins to the same vicinity as each other. Determining if this is the case would be an important insight into the mechanism of the regulation by PMCA2 of signal transduction pathways

Initially it would be important to determine if the low calcium microenvironment causes the decrease in calcineurin and eNOS activity or whether the interaction with PMCA2 results in direct inhibition of activity by another process. By creating a functionally deficient PMCA2 mutant where the calcium extrusion activity of PMCA2 is reduced without altering the structure of the protein the inhibitory effect of PMCA2 on calcineurin and eNOS could be assessed.

Future work involves defining whether PMCA2, eNOS and calcineurin exist in a ternary complex and how their interaction with PMCA2 results in their inhibition. Hypothetically the activation of eNOS depends on the dephosphorylation of the negative regulatory threonine residues and phosphorylation of the positive regulatory serine residues. This dephosphorylation of the threonine residue has been shown to involve calcineurin (Harris et al., 2001) implicating that PMCA2 may dually inhibit the activity of eNOS by tethering eNOS to a low calcium microenvironment and inhibiting the activity of calcineurin. The development of a small cell penetrating peptide encompassing the region of PMCA2 involved in the interaction between PMCA2 and calcineurin and eNOS may cause the disruption of the ternary complex and release calcineurin and eNOS from the inhibition of the low calcium microenvironment and also allow eNOS activation by calcineurin. This would be a useful therapy if it could be cell specific for endothelial cells in diseases where NO levels are low. Releasing eNOS from endogenous inhibition may increase NO production. This increased NO would be important for the activation of angiogenic pathways that are often dysfunctional in diseases such as atherosclerosis.

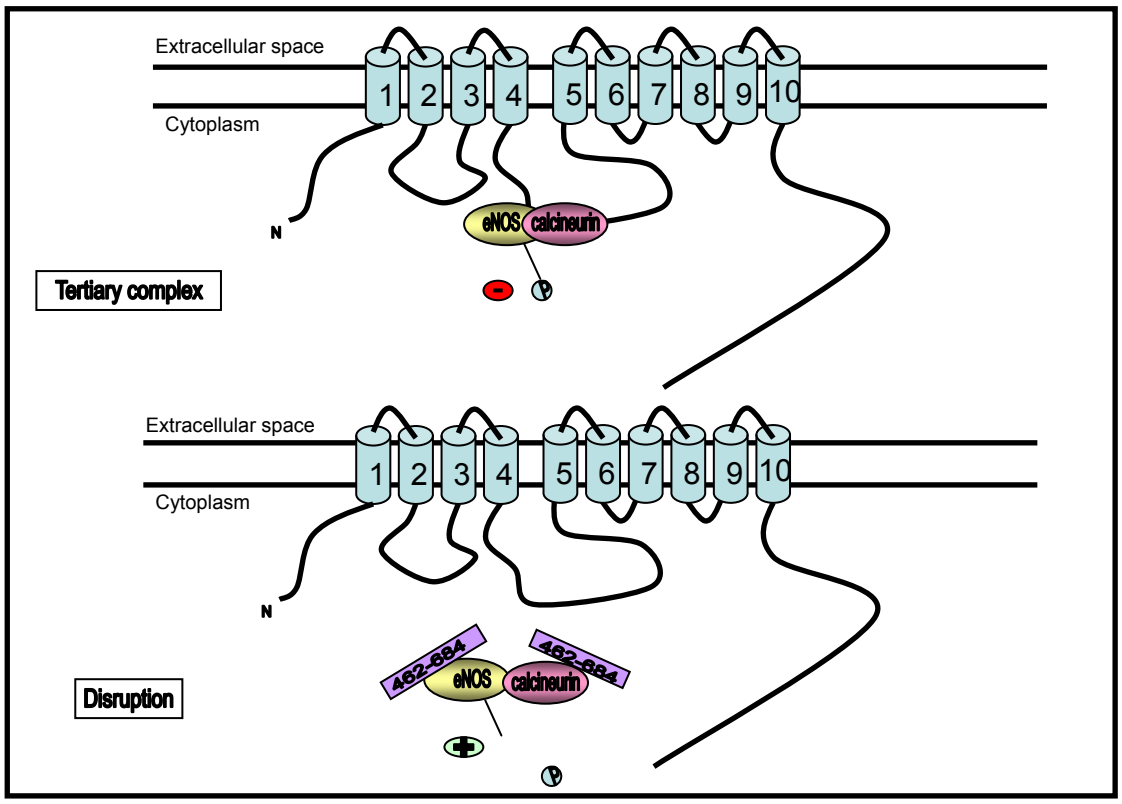

Fig. 5.3.1 Hypothetical PMCA2, calcineurin, eNOS ternary complex formed in endothelial cells and disruption of this complex by a cell penetrating peptide encompassing the interaction region of PMCA2 as a therapy for disease involving dysfunctional endothelial cells caused by low NO levels.

Also co-localisation experiments using confocal imagery could be performed to visually demonstrate that PMCA, eNOS and calcineurin exist as a ternary complex by providing an overview of their localization within endothelial cells. This could be performed in endothelial cells as each protein is endogenously expressed in these cells, using antibodies specific for each protein that are conjugated with different fluorescent dyes. Another method to demonstrate the hypothesis that calcineurin and eNOS interact would be to perform immunoprecipitation with anti-calcineurin and Western blot with anti-eNOS analysis.

5.4 Conclusion

The potential avenues for this research are vast with the possible involvement of PMCA in calcineurin-mediated apoptosis and control of NO production by eNOS. Since PMCA is involved in these important pathways is essential to establish its involvement in other pathways and the degree to which PMCA actually regulates them. This information would be essential for the production of therapeutic drugs and simply defining signalling pathways involved in these processes for future reference.

CONCLUDING REMARKS

This thesis has clearly demonstrated that PMCA is a novel regulator of calcium/calmodulin-dependent signal transduction pathways in mammalian cells. This work has shown that PMCA interacts with calcium/calmodulin-dependent proteins in an isoform-specific manner. PMCA isoform 2 region 462-684 of the large intracellular loop was found to interact with calcineurin (region undefined) and eNOS at region 735-940. The calcineurin-PMCA2 interaction was described in; mammalian cells HEK293, breast adenocarcionoma cells MCF7 and endothelial cells HUVEC. The eNOS-PMCA2 interaction was described in mammalian cells HEK293 and endothelial cells HUVEC. PMCA2 interacts with both proteins resulting in the significant inhibiton of their subsequent signalling pathways. The overexpression of F-PMCA2(462-684) resulted in the disruption of the endogenous interaction between PMCA2 and eNOS in endothelial cells HUVEC. These results demonstrate the importance of PMCA2 in the regulation of calcium/calmodulin-dependent signal transduction pathways in breast and endothelial cells and represent a novel and potential therapeutic avenue to pursue in the treatment of many diseases.

APPENDIX

A1. Primary antibody specificity

To demonstrate the specificity of the primary antibodies used throughout this work, a full length picture of the western blots for each PMCA isoform and eNOS are included.

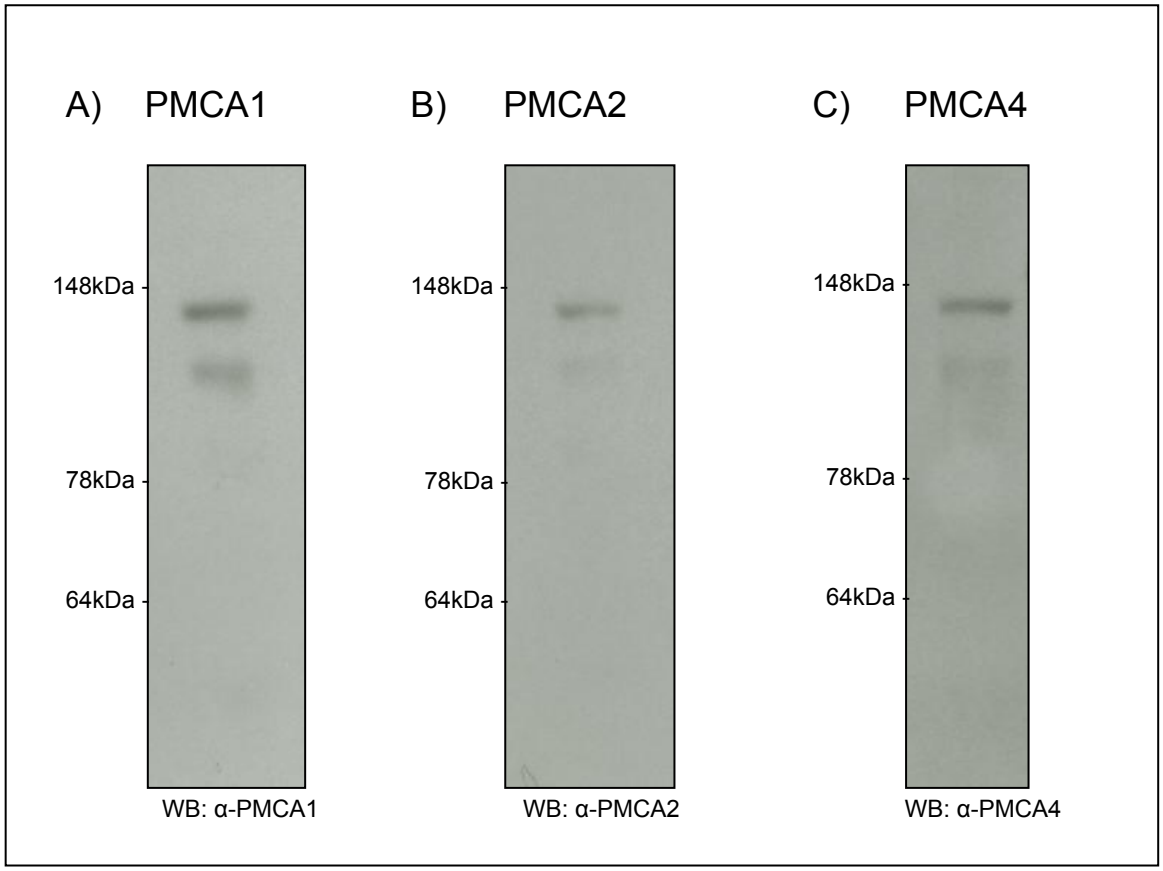

Fig. A1.1 Full length pictures of whole HUVEC protein lysates on Western blots probed with PMCA antibodies to demonstrate the specificity of the primary antibodies. Western blots of proteins immunoprecipitated with 5F10 (Abcam) were probed with antibodies specific for PMCA1, -2, or -4 (Swant). A) PMCA1 (WB: α-PMCA1), B) PMCA2 (WB: α-PMCA2) and C) PMCA4 (WB: α-PMCA4). Secondary antibody α-Rabbit-hrp (Sigma).

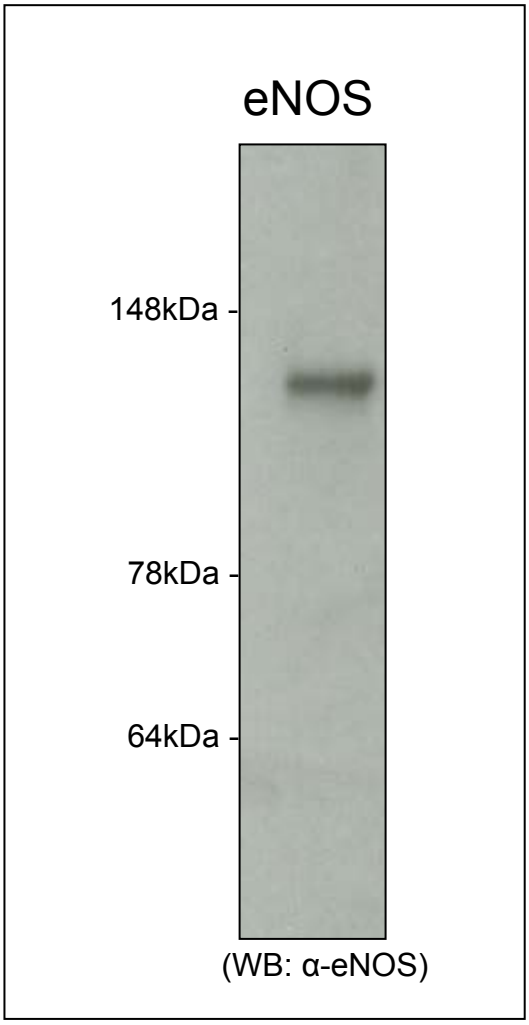

Fig. A1.2 Full length picture of whole HUVEC protein lysates on a Western blot probed with α-eNOS antibody to demonstrate the specificity of the primary antibody. Western blot of eNOS was probed with an antibody specific for eNOS (Zymed) (WB: α-eNOS). Secondary antibody α-Mouse-hrp (Sigma).

A2. Materials

Amersham
GE Healthcare UK Ltd,
Amersham Place,
Little Chalfont,
Buckinghamshire.
HP7 9NA.

cGMP enzymeimmunoassay biotrak (EIA) system

ATCC
LGC Standards,
Queens Road,
Teddington,
Middlesex,
TW11 0LY.

HEK293
MCF-7

Clontech
The Dandy Building,
Edmund Halley Road,
Oxford Science Park,
Oxford.
Oxen
pNFAT-TA-Luc

Geneflow
Geneflow Ltd,
Fradley Business Centre,
Wood End Lane,
Fradley ,
Staffordshire.
WS13 8NF.

Running buffer
Transfer buffer
EZ chemiluminescence kit

Invitrogen Ltd,
3 Fountain Drive,
Inchinnan Business Park,
Paisley,
UK.
PA4 9RF.

Lipofectamine
Opti-mem
Electrophoresis grade agarose
TAE buffer
Ethidium Bromide
Loading buffer (AGE)
DNA ladder (1kB)
Agar powder
LB powder
Seeblue plus2 protein marker
Loading buffer (PAGE)
$pcDNA_3$

Kodak,
Kodak Limited,
Hemel One,
Boundary Way,
Hemel Hempstead,
Herts.
HP2 7YU.

Autoradiographic film

Lonza
Lonza Wokingham Ltd,
1Ashville way,
Wokingham,
Berkshire.
RG412PL.

EGM-2
HUVEC
Nucleofector solution

Marvel
Premier foods,
Central technical,
The Lord Rank centre,
Lincoln road,
High Wycombe.
HP12 3QS.

Milk powder

Millipore
Millipore (UK) Ltd,
3-5 The Courtyards,
Hatters Lane,
Watford,
Herts.
WD18 8YH.

Nitrocellulose membrane

National Diagnostics
AGTC Bioproducts t/a National Diagnostics UK,
Itilngs lane,
Hessle.
HU139 LX.

Bis-acrylamide

Pierce
Thermo Fisher Scientific,
p/a Perbio Science,
Unit 9,
Atley Way,
North Nelson Industrial Estate,
Cramlington,
Northumberland.
NE231WA.

BCA protein assay kit

Promega UK Ltd,
Delta House,
Southampton Science Park,
Southampton,
Hampshire.
SO16 7NS.

Restriction enzymes and buffers
PCR gel clean up wizard kit
Alkaline phosphatase
Alkaline phosphatase buffer
Ligase buffer
JM109 E.Coli
Bacteriophage T4 DNA ligase
Luciferase lysis reagent
Luciferase substrate

QIAGEN,
QIAGEN HOUSE,
Fleming Way,
Crawley,
West Sussex,
RH10 9NQ.

QIAGEN maxiprep kit
QIAGEN miniprep kit

Roche Diagnostics Ltd,
Charles Avenue,
Burgess Hill,
RH15 9RY.

High fidelity PCR master mix
Agarose A beads

Sigma-Aldrich Company Ltd.
The Old Brickyard,
New Road,
Gillingham,
Dorset.
SP8 4XT.

A23487 calcium ionophore
Ampicillin powder
Aprotinin
APS powder
Butanol
DMEM
DMSO
Ethanol
α-Flag®-M2 affinity gel agarose beads
FBS
Flag peptide
Glycine
HEPES buffer
Hydrochloric acid
IBMX
IGEPAL
Isopropanol
L-Glutamine

KCl powder
L-Arginine powder
LB powder
Leupeptin Hemisulphate powder
Methanol
NaAc
NaCl
NEM
P3xFlagcmv7.1
pFlag5b
PBS
Penicillin/streptomycin
Pepstatin A
PMA
PMSF
Recombinant calcineurin
Recombinant eNOS
RPMI
SDS
Sodium Deoxycholate
Superoxide dismutase
TE buffer
TEMED
Tris base
Trypsin-EDTA
TWEEN20

VHBio,
Unit 11B Station Approach, Team Valley Trading Estate,
Gateshead.
NE11 0ZF.

Primers

A3. Solutions

Freezing solution

FBS (90%)

DMSO (10%)

Luria broth

dH_2O

LB powder (25g/L)

+/- Ampicillin (100µg/ml)

LB/Agar

dH_2O

LB powder (25g/L)

Agar powder (15g/L)

Ampicillin (100µg/mL)

Resolving gel

6% gel

dH_2O

4x 1.5M Tris buffer pH 8.8 (28.8%)

10% of APS (0.71%)

TEMED (0.43%)

Bis-acrylamide (20%)

12% gel

dH_2O

4x 1.5M Tris buffer pH 8.8 (28.8%)

10% of APS (0.71%)

TEMED (0.43%)

Bis-acrylamide (40%)

RIPA buffer

dH_2O

PBS (1%)

Igepal (1%)

Sodium Deoxycholate (0.5%)

SDS (0.1%)

20µM PMSF (0.05%)

Pepstatin A (500ng/mL)

Leupeptin (500ng/mL)

Aprotinin (1µg/mL)

Running buffer

Tris base (0.25M)

Glycine (1.92M)

SDS (1%)

Stacking gel

dH_2O

Bis-acrylamide (14%)

4x 1.5M Tris buffer pH 6.8 (25%)

10% APS (0.1%)

TEMED (0.06%)

TBS

dH_2O

NaCl (10g/L)

Tris base (3.75g/L)

KCl (0.25g/L)

TBS-T

dH_2O

NaCl (10g/L)

Tris base (3.75g/L)

KCl (0.25g/L)

Transfer buffer

Tris base (0.2M)

Glycine (1.5M)

Methanol (20%)

Tris buffer

dH_2O

Tris base (121.14g/mol)

1M hydrochloric acid (HCl)

TWEEN20 (0.1%)

REFERENCES

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